Frontispiece

Rihaakuru

Control of Histamine in *Rihaakuru*: Emerging Approaches

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3. Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., & Meerdink, G. (2011b). Biogenic amines and potential histamine - Forming bacteria in *Rihaakuru* (a cooked fish paste). *Food Chemistry*, *128*(2), 479-484.

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3. Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., Meerdink, G., & Morton, R. H. (2011). Degradation of histamine in tuna soup by diamine oxidase (DAO). First International Conference on Food and Environment- The quest for a sustainable future, 21-23 June, New Forest, UK.

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4. Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., Meerdink, G., & Morton, R. H. (2011). Modelling the degradation of histamine in tuna soup by diamine oxidase (DAO). NZMS Annual Conference, 23-25 November, Palmerston North, New Zealand.

Other publications

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., Meerdink, G. (2009). The development and control of histamine production in a fish paste product (*Rihaakuru*) from the Maldives. Food New Zealand, F-files, p. 19.

Abstract

Rihaakuru is a cooked fish paste from the Maldives, consumed as a condiment with rice and other food. The product is unique to the Maldives and there is no information on the composition, characteristics and safety of this product. Histamine contamination has been suspected due to symptoms sometimes seen following consumption. This research established that *Rihaakuru* is a nutritious and shelf-stable product. *Rihaakuru* is generally produced from poor quality fish therefore presence of biogenic amines was suspected. This study confirmed that *Rihaakuru* contained up to ten different biogenic amines, with histamine in excess of 500 ppm. This may cause histamine poisoning with symptoms such as skin rashes, vomiting and fever. The product examined in this study contained a few weak histamine forming bacteria. Most of the histamine is likely to be produced by bacteria in the raw fish. These bacteria are likely to die during the manufacture of *Rihaakuru.* Histamine in *Rihaakuru* decreased by 30-70% during storage at -80°C, 4°C and 30°C for 10 months. This showed that the histamine hazard in *Rihaakuru* is unlikely to increase and may decrease during long term storage. Traditional control of histamine in food is through refrigeration of raw material. In the case of the fish used to manufacture *Rihaakuru,* refrigeration is not available or limited. Histamine oxidizing bacteria and enzymes were identified as emerging approaches to degrade pre-formed histamine. Histamine oxidizing bacteria (*Lactobacillus sakei* [AGR 37, AGR 46, Lb 706] and *Vergibacillus halodonitrificans* Nai18) tested in this study degraded histamine by 30-50%. The histamine oxidizing enzyme, diamine oxidase (DAO) completely degraded 500 mg/L of histamine at pH 6 and salt 1% in buffer and in the tuna soup used to manufacture Rihaakuru. A regression model was developed that predicted the rate and amount of histamine removal by DAO under varied pH and salt concentration. This model may be used to determine conditions that will reduce histamine in other foods that have similar characteristics to the tuna soup used to manufacture *Rihaakuru*.

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My mother, Fathimath Agila, without whom none of this would have been possible and to her I dedicate this thesis.

Table of Contents

List of Abbreviations

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
CCD	central composite design
CHD	coronary heart disease
CHO	carbohydrates
DAO	diamine oxidase
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	ethylene diamine tetra acetic acid
EPA	eicosapentaenoic acid
ETE	eicosatrienoic acid
FDA	Food and Drug Administration
FID	Flame Ionization Detector
GAM	Gifu Anaerobic Medium broth
GC	gas chromatography
GDL	glucono-delta-lactone
GE	Gross Energy
HDC	Hisitidine Decarboxylase
HHP	high hydrostatic pressure
HPLC	high performance liquid chromatography
ICMSF	International Commission on Microbiological Specification for Foods
MAOI	monoamine oxidase inhibitors
MAP	modified atmosphere packaging
MPN	most probable number
MRS	deMan, Rogosa and Sharpe broth
MS	mass-spectroscopy
NOAEL	no observed adverse effect level
NZAID	New Zealand Agency for International Development
NZDS	New Zealand Development Scholarship

NZIFST	New Zealand Institute of Food Science and Technology
NZMS	New Zealand Microbiological Society
PCR	polymerase chain reaction
PUFA	polyunsaturated fatty acids
RDI	recommended daily intake
rDNA	Ribosomal DNA
RSM	response surface model
SBMB	New Zealand Society for Biochemistry and Molecular Biology
sfam	Society for Applied Microbiology
TCA	trichloroacetic acid
TDF	Total Dietary Fibre
TLC	thin layer chromatography
TMAH	tetramethylammonium hydroxide
TSA	trypticase soy agar
TSB	trypticase soy broth
TSBH	histidine tripticase soy broth
UHT	ultra high temperature
USA	United States of America
USDA	United States Department of Agriculture
UV	ultra-violet
WHO	World Health Organization

List of Tables

List of Figures

Chapter 1

Introduction

1.1 Background

Rihaakuru, a traditional side dish of the Maldives, is generally made using low quality tuna, which means that histamine is suspected in the product. If the histamine concentration exceeds 500 ppm, *Rihaakuru* may cause food poisoning. The Maldives regulatory authorities provide no guidelines for the standardization of *Rihaakuru* and this is unlikely to change without scientific evidence to support the enforcement of guidelines. Thus this thesis aims to provide scientific evidence that can be used to support the development of guidelines for the safe production of *Rihaakuru*.

1.2 Thesis hypothesis and objectives

The hypothesis of this study was that histamine in *Rihaakuru* can be reduced to a safe level (< 100 ppm).

The objectives were:

1. To characterize *Rihaakuru* in terms of compositional, physiochemical analysis and isolation of bacterial flora (**Chapter 3**).

2. To characterize biogenic amines from *Rihaakuru* and identify potential histamine forming bacteria (**Chapter 4**).

3. To determine histamine stability in *Rihaakuru* over time (**Chapter 5**).

4. To investigate histamine degradation by bacteria and enzyme (diamine oxidase, DAO) in order to select the most effective method to degrade histamine in *Rihaakuru* (**Chapter 6**).

5. To examine the ability of DAO to degrade histamine in *Rihaakuru* (based on Chapter 6) using model (phosphate buffer) and real (tuna soup used to manufacture *Rihaakuru*) systems and find the predicted optimum rate and amount of histamine degradation using regression modelling (**Chapter 7**).

1.3 Preface

Although *Rihaakuru* is consumed daily in the Maldives, little is known about the characteristics of this product. This study reports the physiochemical composition, bacterial flora and nutritional content of the product (**Chapter 3**). The histamine concentration of the product was measured and reported, confirming the potential safety hazard (**Chapter 4).** The histamine in the product was shown to decrease with long-term storage (**Chapter 5). Chapters 6-7** investigated two methods to remove histamine from the product - histamine degrading bacteria and diamine oxidase (DAO). DAO was found to be the most effective method but the effect of the pH and salt concentration in *Rihaakuru* on DAO activity was a potential concern. Therefore, 15 factorial designed experiments firstly in buffer and later in tuna soup used to manufacture *Rihaakuru*, determined the optimum conditions for the degradation of histamine in *Rihaakuru* and a model prepared from this data enabled the effect of variations in the natural properties of the product on DAO activity to be determined (**Chapter 7**). The general discussion and future directions of the study which included the major findings and potential impacts of this study are described in **Chapter 8.** Supplementary data to **Chapters 3** and **7** are provided under **Appendix I**. **Appendix II** contains the first page of published papers that stand for **Chapters 2** through **4**.

Anoverview of the study and relationships between the chapters is illustrated in Figure 1- the light blue boxes show the knowledge gaps and relevant chapters that fill the gaps are adjacent to them. The diagram represents **Chapters 2** through **8**.

This thesis is written based on submitted publications. **Chapters 2-4** are published in peer reviewed journals, part of **Chapter 7** is published in a peer reviewed conference proceedings, and **Chapters 5-7** have been submitted as separate papers to journals. All the chapters with the exceptions of Chapters **1, 4** and **8** have been presented in national or international conferences as poster or oral presentations.

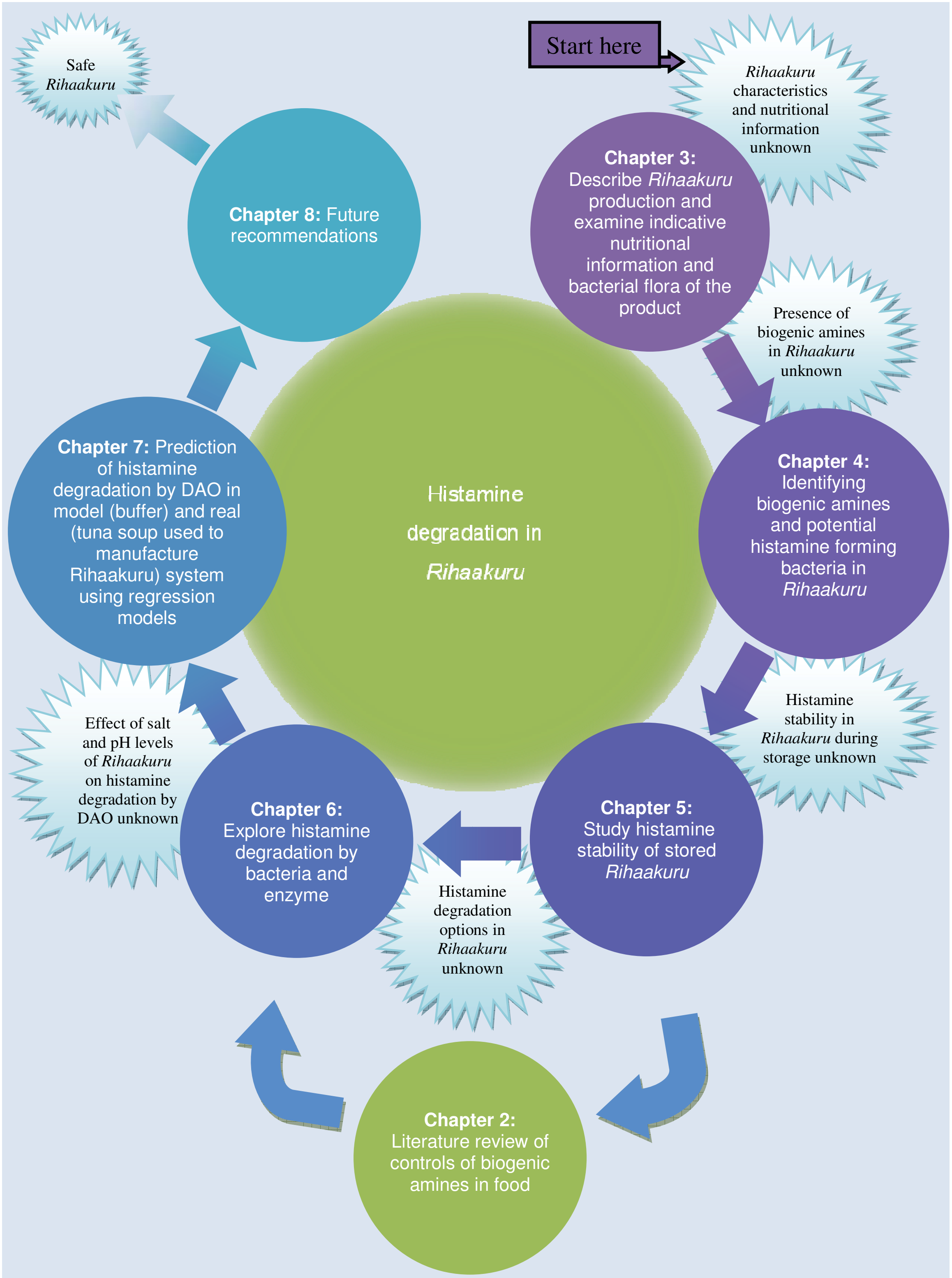

Figure 1: Overview of the thesis chapters and their relationship to knowledge gaps

Chapter 2

Literature Review

The review presented in this chapter has been published in the following peer reviewed publication:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., & Meerdink, G. (2010). Control of biogenic amines in food-existing and emerging approaches. *Journal of Food Science, 75*(7), R139-R150.

2.1 Abstract

Biogenic amines have been reported in a variety of foods, such as fish, meat, cheese, vegetables and wines. They are described as low molecular weight organic bases with aliphatic, aromatic and heterocyclic structures. The most common biogenic amines found in foods are histamine, tyramine, cadaverine, 2- phenylethylamine, spermine, spermidine, putrescine, tryptamine, and agmatine. Additionally octopamine and dopamine have been found in meat and meat products, and fish.

The formation of biogenic amines in food by the microbial decarboxylation of amino acids, can result in consumers suffering allergic reactions, characterized by difficulty in breathing, itching, rash, vomiting, fever, and hypertension. Traditionally, biogenic amine formation in food has been prevented, primarily by limiting microbial growth through chilling and freezing. However, for many fishing based subsistence populations, such measures are not practical. Therefore, secondary control measures to prevent biogenic amine formation in foods or to reduce their levels once formed need to be considered as alternatives. Such approaches to limit microbial growth may include hydrostatic pressures, irradiation, controlled atmosphere packaging, or the use of food additives. Histamine may potentially be degraded by the use of bacterial amine oxidase or amine-negative bacteria. Only some will be cost effective and practical for use in subsistence populations.

Keywords: biogenic amines, scombroid poisoning, high hydrostatic pressure (HHP), irradiation, food additives, packaging, temperature, starter cultures.

2.2 Introduction

Biogenic amines and polyamines have been reported in variety of foods, such as fish, meat, cheese, vegetables and wines and are described as organic bases with aliphatic, aromatic and heterocyclic structures (Lorenzo et al., 2007). Biogenic amine formation through the microbial decarboxylation of amino acids is dependent on the specific bacterial strain(s) present, the level of decarboxylase activity, and the availability of the amino acid substrate (De Las Rivas et al., 2008; Suzzi & Gardini, 2003). Histaminolytic (histamine oxidizing) bacteria may allow an equilibrium to develop between histamine production and destruction in foods containing high amounts of histamine (Ienistea, 1971). The most common biogenic amines found in foods are histamine, tyramine, cadaverine, 2-phenylethylamine, spermine, spermidine, putrescine, tryptamine, and agmatine. Additionally octopamine and dopamine have been found in meat and meat products, and fish (Hernandez-Jover et al., 1996). Polyamines such as putrescine, cadaverine, agmatine, spermine and spermidine are naturally present in food, and are involved in growth and cell proliferation (Hernandez-Jover et al., 1997; Kalac, 2009; Kim et al., 2009). These amines in the presence of nitrites can be potential carcinogens when converted to nitrosamines (Kim et al., 2009). Nitrosamines from polyamines may not necessarily pose a health risk as toxicity is reached only after consumption of large amounts, more than expected in a daily meal (Kalac, 2009). The aromatic biogenic amines, tyramine and 2- phenylethylamine have been reported to be initiators of dietary induced migraine and hypertensive crisis (Stratton et al., 1991). Tyramine, 2- phenylethylamine and putrescine are versoactive amines and increase blood pressure which can lead to heart failure or brain haemorrhage (Kalac, 2009; Mohan et al., 2009; Til et al., 1997).

Histamine poisoning (scombroid poisoning) is a worldwide problem (Russell & Maretic, 1986) that occurs after the consumption of food containing biogenic amines, particularly histamine at concentrations higher than 500 ppm (Gonzaga et al., 2009). Histamine poisoning manifests itself as an allergen-type reaction characterized by difficulty in breathing, itching, rash, vomiting, fever, and hypertension. People having deficient natural mechanisms for detoxifying biogenic amines through genetic reasons or through inhibition due to the intake of anti-depression medicines, such as monoamine oxidase inhibitors

(MAOI) are more susceptible to histamine poisoning (Hernandez-Jover et al., 1997; Yongmei et al., 2009). Histamine alone may not cause toxicity at a low level, but the presence of other biogenic amines such as putrescine and cadaverine, at concentrations five times higher than histamine, enhance the toxicity of histamine (Emborg & Dalgaard, 2006; Hernandez-Jover et al., 1997; Stratton et al., 1991) through the inhibition of histamine oxidizing enzymes. Oral toxicity levels for putrescine, spermine, and spermidine are 2000, 600 and 600 ppm, respectively. The acute toxicity level for tyramine and cadaverine is greater than 2000 ppm. The no observed adverse effect level (NOAEL) is 2000 ppm for tyramine, putrescine and cadaverine, 1000 ppm for spermidine and 200 ppm for spermine (Til et al., 1997). Tyramine alone at high levels can cause an intoxication known as the cheese reaction, which has similar symptoms to histamine poisoning.

When legumes were boiled, the biogenic amines transferred completely into the boiling water so by this means any hazard could be eliminated. However, sprouted legumes behaved differently when boiled, with the biogenic amine concentration being only reduced slightly (Shalaby, 2000). This indicates that although biogenic amines in some type of legumes can be eliminated through boiling, boiling is not effective in eliminating biogenic amines in sprouted legumes. The effectiveness of biogenic amines elimination via boiling on other food products, each type requires experimentation. However, biogenic amines are reported as heat stable compounds (Tapingkae et al., 2010b) and cooking or prolonged exposure to heat, will not eliminate the toxin (Duflos, 2009; Gonzaga et al., 2009; Shalaby, 1996).

Factors influencing biogenic amine production are storage conditions (Komprda et al., 2001), manufacturing processes (De Las Rivas et al., 2008), manufacturing practices (Komprda et al., 2001) the proportion of the microbial population with decarboxylase activity (Santos, 1996), raw material quality (Maijala et al., 1995b), and the availability of free amino acids (Maijala et al., 1995a).

Biogenic amines can be controlled with the use of existing methods and emerging methods. The existing method in this paper refers to temperature, which has been well established in control of biogenic amines. The emerging method in the paper refers to other methods to date used for controlling or eliminating biogenic amines in food than temperature or with the combination of temperature. The emerging methods include modified atmosphere packaging (MAP), irradiation, high hydrostatic pressure (HHP), and microbial modelling and addition of preservatives.

Biogenic amine formation can be controlled through inhibiting microbial growth or inhibiting the decarboxylase activity of microbes (Wendakoon & Sakaguchi, 1995) . The prevention of biogenic amine formation in food, has therefore been achieved using temperature control, using high quality raw material, good manufacturing practice, the use of non-amine forming (amine-negative) or amine oxidizing starter cultures for fermentation (Dapkevicius et al., 2000; Nieto-Arribas et al., 2009), the use of enzymes to oxidize amines (Dapkevicius et al., 2000), the use of microbial modelling to assess favourable conditions to delay biogenic amine formation (Emborg & Dalgaard, 2008a, 2008b; Neumeyer et al., 1997), packaging techniques (Mohan et al., 2009), HHP (Bolton et al., 2009), irradiation(Kim et al., 2003), and food additives (Mah & Hwang, 2009a). Emerging approaches to control histamine production involve the combined effect of an existing method, such as the combination of HHP and amine-negative starters (Latorre-Moratalla et al., 2007). However, optimisation of such an approach is required.

This review will identify and discuss techniques that can be used to limit amine formation or enhance their degradation.

2.3 Existing methods for biogenic amine control in food

Biogenic amine formation is temperature dependent (Shalaby, 1996) it is decreased at low temperatures (Duflos, 2009; Prester et al., 2009) through inhibition of microbial growth and the reduction of enzyme activity (Arnold et al., 1980; Bremer et al., 1998; Chander et al., 1989; Du et al., 2002; Mah & Hwang, 2009b). Biogenic amine formation in food can therefore be controlled by strict adherence to the cold chain (Bover-Cid et al., 2006; Bremer et al., 2003; Dalgaard et al., 2006). Biogenic amine forming bacteria such as *Morganella morganii* and *Proteus vulgaris* in skipjack tuna (*Katsuwonus pelamis*) were inhibited through chilling (Arnold et al., 1980; Ruiz-Capillas & Jiménez-Colmenero, 2004).

Maintaining the cold chain in foods that already contain high levels of biogenic amines will generally stabilize the levels of biogenic amines although in some cases there may be a slight increase over time (Chen et al., 2010; Gonzaga et al., 2009). For example, yellowfin tuna stored at 0 °C and 22 °C up to 9 days, showed an increase in histamine of 15 ppm at 0 °C and 4500 ppm at 22 °C (Du et al., 2002). Freezing is more effective than cooling in preventing biogenic amine production (Arnold & Brown, 1978).

High temperature treatments can also be used to extend the shelf life of food. A thermal regime designed to kill the bacterial species responsible for histamine formation, can prevent the subsequent formation of histamine. For a *Hafnia alvei* strain implicated in histamine production in hot smoked Kahawai, at temperatures between 54 - 58 ºC, the D-values (the time required to kill 90% of the contaminating bacteria) ranged from 51 to 20 seconds (Bremer et al., 1998). For *M. morgani* in hot smoked Kahawai at temperatures between 58 - 62 ºC, the D-values were between 15 and 1.5 s (Osborne & Bremer, 2000). Although heating can destroy the histamine producing bacteria in food, if recontamination and temperature abuse occurs after thermal processing, histamine formation may still occur in the thermally processed product.

However, as discussed above histamine is heat stable so applying heat after histamine has formed in the product will not ensure its safety. For example, fish paste (*Rihaakuru*, Maldives local dish) is made through prolonged cooking (maximum 100 °C), which eliminates all the potential bacteria responsible for histamine formation. However, *Rihaakuru* often contains high levels of histamine (> 1000 ppm) (authors unpublished data) as the histamine is believed to be formed in fish well before the cooking step and heat does not destroy histamine.

2.4 Emerging methods for biogenic amine control

It is not always possible to control biogenic amine production through temperature alone, since some bacteria produce biogenic amines at temperatures below 5 °C (Emborg & Dalgaard, 2006; Emborg et al., 2005). In addition, in some societies, refrigeration is not readily available. In such circumstances, emerging methods of control need to be considered however, little work has been done on these.

Emerging methods as control measures include the addition of starter cultures that degrade histamine, the application of hydrostatic pressures, irradiation, packaging, using food additives and preservatives, and altering conditions based on microbial modelling of histamine producing bacteria. The majority of these methods are not new in terms of food preservation, but are not commonly used in controlling biogenic amines. The use of enzymes, such as diamine oxidase (DAO) that degrade biogenic amines, and the use of bacteria that posses this enzyme, are the only potential tools to degrade already formed biogenic amines and are not currently recognized preservation methods.

The formation of biogenic amines is associated with food spoilage, suggests poor hygienic practices and may therefore indicate other food safety issues. Any attempts to control biogenic amines must take into account the factors leading to the formation of the biogenic amine and ensure other food safety issues are not being overlooked. Products where a secondary control approach is justified are those that are microbiologically stable. An example is the fish paste product from the Maldives, *Rihaakuru*, with a maximum water activity of 0.8. Temperature abused fish that has been rejected from fish factories, is used as the raw ingredient for *Rihaakuru* – a product made through prolonged cooking, that once produced, is stable at ambient temperature (25 to 30 °C) for over a year. Although *Rihaakuru* has nutritional benefits, rich in protein and omega 3 sources, a health concern is potential for scombroid poisoning due to high biogenic amines contents. Maintaining the cold chain is not a practical solution due to the cost of refrigeration being out of reach for the artisan fishers. One option to ensure the safety of products like *Rihaakuru* is to destroy the biogenic amines in the product but this has not been investigated. Most approaches to control histamine in a food like *Rihaakuru* focus on delaying biogenic amine formation (Emborg & Dalgaard, 2008a; Fletcher et al., 1999; Joosten & Nunez, 1996). Methods to destroy biogenic amines, particularly histamine have not been seriously considered as the sensory quality of foods with high biogenic amines is often unacceptable and biogenic amines are actually used as a freshness indicator in many foods (Pons-Sanchez-Cascado et al., 2006). However, with *Rihaakuru*, the final product is microbiologically stable and biogenic amines formed in the fish before processing do not appear to be associated with sensory defects in the final product. The concern with histamine in *Rihaakuru* is food safety and prevention through normal handling through refrigeration of raw fish is impractical. This review also examines options for to the destruction of histamine in microbiologically stable foods such as *Rihaakuru*.

2.5 Methods for delaying biogenic amines accumulation

2.5.1 Application of food additives and preservatives

Additives and preservatives can reduce the formation of biogenic amines (Table 1) in products such as mackerel by inhibiting bacterial growth and amine formation (Kang & Park, 1984). Sodium sorbate may limit the formation of biogenic amines and sodium hexametaphosphate at 2% has been shown to delay histamine production (Kang & Park, 1984; Shalaby, 1996; Shalaby & Rahman, 1995). Citric acid, succinic acid, D-sorbitol and malic acid inhibited decarboxylase activity and the resulting histamine formation in mackerel stored for 10 days at 25 °C (Shalaby, 1996). Citric acid use (1%) during pickled cabbage fermentation produced a slight decrease in biogenic amines at a salt level of 6, 8, or 10% (Yuecel & Ueren, 2008).

Potassium sorbate has also been found to extend the shelf life of seafood (Shalini et al., 2001). Sausage containing potassium sorbate, and ascorbic acid showed a significant reduction in biogenic amine accumulation (Bozkurt & Erkmen, 2004). Sodium nitrites (45 to 195 ppm) in sausage decreased biogenic amine production, (Kurt & Zorba, 2009). This confirms the findings of Bozkurt and Erkmen (2004) that sodium nitrite and sodium nitrate inhibit biogenic amine production. The addition of 0 to 1% glucono-delta-lactone (GDL) into meat decreased histamine and putrescine production through a pH drop in meat (Maijala et al., 1993). The addition of sugar may also slightly reduce biogenic amine formation(Bover-Cid et al., 2001a). When glycine was applied to Myeolchi-jeot, (a salted and fermented anchovy product) the overall production of biogenic amines was reduced by 63 to 73%. The authors concluded that glycine inhibits the amine forming activity of microorganisms. Biogenic amines in other fermented fish products may be reduced using glycine as a food additive (Mah & Hwang, 2009a).

Naturally occurring specific inhibitory substances in spices and additives have also been shown to inhibit biogenic amine formation (Komprda et al., 2004). Such substances include curcumin (turmeric), capsaicin (red pepper), and piperine (black pepper) (Shakila et al., 1995; Wendakoon & Sakaguchi, 1992). The disadvantage of these substances is the considerable loss in efficacy that occurs during cooking (Suresh et al., 2007). Among these substances, capsaicin was found more heat stable than curcumin and piperine (Srinivasan et al., 1992). However, capsaicin is a pungent component, and excites primary sensory neurons (Someya et al., 2003). The most active component of turmeric is curcumin, an analogue of 6-gingerol. The turmeric yellow colour appearance is due to curcuminoids. Curcumin has been used as a food additive, spice and as a medicinal herb (Bhutani et al., 2009). Curcumin levels of 8 g/day may be tolerable with approximate consumption being 0.1 g/day. It is a potent antioxidant 10 times more powerful than vitamin E (Shishodia et al., 2005).

Table 1: Biogenic amines reduction through food preservatives

Food type	Additives applied	Storage condition	storage time	Biogenic amines reduced	Reference
Meat	Glucono-delta-lactone (GDL) 0%, 0.5%, 1.0%	20-22 °C	7 days	Significant reduction of histamine (dropped from 126 ppm to 7 ppm at 1.0% GDL) and putrescine (dropped from 236 ppm to 147 ppm)	(Maijala et al., 1993)
Indian mackerel (whole)	10% (weight of fish) Curcumin (turmeric), capsaicin (red pepper), piperine (black pepper)	5°C	8 days	All spices reduced biogenic amines [histamine (dropped from >200ppm to 13ppm), cadaverine (approximately dropped from 200ppm to 100ppm) putrescine(approximately dropped from 100 ppm to25 ppm) , and tyramine (approximately from 200ppm to <100ppm)). Turmeric, pepper and cardamom treatment more effective.	(Shakila et al., 1995)
Slightly fermented sausages	Sugar (glucose, lactose) between 4000-20000ppm	4°C and 19°C	20 days	Cadaverine Tyramine	(Bover-Cid et al., 2001a)

Food type	Additives applied	Storage condition	storage time	Biogenic amines reduced	Reference
Fermented sausage (Sucuk)	Potassium pyrophosphate (2500 ppm), di-potassium hydrogen phosphate (2500 ppm), ascorbic acid (500 ppm), alpha-tocopherol (200 ppm), potassium sorbate (200 ppm)	Temperature: 20°C, 30C and 40°C % relative humidity: 50, 65 and 80	60 days	Amines reduced were histamine (dropped from 242 to 35 ppm at 80% relative humidity and at 30°C), putrescine (dropped from 378 ppm to 12 ppm at 65% relative humidity and at 40°C), tryptamine (dropped from 60 ppm to 14 ppm at 50% relative humidity and at 20°C)	(Bozkurt & Erkmen, 2004)
Myeolchi-Jeot (fermented anchovies)	5% garlic extract (dissolved in ethanol)	25°C	10 weeks	Histamine and tyramine reduced by 20.8% and 31.2% respectively. Overall amines reduced by 8.7% compared to control	(Mah et al., 2009)
Myeolchi-Jeot (fermented anchovies)	5% glycine (weight basis), sodium chloride (20%)	25°C	10 weeks	Overall biogenic amines (putrescine, cadaverine, histamine, tyramine, spermidine) reduced between 63% and 73% compared to control	(Mah et al., 2009)

Components of spices, such as thymol may inhibit biogenic amine formation (Singh et al., 1999). Thymol is a phenolic monoterpene, naturally found in essential oils, that has antioxidant and antimicrobial properties. It is a major component of thyme and oregano (Lee et al., 2008). However thymol, having unpleasant pungent flavour, may not be accepted by consumers as an ingredient for food formulation (Lee et al., 2008).

Ginger, garlic, green onion, red pepper, clove and cinnamon, have been shown to delay biogenic amine production in Myeolchi-jeot (Mah et al., 2009). The addition of 5% garlic during Myeolchi-jeot ripening reduced the biogenic amine level by 8.7%. Garlic is one of the most popular herbs in the world used as a flavouring agent in food. Allicin is the most active ingredient in garlic, formed from allin by the enzyme alliinase when the garlic clove is crushed (Batra & Rajeev, 2007). Ginger, lowers blood pressure, and may cure hypertension and palpitations (Ghayur et al., 2005) and it possess antibacterial and antifungal activity (Chrubasik et al., 2005). The 6-gingerol, pungent constituent of ginger (Young et al., 2005), is known to enhance gastrointestinal transport (Batra & Rajeev, 2007). The 6-gingerol also been shown to have some inhibitory effect on biogenic amine formation (Singh et al., 1999).

The effect of spices has been measured on specific bacteria that produce biogenic amines. Ethanol extracts of allspice, sage, cloves, cinnamon and nutmeg were found to delay biogenic amine formation by *Enterobacter aerogenes*. The inhibitory effect was improved with the addition of sodium chloride. Cinnamic aldehyde, a component of cinnamon, and eugenol, a compound of cloves were found to be the most effective inhibitors of biogenic amine formation by specific bacteria, *Enterobacter aerogenes*(Wendakoon & Sakaguchi, 1995). Histamine formation by *M. morganii* was delayed in the presence of 0.5% potassium sorbate (Shalaby, 1996) and by the essential oil of lemongrass (Sangcharoen et al., 2009). Histamine formation in *Klebsiella pneumonia* was delayed by sorbate at 0.5% (Shalaby, 1996). *Bacillus licheniformis,* an isolate from Myeolchi-jeot, is a strong biogenic amine former. Glycine (10%) was shown to reduce the histamine, cadaverine and putrescine of *B. licheniformis*by 93, 78 and 32% respectively and reduce tyramine and spermidine production by 100% (Mah & Hwang, 2009a).

Although studies have shown the inhibitory effects of food additives and preservatives on biogenic amine accumulation, few authors have highlighted their potential negative effects. For example, the presence of preservatives has been reported to increase biogenic amine formation during sausage production (Komprda et al., 2004). Recently it was found that curcumin inhibits diamine oxidase (Bhutani et al., 2009), which may inhibit biogenic amine reduction. When sodium sorbate and sodium hexametaphosphate were applied to sardines a putrefactive odour was observed within 2 days at chill storage (Kang & Park, 1984). Other disadvantages of preservatives use is a lack of available knowledge on their effectiveness against biogenic amines in foods and the lack of consumer acceptance (Bjornsdottir, 2009).

In summary, food additives and preservatives that work well in food require further investigation into the effectiveness in delaying biogenic amine production. Food additives that have shown a positive effect on delaying biogenic amine formation need to be tested in variety of food systems.

2.5.2 High hydrostatic pressure

HHP is a non-thermal preservation method that damages cell membranes of micro-organisms resulting in inactivation or sub-lethal injury (De Las Rivas et al., 2008). Through inactivation of micro-organisms, HHP extends shelf life while retaining the original flavour and characteristics of food (Patterson, 2005). HHP treated foods are commercially available in the US (for example, guacamole, oysters), Japan (for example, fruit jam), and Spain (for example, cooked and vacuum packed ham) (Patterson, 2005). HHP has been applied to many other foods including cheese (Novella-Rodriguez et al., 2002), sausage (Latorre-Moratalla et al., 2007; Ruiz-Capillas et al., 2007), fish (Bolton et al., 2009), and sauerkraut (Peñas et al., 2010).

When HHP is applied to raw material or the end products of fermentation, a reduction in the number of bacteria may inhibit biogenic amine formation (Table 2). For example, when HHP (200 MPa) was applied to meat batter raw material for sausage fermentation it inhibited the growth of *Enterobacteria* and simultaneously delayed the accumulation of putrescine and cadaverine (Latorre-Moratalla et al., 2007). Inhibition of biogenic amine formation depends on the level of pressure applied. For instance, during cheese ripening, a low pressure treatment of 50 MPa for 72 h increased biogenic amine content while a high pressure treatment of 400 MPa for 5 min plus 50 MPa for 72 h showed a slight decrease (Novella-Rodriguez et al., 2002).

Treating fermented sausage with high pressure (350 MPa/15 min) reduced lactic acid bacteria (20.1%) and reduced cadaverine (12.5%), putrescine (8.7%) and tyramine (17%) levels during 160 days chilled storage compared to sausage not treated with HHP (Ruiz-Capillas et al., 2007). Histamine forming bacteria and histidine decarboxylase activity in yellowfin tuna and mahi-mahi fish can be reduced by applying HHP between 300 - 400 MPa without affecting the quality of the fish (Bolton et al., 2009).

HHP (300 MPa at 40 °C for 10 min) applied during Saukraut fermentation, extended the shelf life through microbial reduction (Peñas et al., 2010). Although this study did not analyze biogenic amines, the product is known to contain biogenic amines (Shalaby, 1996).

Overall there is limited information on the efficacy of HHP treatment on the control of biogenic amines through the treatment of raw materials (Latorre-Moratalla et al., 2007) with evidence of both increased and decreased biogenic amine formation (Latorre-Moratalla et al., 2007; Novella-Rodriguez et al., 2002; Ruiz-Capillas et al., 2007). It is possible that HHP affects the enzymes as well as the bacteria that cause biogenic amine formation although this aspect has not been studied.

2.5.3 Irradiation

Irradiation to extend the shelf life of food was introduced in the 1950’s (Mbarki et al., 2009). Irradiation has been used in the food industry to prolong shelf life and ensure safety of foods, reducing the use of chemical preservatives (Ahn et al., 2002a; Ahn et al., 2002b; Loaharanu, 1989; Radomyski et al., 1994; Thayer, 1994).

Irradiation may control biogenic amine formation in foods (Table 3), by radiolysis of biogenic amines (Mbarki et al., 2009) and by reducing the number of bacteria responsible for biogenic amine production (Kim et al., 2003). Radiolytic degradation of biogenic amines was demonstrated in a model system. Histamine, cadaverine, putrescine, spermidine, spermine, tryptamine, tyramine and agmatine standards were irradiated at 2.5, 5, 10, 20 and 25 kGy after being dissolved in distilled water at concentrations of 100 ppm. The degradation observed was between 5 to 100%, overall showing 95% degradation of all amines at 20 kGy. Significant degradation of spermine, spermidine and putrescine occurred above 5 kGy (J. H. Kim et al., 2004).

Table 2: Biogenic amines reduction through high hydrostatic pressure

Food type	**HHP applied**	**Storage condition**	**Storage time**	**Biogenic amines reduced**	**Reference**
Goat cheese ripening	400 MPa for 5 min and 50 MPa for 72 h at 14 °C	Ripened at 14 °C and 86% RH	28 days	Tyramine dropped from 10.3 ppm to 1.6 ppm	(Novella-Rodriguez et al., 2002)
meat batter, raw material for sausage fermentation	200 MPa at 17 °C for 10 min	12C, RH>95% for 10 days, RH 80% till end of ripening.	21 days	Putrescine, and cadaverine level decreased (88% and 98% reduction compared to control)	(Latorre-Moratalla et al., 2007)
Dry-cured sausage (Chorizo)	350 MPa for 15 min at 20 °C	2 °C	160 days	Decreased in tyramine (17%), putrescine (8.7%) and cadaverine (12.5%)	(Ruiz-Capillas et al., 2007)
Yellowfin tuna and mahi-mahi	300-400 MPa for 5 min	4.4 °C	12 days	Reduce histamine producing bacteria (Morganella morganii) and its histidine decarboxylase activity	(Bolton et al., 2009)

Table 3: Biogenic amines reduction through irradiation

Food type	Irradiation condition	Storage condition	Storage time	Biogenic amines reduced	Reference
Distilled water containing 100 ppm of biogenic amines	Applied doses: 0, 2.5, 5, 10, 15, 20, 25 kGy (best reduced at 25) Source strength: 100 kCi dose rate: 5 kGy/h at 12 °C	-	-	At 20 kGy putrescine, spermidine, phenylethylamine, spermine, and histamine were completely destroyed. At 25 kGy the remaining amines; cadaverine, tryptamine, tyramine and agmatine were completely destroyed.	(J. H. Kim et al., 2004)
Pepperoni sausage (fermented)	Applied doses: 0, 5, 10, 20 kGy (best reduced at 20) Source strength: 100 kCi 5 dose rate: kGy/h at 12 °C	air packaged and stored at 4 °C	4 weeks	Decreased amines at 20 kGy: putrescine (from 2.6 ppm to complete destruction), tyramine (dropped from 0.9 ppm to 0.2 ppm), spermine (dropped from 9.6 ppm to 4.2 ppm) and spermidine (dropped from 11.8 ppm to 8.4 ppm)	(Kim et al., 2005a)
Low-salt fermented Soybean paste (with 6% and 8% salt)	Applied doses: 5, 10, 15 kGy (best reduced at 15) Source strength: 100 kCi Dose rate: 5 kGy/h at 13 °C	25 °C	12 weeks	Putrescine (dropped from 3124ppm to 797.3 ppm at 8% salt and 15 kGy),	(Kim et al., 2005b)

Food type	Irradiation condition	Storage condition	Storage time	Biogenic amines reduced	Reference
Beef and pork	Applied doses: 0, 0.5, 1, 2 kGy (best reduced at 2) Source strength: 100 kCi dose rate: 83.3 Gy/min at 12 °C	4 °C	20 h	Decreased amines at 2 Gy: putrescine (dropped from 4.7 ppm to 2 ppm in beef, and 2.3 ppm to 0.3 ppm in pork), tyramine,(dropped from 24.7 ppm to 9.3 ppm in beef, and 1.3 ppm to 0.8 ppm in pork) and spermine (dropped from 28.4 ppm to 22.4 ppm in beef, and 31.3 ppm to 25.9 ppm in pork)	(J. S. Min et al., 2007)
Vacuum packed Chub mackerel (*Scomber japonicus*)	Applied dose: 1.5 kGy	At 1 °C with air circulation	14 days	Significant reduction of histamine (dropped from 50.91 ppm to 2.87 ppm)	(Mbarki et al., 2009)

However, as the authors have noted, the study is based only on a model system, the application to a food system requires further investigation. The high dosage use may affect the sensory quality of the food. Irradiation at 10 kGy is considered safe to apply to any food product (WHO, 1994), but levels higher than this require studies on the sensory characteristics and safety of treated food. Shelf life extension of food products treated with irradiation has been applied to many foods including pork and beef (J. S. Min et al., 2007), sausage (Kim et al., 2005a), soybean paste (Kim et al., 2003; Kim et al., 2005b), chicken (J. S. Min et al., 2007)and fish (Mbarki et al., 2009; Schirmer et al., 2009).

Ground pork and beef inoculated with *Alcaligenesfaecalis, Bacillus cereus* and *Enterobacter cloacae* were treated with gamma irradiation doses of 2 kGy. The total amount of biogenic amines (histamine, tyramine, spermidine, beta-phenylethylamine, tryptamine, cadaverine, and putrescine) formed during 24 h storage at 4 °C was reduced by the treatment (J. S. Min et al., 2007). Levels of tyramine, spermidine, spermine and putrescine were effectively reduced in pepperoni sausage by gamma- irradiation (5, 10, 20 kGy) (Kim et al., 2005a). Gamma irradiation at 5, 10 or 15 kGy reduced putrescine, cadaverine, agmatine, histamine, tryptamine, spermine and spermidine during fermentation of low-salt fermented soy paste (Kim et al., 2005b). Irradiation followed by vacuum packing of chub mackerel (*Scomber japonicus*) in chilled storage slowed the formation of biogenic amines (Mbarki et al., 2009).

While irradiation delays the formation of some biogenic amines, there are reports of irradiation enhancing the formation of other biogenic amines (Kim et al., 2003; Wei et al., 2009). Irradiation of Korean fermented soybean paste did not produce a significant difference in biogenic amine content compared with the control, although the concentration of histamine, tyramine, spermidine and putrescine decreased, during fermentation. Possible explanations for the latter include a reduction of micro-organisms by irradiation, or some of the pre formed biogenic amines may have been utilized as substrates by microbes during fermentation (Kim et al., 2003; Nout, 1994). Biogenic amines in raw chicken breast and thigh meat were reduced using irradiation at a dose of 2 kGy, even though some of the biogenic amines (histamine, spermidine and spermine) were increased, perhaps because irradiation changes the structure and physiological properties of enzymes which form biogenic amines (J. S. Min et al., 2007). Prior to ripening, Chinese Rugoa ham was irradiated with a dose of 5 kGy, producing a degradation of spermine, putrescine and tyramine, but formation of tryptamine, spermidine, phenylethylamine and cadaverine increased compared to controls after irradiation. The increase of the latter may be due to the ham being ripened after irradiation and the growth of decarboxylating micro-organisms during the ripening process (Wei et al., 2009).

There is some consumer resistance to the use of irradiation and this includes taste problems (Mbarki et al., 2009). The biogenic amine reduction in foods seems to be more effective at high doses of irradiation (Mbarki et al., 2008). However, high doses are most likely to result in what has been described as ‘irradiation taste’ (Schirmer et al., 2009). It may also be possible that irradiation also inhibits the decarboxylase enzyme activity, however, this requires investigation. Radiolytic products of biogenic amines in irradiated food and their biological effect need to be studied (J. H. Kim et al., 2004). In summary, irradiation has potential use in delaying biogenic amine accumulation, but the method requires further study.

2.5.4 Packaging

Preservation through packaging usually involves changing the gaseous mixture of the environment surrounding the product. This may delay the production of biogenic amines, due to inhibition of the micro-organisms or the enzymes producing biogenic amines. The histidine decarboxylase enzyme was reported to be more effective in the absence of oxygen while histaminases (such as DAO), the enzyme that oxidizes histamine, was found effective, only in the presence of oxygen (Kapeller-Adler, 1941). However, both anaerobic and aerobic bacteria are capable of producing biogenic amines, and as well as degrading biogenic amines so finding a balance that will control microbial growth and enzyme activity may be difficult.

There are reports on the successful control of biogenic amines through packaging (Table 4). These include vacuum packaging of salmon (Mbarki et al., 2009), MAP of fish (Dalgaard et al., 2006; Emborg et al., 2005), chicken (Balamatsia et al., 2006; Patsias et al., 2006), sausage (Kim et al., 2005a) and active packaging of seer fish (Mohan et al., 2009).

In active packaging, different gas scavengers are used (oxygen, carbon dioxide) to control the environment within the pack. Oxygen scavengers eliminate oxygen in the head space and product < 0.01% (Mohan et al., 2009). Mohan, et al. (2009)found that the presence of oxygen/air increased biogenic amine production in Seer fish (*Scomberomorus commerson*) steaks and by removing oxygen (99%) with oxygen scavengers (active packaging), biogenic amines in the fish were lower and shelf life was extended from 12 days (air) to 20 days. Biogenic amine producers were apparently from aerobic bacteria that possess the decarboxylase activity, thus removal of oxygen inhibited aerobic bacteria and delayed biogenic amine accumulation.

Vacuum packaging extends the shelf life of food compared to air packaging (González-Montalvo et al., 2007). Recently a novel packaging method was developed (Schirmer et al., 2009) that involves combining organic acids with CO_2 from the headspace dissolving into the product until a vacuum is formed (‘CO_2-vacuum packed’ products). This was used on salmon as an effective method to inhibit microbial growth and extend shelf life. Microbes that were reduced included *Photobacterium phosphorium* which has been reported as an active histamine former able to grow under normal MAP conditions producing more than 1000 ppm histamine (Dalgaard et al., 2006; Emborg et al., 2005; Kanki et al., 2004; Tao et al., 2009). MAP extends the shelf life of food longer than vacuum packing (Özogul et al., 2004). Histamine content in vacuum packed tuna was > 7000 ppm, and the bacteria responsible for were suspected to be either *P. phosphoreum* or *M. morganii-Morganella psychrotolerans.* Histamine production was controlled when MAP with a gas mix of 40% CO_2 / 60% O_2 was applied to tuna stored for 28 days at 1.0 °C (Emborg et al., 2005). This method may have controlled histamine formation by the inhibition of the growth of the psychrotrophic histamine producing bacteria *P. phosphoreum* and *M. morganii-M. psychrotolerans*. Therefore, it was suggested to use MAP with the above gas mixture for lean fish, such as tuna loins, to avoid possible scombroid poisoning.

Table 4: Biogenic amines reduction through packaging

Food type	**Irradiation condition**	**Storage condition**	**Storage time**	**Biogenic amines reduced**	**Reference**
Yellowfin tuna (*Thunnus albacares*)	MAP (40% CO_2 / 60% O_2)	1 °C	28 days	no histamine formed/strong inhibitory effect to histamine production and growth of *Morganella morganii* and *Photobacterium phosphorium*	(Emborg et al., 2005)
Garfish	MAP (40% CO_2 and 60% N_2)	0 and 5 °C	38 days	Reduced histamine formation in thawed MAP garfish	(Dalgaard et al., 2006)
Breast chicken meat	MAP (30% CO_2, 70% N_2)	4 °C	17 days	Slight decrease in cadaverine (223.7 ppm in MAP and 252.7 ppm in air packaging) and putrescine (354 ppm in MAP and 409.6 ppm in air packaging) compared to air packaging	(Balamatsia et al., 2006)
Precooked chicken meat	MAP (30% CO_2, 70% N_2)	4 °C	23 days	Reduced putrescine (90.4 ppm under MAP at 23rd day, 202.6 ppm at 23rd day under air) and tyramine (8.8 ppm under MAP at 23rd day, 18.8 ppm at 23rd day under air)	(Patsias et al., 2006)
Chub mackerel (*Scomber japonicus*)	Vacuum packaged	1°C	7 d	Slight reduction (on 7th d of storage), of biogenic amines; histamine (dropped from 57.22 to 47.66 ppm), cadaverine (dropped from 18.93 to 10.07 ppm), spermidine (dropped from 10.29 to 6.94 ppm), putrescine (dropped from 21.13 to 13.52 ppm)	(Mbarki et al., 2009)

Food type	Irradiation condition	Storage condition	Storage time	Biogenic amines reduced	Reference
Seer fish (*Scomberomorus commerson*)	Packed in pouches (a multilayer film of ethylene-vinyl alcohol) with O_2 scavenger sachets	0 to 2°C	30 d	Delayed formation of putrescine (on 15^{th} d in air pack contained 14.62 ppm and on 30^{th} d fish held in O_2 scavenger pack contained 11.1 ppm); cadaverine (on 15^{th} d in air pack contained 14.77 ppm and 2.16 ppm in O_2 scavenger pack); histamine (air pack reached 6.9 ppm on the 15^{th} d storage while the same level reached on the 30^{th} d in O_2 scavenger pack); tyramine (1.7 ppm on 15^{th} day in air pack and 0.07 ppm on 30^{th} d in O_2 pack).	(Mohan et al., 2009)

Dalgaard, et al. (2006), demonstrated the synergistic effect of MAP with a gas mixture of 40% CO_2/60% N_2 and freezing and thawing to control histamine production in chilled garfish by *P. phosphoreum* which had produced histamine > 1000 ppm at chilled storage under air and MAP. When the garfish was frozen, thawed and stored at 5 °C, the shelf life was 70% longer under the MAP gas mix and histamine production was reduced compared with storage in air. The authors considered that this was because the *P. phosphoreum* responsible for histamine production was inactivated by freezing and thawing (Dalgaard et al., 2006). MAP cod fillets with 500 ppm Na_2CaEDTA (antimicrobial) reduced *P. phosphoreum* by 40% and extended shelf life by 40% at 0 °C (Dalgaard et al., 1998). Since *P. phosphoreum* is able to form > 1000 ppm histamine below 5 °C (Dalgaard et al., 2006), this method may reduce histamine content in cod through the inhibition of the bacteria.

Chicken breast meat stored under MAP (30% CO_2 / 70% N_2) was evaluated for shelf life up to 17 days at 4 °C (Balamatsia et al., 2006). On the 17th day of storage, the histamine level only reached 26.8 ppm, the delay may be due to specific types of histamine producing bacteria, such as *Enterobacteriaceae* having difficulty growing under MAP and when the total bacterial level reached 10^7 cfu/g on the 11th day of storage, the histamine was detected (5.4 ppm). However, cadaverine and putrescine in chicken under MAP was only slightly less (223.7 ± 12.0 ppm, and 354.0 ± 17.2 ppm, respectively) than chicken breast meat in aerobic packaging (252.7 ± 12.8 ppm, and 409.6 ± 18.4 ppm respectively). Cadaverine and putrescine may be reduced under MAP, due to lactic acid bacteria, which may either utilize these amines as substrates or may have oxidizing enzymes that degraded these amines or delayed the accumulation. Some lactic acid bacteria have been shown to degrade biogenic amines (Dapkevicius et al., 2000).

Patsias et al. (2006) studied precooked chicken meat under air and MAP (30% CO_2 / 70% N_2) at 4 °C for up to 23 days. When the biogenic amines levels were compared after 23 days of storage under MAP, putrescine and tyramine were reduced compared with packaging under air.

J. H. Kim, Ahn, et al. (2005a) found the use of MAP with a gas mixture of 25% CO_2 / 75% N_2 gas did not reduce the production of biogenic amines in pepperoni. Other packaging types, air and vacuum were found more effective than MAP, although in general, each packaging type had a different affect on individual amines. The effect of MAP on the suppression of biogenic amine formation in pepporini, requires more studies using different gas mixtures such as those used by Dalgaard, et al. (2006) on fish and Patsias, et al. (2006) on chicken.

In summary, compared to air packaging, active packaging, vacuum packaging and MAP, inhibit or delay formation of biogenic amines more effectively, through inhibition of biogenic amines forming bacteria or enzyme activity, but the success of inhibition largely depends on the type of microflora, and its environmental conditions such as temperature, and also the gas mix used in case of MAP. It may also be product specific.

2.5.5 Microbial modelling

Microbial modelling can be used to study the growth and inactivation of micro-organisms (Van Boekel, 2002; Xiong et al., 1999; Zwietering et al., 1990) with the aim of controlling growth and predicting risk factors (Neumeyer et al., 1997; Ross, 1996; Seo et al., 2007). Modelling micro-organisms responsible for biogenic amine formation (Emborg & Dalgaard, 2008a, 2008b; Gardini et al., 2008) has been used to explore options for biogenic amine control.

Temperature, time and pH affect biogenic amine production and these could be modelled for particular microbial species in specific foods. Such models may help design conditions to limit amine production. However, the drawback of this method is that there are many known bacterial species capable of producing biogenic amines already known and probably others yet to be found therefore generic modelling to account for all these species would be complex, time consuming and tedious. Currently available models for biogenic amine producing bacteria include those of Emborg and Dalgaard (2008a) on *M. psychrotolerans* and *M. morganii* in fish, and Gardini, et al. (2008) for *Enterococcus faecalis* EF37in sausage.

Emborg and Dalgaard (2008a) developed a mathematical model for the histamine forming bacteria, *M. psychrotolerans* and *M. morganii* and identified the conditions to inhibit the growth of these bacteria through heat in canned tuna meat, thawed garfish meat, tuna juice and broth. The mathematical equations of the model (equations 1 to 3) have subsequently been incorporated into freely available software (Dalgaard, 2009). The main parameter was the effect of temperature on the growth and inactivation of *M. psychrotolerans* and *M. morganii*.

$$\mathrm{Log}\,N_t = \mathrm{Log}\,N_0 \qquad t < t_{\mathrm{lag}}$$

$$\mathrm{Log\,N_t} = \mathrm{Log}\left(\mathrm{N_{max}}/\left(1+\left(\left(\frac{\mathrm{N_{max}}}{\mathrm{N_0}}\right)^{\mathrm{m}}-1\right)\exp\left(-\mu_{\mathrm{max}}.\,\mathrm{m}\left(\mathrm{t}-\mathrm{t_{lag}}\right)\right)\right).1/\mathrm{m}\right) \qquad t \geq t_{lag} \qquad (1)$$

$$\mathrm{His_t} = \mathrm{His_0} + \mathrm{Y_{His/CFU}}.\,(\mathrm{N_t}-\mathrm{N_0}).\,1000 \qquad (2)$$

$$\sqrt{\mu_{\mathrm{max}}} = \mathrm{b.}\,(\mathrm{T}-\mathrm{T_{min}}).\left(1-\exp\left(\mathrm{c.}\,(\mathrm{T}-\mathrm{T_{max}})\right)\right) \qquad (3)$$

Where, μ_{max} = maximum specific growth rate, N_{max} = the maximum cell density, $Y_{His / CFU}$= yield factor for histamine formation, N_0 = actual initial concentration, t_{lag} = 2.55 ln(2)/ μ_{max} = lag time, N_t = cell concentration at time t, m = parameter to characterize growth dampening when the cell concentration N_t approaches the maximum cell concentration(N_{max}), His_t and His_0 = concentration of histamine (ppm) at time t and 0 and N_t and N_0(cfu/g or cfu/ml) = corresponding cell concentration, b and c = constants, T = temperature, and T_{min} and T_{max}= the theoretical minimum and maximum temperatures, respectively (Emborg & Dalgaard, 2008a p. 236).

The model predicted the time for histamine to reach 100 ppm was longer than the observed value and to reach 500 and 2000 ppm was shorter than the observed value. Therefore, the model could be used to help control the formation of histamine in food to < 500 ppm, but not down to 100 ppm (Emborg & Dalgaard, 2008a). Emborg and Dalgaard (2008b) also modelled the growth of the histamine forming bacteria, *M. psychrotolerans* examining the effects of pH, water activity, temperature and carbon dioxide. The model detailed histamine formation and the growth of bacteria under different storage conditions and different product characteristics (sodium chloride [NaCl], water activity, pH) of tuna meat. The parameters included in the model were temperature (0 – 20 ºC), atmosphere (0 - 100% CO_2), pH (5.4 - 6.5) and NaCl (0.0 -

6.0%). This model provided a slightly conservative (fail - safe) prediction for the time when the histamine concentration is toxic, between 500 and 2000 ppm, even though the predictions were not very accurate. The kinetic approach used in this model maybe used to develop models for other histamine forming bacteria in different storage conditions and with different fish (Emborg & Dalgaard, 2008b).

Gardini, et al. (2008) modelled *E. faecalis EF37* for biogenic amine formation in dry fermented sausages. Fermentation was carried out for 30 days, and samples were analyzed on days 3, 5, 19 and 30. The parameters included were glucose (0, 700, 1400 ppm), temperature (15, 20, 25 °C), and NaCl (0, 2.5, 5%). The Enterococcal count reached 10^5 cfu/g after 30 days fermentation in all the conditions. Sodium chloride at 5% reduced tyramine to a negligible amounts (< 1 ppm) while the tyramine level with 0% NaCl was > 200 ppm. The authors found that the most important variable in preventing tyramine formation was the salt content. Temperature and glucose had negligible effects on tyramine accumulation. The level of tyrosine decarboxylase (tdc) decreased when sodium chloride levels were > 2%. The tdc activity increased with an increase in temperature (20 – 25 °C) (Gardini et al., 2008).

In summary mathematical modelling has been used on biogenic amine forming bacteria: *M. morgani*, *M. psychotolerans* and *E. faecalis*. Parameters studied included, water activity, temperature, salt content, pH, glucose and carbon dioxide. For *E. faecalis* growth and activity in sausage, > 2% salt content decreased biogenic amines. A correlation was found between the tdc level and tyramine formation: that is as tdc increased the tyramine level also increased during the fermentation of sausage. Since limited modelling on biogenic amine forming bacteria has been reported, there is an opportunity to develop new models or improve current models through further studies.

2.5.6 Starter cultures

Starter cultures used in fermentation can also delay the formation of biogenic amines (Bover-Cid et al., 2001b; Bover-Cid et al., 2001c; Latorre-Moratalla et al., 2007; Mah & Hwang, 2009b; Spicka et al., 2002). Starters used for fermented foods are either amine-negative (not able to decarboxylate amino acid into biogenic amines) or amine oxidizing (oxidize biogenic amines into aldehyde, hydroden peroxide and ammonia) bacteria

(Bover-Cid et al., 2000a; Suzzi & Gardini, 2003). These bacteria require optimal growth conditions to dominate over biogenic amine producing (Xu et al., 2010) and other contaminant bacteria (Hu et al., 2008; Maijala et al., 1995a; Maijala et al., 1995b). Typical fermented foods where the effect of starters on biogenic amines have been studied include sausages (Bover-Cid et al., 2000a; Bover-Cid et al., 2000b; Latorre-Moratalla et al., 2007), cabbage (Spicka et al., 2002), cheese (Fernández-García et al., 2000; Nieto-Arribas et al., 2009), wine (Hernández-Orte et al., 2008) and vegetables (Tamang et al., 2009).

A number of bacteria have been found to have negative decarboxylase activity or possess enzymes that oxidize biogenic amines in food (amine-negative bacteria) Artisanal Manchego cheese isolates, of *Lactobacillus plantarum* and *Lactobacillus paracasei subsp. paracasei* were found to be amine-negative bacteria except for one isolate from the latter, found producing tyramine. These amine-negative organisms were suggested as potential starters for cheese production (Nieto-Arribas et al., 2009). Amine-negative starters, *Staphylococcus xylosus* and *Lactobacillus carvatus*delay putrescine and cadaverine formation during the ripening and storage of dry fermented sausages (Bover-Cid et al., 2001b). The inoculation of amine-negative mixed starters, *Pediococcus acidilactici*, *Staphylococcus carnosus*, *Lactobacillus sake*, *S. xylosus* into cold smoked fish, can help control biogenic amines (Petäjä et al., 2000). Amine-negative mixed starters of *Staphylococcus carnosus*, *Lactobacillus sakei*, and *S. xylosus* have also been used during the fermentation of dry sausage and were found to suppress biogenic amine accumulation (Bover-Cid et al., 2001b). Mixed starters of *L. plantarum*, *Pediococcus pentosaceus*, *S. xylosus*, *Lactobacillus casei* inhibited formation of biogenic amines, and suppressed the contaminant micro-organisms in silver carp sausages (Hu et al., 2007).

Mixed starters produce a synergistic effect in the control of biogenic amines (Hu et al., 2007). The use of mixed starters results in a large pH decrease (Hu et al., 2007) which may be an additional factor contributing to reducing biogenic amine accumulation.

Effective control of biogenic amines may require a combination of several factors (Latorre-Moratalla et al., 2010). For example, the control of biogenic amines with starters is likely to be most effective with good quality raw material (Bover-Cid et al., 2000b; Hu et al., 2007; Petäjä et al., 2000).

2.6 Methods for oxidizing/degrading formed biogenic amines

Even though many methods are available, as described above, for delaying biogenic amine accumulation, few methods are available for degrading biogenic amines. Such methods include the use of oxidizing micro-organisms, such as biogenic amine oxidizing bacteria, and enzymes such as DAO. Biogenic amine degrading bacteria could be introduced into a food processing step to degrade the biogenic amines in the food, or the bacteria could be used as a starter for fermented foods. Bacteria described as biogenic amine oxidizers include *Micrococcus varians*(Leuschner & Hammes, 1998b), *Natrinema gari*(Tapingkae et al., 2010b)*Brevibacterium linen*(Leuschner & Hammes, 1998a), *Vergibacillus sp* SK33 (Yongsawatdigul et al., 2007), *Lactobacillus sakei*, *Lactobacillus curvatus*(Dapkevicius et al., 2000), and *S. xylosus*(Mah & Hwang, 2009b). *Arthrobacter crystallopoietes* KAIT-B-007 contains the amine oxidizing enzyme (DAO) which is specific to histamine oxidation. Although this enzyme was isolated and the activity studied (Sekiguchi et al., 2004), the source bacteria has not been studied for biogenic amine degradation in food. *M. varians* having tyramine oxidase, degraded tyramine during sausage fermentation (Leuschner & Hammes, 1998b). *Natrinema gari*, an extremely halophilic archaea isolated from anchovy fish sauce, was reported to degrade histamine in high salt media. The optimum temperature and pH for the degradation was between 6.5 - 8.3 and 40 – 55 °C, respectively and the sodium chloride concentration was 3.5 – 5 M(Tapingkae et al., 2010b). However, no studies of this bacterium have been done in food. *B. linen* reduced histamine by 70% and tyramine by 55% in Munster cheese over 4 weeks of ripening (Leuschner & Hammes, 1998a). Mah and Hwang (2009b) studied biogenic amine reduction in Myeolchi-jeot, a salted and fermented anchovy (*Engraulis japonicas*) by applying starter cultures during ripening. *S. xylosus* No. 0538 degraded histamine and tyramine by 38% and 4% respectively and the total biogenic amine level was decreased by 16%.

The histamine level in fish sauce has been regulated in Canada and USA, with the maximum allowable limit set at 200 ppm and 500 ppm, respectively (Brillantes et al., 2002). Per meal, an intake of 40 mg of biogenic amines is considered toxic (Nout, 1994). Histamine poisoning incidents due to fish sauce may have occurred, but may not be reported as the symptoms are similar to those of food allergies (Tsai et al., 2006). Histamine content in Thai fish sauce ranges between 200 - 600 ppm (Brillantes

&Samosorn, 2001). Inoculation of 10% (w/w) *Virgibacillus sp* SK33in Thai fish sauce fermentation, reduced histamine production by 50% (117.6 ± 0.07 ppm) from an initial level of 215.3 ± 4.41 ppm histamine (Yongsawatdigul et al., 2007). However, the tyramine level increased from an initial level of 49.6 ± 0.93 ppm to 90.6 ± 1.45 ppm in both the control and in the fish sauce containing the starter culture, respectively. The reasons for the increase in tyramine need to be determined as do the effects of other factors such as sodium chloride, pH and temperature on the biogenic amine content of fermented fish sauce (Yongsawatdigul et al., 2007). It is possible that *Virgibacillus sp* SK33may also be used successfully to control biogenic aminesin other foods.

Lactobacillus spp are also able to reduce biogenic amines. Dapkevicius, et al. (2000) studied the ability of lactic acid bacteria isolated from mackerel fish paste to degrade biogenic amines. Five cultures (*Lactobacillussakei* 15.05, *L. sakei* 15.18, *L. sakei* 15.36, *L. sakei* 15.39 and *Lactobacillus. curvatus* 15.35) were found to degrade histamine (20 - 54%) in deMan, Rogosa and Sharpe (MRS) broth containing 50 ppm histamine, and two cultures (*L. sakei* 15.18, and *L. sakei* 15.36) degraded histamine (50 - 54%) in the fish slurry (containing 10 ppm histamine) (Dapkevicius et al., 2000). *L. sakei* 15.18 and *L. sakei* 15.36 are potential starters to degrade histamine during food fermentation. Diamine oxidase (DAO) was also studied by the same authors for the potential for histamine degradation in broth and fish slurry.

Diamine oxidase is another option for biogenic amine degradation. The ability of DAO to degrade histamine in both phosphate buffer (pH 7.0), and ensiled fish slurry (pH 4.5) was studied (Dapkevicius et al., 2000). DAO was investigated by applying the similar conditions found in fish silage to fish slurry; 2% NaCl, 12% sucrose, 0.05% cysteine. DAO degraded histamine (approximately 40% compared with a control) in fish slurry incubated at 30 ºC with starting pH of 6.4. There was no effect on histamine degradation by DAO with 12% sucrose and 2% NaCl. The addition of 0.05% cysteine decreased histamine degradation and degradation did not occur at pH 4.5. The optimum temperature for DAO activity is 37 °C. DAO activity needs to be investigated in a variety of foods to determine the effectiveness of the enzyme in degrading biogenic amines in different food matrices. A factorial designed experiment combining key factors such as temperature, pH and DAO concentration on the degradation of biogenic amines in food will be useful in recommending DAO for use in specific foods.

The use of bacteria with amine oxidising activity or oxidising enzymes to reduce biogenic amine levels in foods is a potential control measure where it is difficult to control biogenic amine levels through the traditional means of refrigeration, and to eliminate already formed biogenic amines in food.

2.7 Conclusion

The existing method for controlling biogenic amines in food is refrigeration. However, since some bacteria that form biogenic amines can grow below 5 °C, refrigeration alone is not always controlling biogenic amines and thus emerging control measures need to be considered. Emerging control measures for delaying biogenic amine formation include HHP, irradiation, packaging, microbial modelling, and the use of food additives or preservatives. These methods only delay biogenic amines formation in food primarily through the inhibition of bacteria or the decarboxylase enzyme activity responsible for amine production. Application of sufficient heat or freezing storage can prevent further development of biogenic amines although product needs to be protected from recontamination in the case of heat and from thawing in the case of freezing.

Refrigeration is not always a feasible option for artisian fishers, thus the microbiologically stable product having high biogenic amines need to be controlled by other means. The use of amine oxidising bacteria and enzymes are the best options.

There are some practical limitations on the use of some of these methods depending on the resources available. The use of the more novel emerging methods and combinations of control measures, often described as hurdle technology, for the control of biogenic amines needs to be further investigated.

2.8 Nomenclature

DAO= diamine oxidase

MAP= modified atmosphere packaging

NOAEL= no observed adverse effect level

MAOI= monoamine oxidase inhibitors

HHP= high hydrostatic pressure

NaCl = sodium chloride

GDL = glucono-delta-lactone

2.9 References

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2.10 Additions and Updates to Literature Review:

A number of studies relevant to my research have been published since our review in 2010 (Naila et al., 2010). These are briefly reviewed below along with other literature relevant to this thesis but not covered in the original review.

2.10.1 Control of biogenic amines in food - Existing and emerging approaches

Introduction

In Chapter 2 of this thesis (review by Naila et al., 2010) the existing and emerging approaches to control histamine were extensively discussed (Naila et al., 2010). It was concluded that while prevention of histamine formation should be the first choice of control the use of bacteria or enzymes to degrade pre-formed histamine in food products that are stable in terms of bacterial deterioration was a promising approach worthy of further study. Since the review has been published, a number of studies have been published on the inhibition of biogenic amines through the application of food additives (Lapa-Guimarãesa et al., 2011), starter cultures (Aponte et al., 2010; Kuda et al., 2012; Udomsil et al., 2011; Zaman et al., 2011), plant extracts (Özogul et al., 2011) and food such as *nuka*-paste which contained histamine reducing bacteria have been used to reduce histamine in fish sauce (Kuda & Miyawaki, 2010).

Since the focus of this thesis is mainly on finding suitable methods for histamine control, these recent reports will be further discussed.

Application of food additives and preservatives

Food additives and preservatives have some inhibitory effect on biogenic amines formation (Fuentes et al., 2011; Kuda et al., 2012; Kuley et al., 2011; Lapa-Guimarãesa et al., 2011). When 4 strains of *Tetragenococcushalophilus* (HmF - 7, 10, 78 and 85), isolates of fish-*nukazuke*, were grown in Gifu anaerobic medium (GAM) broth containing 0.5% histidine with and without10% NaCl at 30°C for 4 days, histamine formed was suppressed by 75% in the broth containing salt compared to the broth without salt. The strains in the GAM broth without salt produced 200 ppm of histamine (Kuda et al., 2012).

Similarly, salt concentration also inhibited biogenic amines formation in fish. Brine solution (10-20% NaCl) were found to have inhibitory effect towards histamine, cadaverine and putrescine production in vacuum packed trout fillets stored at 4°C for 42 days. The initial histamine level of the fillet was 26 ppm and after 42 days the sample without salt had 64 ppm and the samples with 10% and 20% salt had 46 ppm and 37 ppm, respectively. (Kuley et al., 2011).

Fuentes et al. (2011) reported the first study that compared biogenic amine inhibition with that of salt and a salt mixture. The researchers used a salt mixture of 50% NaCl and 50% KCl to produce smoked sea bass (*Dicentrarchus labrax L.*) that was stored at 4°C for 42 days. During this incubation period significant inhibitory effect on biogenic amines formation by the salt mixture was observed compared to that of just NaCl. For example, the initial histamine concentration of air packed sea bass was 2.7 ppm and after 42 days the sample salted with NaCl only was 34 ppm while the sample with the salt mixture had 3.5 ppm. This may be because the mixture requires more salt quantity to retain the same water activity level compared to the amount of the single salt; the NaCl added samples (smoked sea bass) lost moisture and weight faster compared to the mixtures (Fuentes et al., 2010).

Sodium benzoate is another food preservative that has shown some inhibitory effect towards histamine formation. The application of the sodium benzoate at 200 ppm in cod (*Gadus morhua*) roe ripening was found to inhibit histamine formation by 80% compared to that of roe ripened without sodium benzoate which gave a histamine concentration of 88 ppm, at pH 6.5 and 17°C for 4 months (Lapa-Guimarãesa et al., 2011).

In summary, salt and sodium benzoate have potential in preventing biogenic amine formation. The mixture of salt seemed to have better inhibitory effect than use of single salt probably due to higher amount of salt in the former. Therefore, with careful selection of food additives and preservatives that have potential to reduce amine production in food are cheaper options of histamine control. However, the environmental factors of food such as the type of microflora, pH and temperature need to be considered due to their influence on biogenic amine formation.

Plant extracts

Plant extracts have been shown to control histamine production in just one study. Rosemary and sage tea extract reduced histamine production from 287 ppm to 60 ppm and 120 ppm, respectively, in sardines stored at 3°C for 20 days (Özogul et al., 2011). The extraction of the plant compounds is a tedious procedure, adding expense to the process. However, further experimentation on various plant extracts may yield a practical solution. Cheaper option maybe to use the plants without extracting the compounds.

Starter cultures

Recently many bacteria have been isolated and identified as histamine reducers and suggested as potential starter cultures for food fermentation. The bacteria isolated and identified were *Tetragenococcushalophilus* (Kuda et al., 2012; Udomsil et al., 2011), *Staphylococcus carnosus* FS19 and *Bacillus amyloliquefaciens* FS05 (Zaman et al., 2011), *Haloarcula marismortui* IR and *Halobacterium salinarium* CER6a (Aponte et al., 2010), *Natrinema gari BCC 24369* (Tapingkae et al., 2010a), and *Lactobacillus plantarum* BCC 9546 (Tosukhowong et al., 2011).

T. halophilus is a potential starter culture for salted and fermented food products. This species has been isolated from fish-*nukazuke* (Kuda et al., 2012) and Thai fish sauce (Udomsil et al., 2011). Before using as a starter culture, the strains need to be tested for histamine production because Kuda et al. 2012 found that some strains of this species can produce histamine to about 200 ppm. These authors demonstrated other strains such as *T. halophilus* HmS-129 were able to reduce histamine in 0.5% histidine GAM broth (Kuda et al., 2012). Udomsil et al. (2011) found that *T. halophilus* MRC 10-1-3 and MCD 10-5-10 reduced the histamine concentration by about 50% when inoculated into anchovies mixed with 25% NaCl and fermented for 6 months to produce fish sauce.

S. carnosus FS19 and *B. amyloliquefaciens* FS05 were found to reduce histamine by 27.7% and 15.4% respectively during the fermentation of Malaysian fish sauce at 35°C for 20 days. These isolates had themselves been obtained from Malaysian fish sauce (Zaman et al., 2011). These bacteria were also shown to degrade histamine by 59% and 29% respectively from the initial value in 0.05 M phosphate buffer (pH 7) grown for 24 h at 37°C under shaking at 150 rpm (Zaman et al., 2010).

H. marismortui IR and *H. salinarium* CER6a inhibited histamine accumulation in salted anchovies (*Engraulis encrasicholus L.*). The fish were stored at 20°C for 150 days after packing in glass jars with alternative layers of fish and salt (2:1) and inoculation with bacteria (Aponte et al., 2010).

N. gari BCC 24369 cells were immobilized in Celite and showed 80% histamine degradation in 50 mM Tris-HCl buffer (pH 7) containing 4.5 M NaCl at 45°C and agitated to 200 rpm for 1h (Tapingkae et al., 2010a).

A commercial starter culture, *L. plantarum* BCC 9546, was found to reduce the accumulation of tyramine in a Thai fermented sausage (Nham) that were incubated for 7 days at 30°C and 50% relative humidity (Tosukhowong et al., 2011).

The above studies confirm that some bacteria are able to degrade histamine but it is not clear whether these bacteria use histamine oxidizing enzyme or another mechanism to degrade histamine.

Food products

Only one study has investigated histamine control using a food product itself.

Histamine in fish sauce was reduced to 600-700 mg/L from the initial value of 1200 mg/L by addition of 20% (w/v) of *nuka*-paste (fermented food with rice bran) (Kuda & Miyawaki, 2010). Interestingly, *T. halophilus* bacteria were isolated from this paste and this species contains both histamine producing and reducing strains (Kuda et al., 2012). Therefore, it may be possible that these bacteria in the paste may play some role in reducing histamine in the fish sauce made from this paste. Using these bacteria in food may be a practical approach to reduce histamine, if they can be made to degrade histamine by 75% or more. However, this needs to be tested in a variety of foods.

Methods for oxidizing/degrading pre-formed biogenic amines

In the review published by Naila et al. (2010) the use of diamine oxidase to degrade histamine in fish slurry used for the manufacture of animal feed (Dapkevicius et al., 2000) was highlighted. Based on this study we subsequently demonstrated histamine degradation by diamine oxidase in tuna soup used for the manufacture of *Rihaakuru* for human consumption (Chapter 7 this thesis; Naila et al., 2011c).

The method of Tapingkae, Parkin, Tanasupawat, Kruenate, Benjakul, and Visessanguan(2010a) may be another option for histamine degradation, where a degrading bacterium (*N. gari BCC 24369)* is immobilization in Celite (diatomaceous earth/ diatomite; white powder obtained from sedimentary rock). While this method may be expensive compared to the use of free bacteria it may well be cheaper than the use of purified enzymes. To date complete histamine degradation has only been reported in studies using purified enzymes(Chapter 7 this thesis; Naila et al., 2011c). Methods using purified and immobilized bacteria have only be able to achieve partial histamine degradation (Tapingkae et al., 2010a) .

Conclusion

More bacteria containing histamine oxidation enzymes are being discovered and these may provide more options for histamine control in food.

2.10.2 Histamine in food

Research on histamine and other biogenic amine in food has been increasing globally due to the widespread toxicity of the compounds such as histamine when consumed at high concentration (> 500 ppm). Just to give few examples: histamine in fresh fish and canned fish were quantified in Brazil (Silva et al., 2011), improved HPLC methods for histamine analysis were developed in Denmark (Šimat & Dalgaard, 2011) which quantified nine biogenic amines including histamine in tuna and herrings , fish sauce was found to contain more than 1000 ppm histamine in Iran (Zarei et al., 2012), and MPN and real time PCR method for the enumeration of histamine producing Gram-negative bacteria in fish was developed in the USA. The latter method could detect active histamine formers such as *Morganella morganii*, *Enterobacter aerogenes*, *Raoultella planticola* and *Photobacterium damselae* from fish tissue, that reduces the need to do tedious work on isolating and identifying histamine formers from a mixed culture (Bjornsdottir-Butler et al., 2011). Monitoring histamine in foods remains an important task so potential outbreak of histamine food poisoning can be controlled.

Histamine detection methods

There are several new methods for the detection of biogenic amines (Awazu et al., 2011; Basheer et al., 2011; Fernandez-No et al., 2010; Nakamura et al., 2011; Rabie et al., 2011; Šimat & Dalgaard, 2011; Stănescu et al., 2010; Tahmouzi et al., 2011; Tao et al., 2011a). Most are based on chromatography and include high performance liquid chromatography (HPLC), gas chromatography (GC) and thin layer chromatography (TLC) (Tao et al., 2011a). Other methods include capillary electrophoresis (Du et al., 2002) and enzyme sensors (Ito et al., 2009). One interesting method described by Stănescu et al. (2010) was to use performant mass spectroscopic (MS) fingerprints to identify biogenic amines. If use of this method becomes cheaper, it would be the most convenient and fastest method compared with other methods available to date. Those authors reported that biogenic amine fingerprinting was quick and accurate and the peaks for histamine, tyramine and cadverine were easily identified. However, the most frequently used method, HPLC, is an expensive method and MS is also believed to be expensive.

For this thesis HPLC was chosen to analyze biogenic amines following a method of Hwang, Chang, Shiua, and Chai (1997) because of its sensitivity and ability to detect histamine and other biogenic amines. We also chose this method as it uses less chemicals and is easier to adopt compared to other methods (Eerola et al., 1993; Hernandez-Jover et al., 1996). This method has been used by many other researchers (Chang et al., 2009; Tsai et al., 2007c; Zarei et al., 2012).

2.10.3 Histamine stability during storage

Literature related to histamine stability during storage is reviewed in Chapter 5.

2.10.4 References

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Aishath Naila

Name/Title of Principal Supervisor: Associate Professor Steve Flint

Name of Published Research Output and full reference:

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Chapter 3

Rihaakuru: Process, Composition and Microflora

The work presented in this chapter represents the following peer reviewed publication:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., & Meerdink, G. (2011b). Chemistry and microbiology of traditional Rihaakuru (fish paste) from the Maldives. *International Journal of Food Sciences and Nutrition, 62*(2), 139-147.

3.1 Abstract

Rihaakuru is a traditional Maldivian side dish consumed mainly with rice. It is a thick brown fish paste, made from tuna after prolonged heating. Samples tested were found to have a low water activity (0.55 to 0.8), slightly acidic pH (5.62 to 6.18) and moderate salt content (1.4 to 1.6%). The product was found to be rich in polyunsaturated fatty acids such as Omega-3, had high protein content (56 to 59%) and an energy level of 13.8 kJ/g. The product had a low microbial count (1.54 to 2.31 $\log_{10}$ cfu/g). The bacteria isolated belonged to the *Bacillaceae* (Genus *Clostridium*and *Bacillus*), *Streptococcaceae* (Genus *Streptococcus*), *Micrococcaceae* (Genus *Staphylococcus*), and *Corynebacterium*. The product appears to be a nutritious and shelf stable product.

Keywords: Maldives, tuna, *Rihaakuru* (fish paste), amino acids, omega fatty acids, proteins.

3.2 Introduction

Rihaakuru, a traditional side dish in the Maldives, is a thick brown tuna based paste consumed on a daily basis. While it has traditionally been produced by individual households, commercial manufacture is starting to become more common. The *Rihaakuru* is prepared by boiling tuna in salted water, removing the cooked fish and concentrating the remaining material by heating until it becomes a thick paste. The raw materials-gutted tuna, sometimes headless, or just chunks-are cooked for about 45 min, in water [1:1, fish: water or water is added until all fish are fully submerged] containing approximately 1% salt, for about 45 min. The scum (filleyo) that forms is continuously removed by scooping off the surface, during the cooking process. The cooked fish is removed and processed into Maldive dried fish, then the remaining soup is filtered using cheese cloth to remove scales, bones and other fish debris that may have remained in the soup. This soup is further cooked on low heat/flame/fire (approximately 70 - 90 °C) with continuous stirring with a wooden/stainless steel spatula until most of the water has evaporated, leaving a thick, brown paste. This takes approximately 4 – 8 h to concentrate to the desired level (determined by dipping a utensil and judging its thickness from the drip/flow) depending on the volume of *Rihaakuru* to be made. This method is a summary from five recipes obtained from different parts of the Maldives and from personal communication with different homemade and commercial manufacturers. The fish are cooked in this manner in large aluminium open pans as a single batch of fish or batches of fish each boiled for 45 min in the same soup. Once the paste temperature falls to ambient temperature (in Maldives, 25 – 30 °C), it is generally bottled into clean glass or plastic bottles with lids, and sold to the general public through retail outlets. The paste is kept in ambient temperature; no special storage condition is required. Commercial manufactures label the shelf life as 6 months, while homemade manufacturers state a year as the shelf life, however through personal experience, it can be kept more than one year if kept under reasonably hygienic conditions. The tuna types mainly used are skipjack (*Katsuwonus pelamis*), yellowfin (*Thunnus albacares*), big eye (*Thunnus obesus*), frigate (*Auxis thazard*) and little tunny (*Euthynnus affinis*) [through personal experience and personal communication with manufactures].

While the production, and quality studies of a wide range of fish paste products have been reported in the literature to our knowledge there have been no publications relating to *Rihaakuru*. Most publications on fish pastes are on fermented products and with few dealing with cooked pastes. Even for the latter, production methods differ from *Rihaakuru*. The following paragraphs summarise the published information on fish paste products.

Cooked pastes include Kamaboko, a Japanese product made from surimi, a fish minced meat leached with water and mixed with polyphosphate, sugar and frozen (Makoto, 2001) and Shio-surimi- a Japanese product made from frozen Alaska pollack surimi, starch (4.5-9%), water (150 or 300%) and sodium chloride. The product is cooked at 80°C for 30 min, with the resulting paste being comparable to tomato ketchup or mayonnaise (Niwa et al., 1990).

Miso-like fermented fish pastes (*sakana miso*) have been manufactured from spotted mackerel (*Scomber australasicus*), lizard fish (*Saurida wanieso*), horse mackerel (*Trachurus japonicas*) and squid (*Todarodes pacificus*) using starter cultures (malt rice-*kome-koji*) which were found to improve the nutritional quality of the pastes (Giri et al., 2009a). The same researchers studied other fermented fish pastes using different *koji* moulds as starters and found little effect on the taste but the nutritional value and sensory characteristics were improved (Giri et al., 2009b).

In Japan, fish pastes have been fortified or their functionality changed by the addition of compounds, such as boiled-dried anchovy powder to increase calcium (Bae & Lee, 2007), *Styela plicata* to enhance anticarcinogenic and antioxidant activity (Park et al., 2006), Propolis to enhance antioxidant and antispoiling properties (Kim et al., 2008), and *Lycii fructus* powder to improve the quality and functionality (Shin et al., 2008). The shelf life of fish paste was found to be extended, with the use of 50 ppm chlorine dioxide, since this decreased the total bacterial, yeast and mould counts (Shin et al., 2007).

In summary, there has been some research on the shelf life and quality on various fish pastes, but these differ from *Rihaakuru*. To our knowledge, *Rihaakuru* is a unique fish paste, as it is made from fish soup, rather than fish flesh, and involves prolonged heating rather than a fermentation process. As this product has not been previously reported in the literature this paper describes the manufacturing process, nutritional composition and bacterial microflora of *Rihaakuru*.

3.3 Materials and Methods

3.3.1 Sampling

Twenty eight samples of *Rihaakuru* were collected from different geographical (North, South and Central) locations in Maldives. There are no regional differences in the method of preparation but the taste (quality) varies. Quality improves when unwanted foam that rises during the initial stages is skimmed immediately- the sooner the foam is picked up, the better the taste (personal experience) and can possibly be explained by preventing microbial growth in the foam layer. The twenty eight samples were tested for total aerobic and anaerobic count, water activity and pH. Due to budget constraints for the project, only two samples were randomly selected, for nutritional analysis, and identification of microflora. The main focus of this project was to find indicative information on the nutritional value and typical bacteria present in the product.

3.3.2 Physicochemical analysis

The pH of a 5g sub-sample of each *Rihaakuru* samples homogenized with 20 ml of distilled water was estimated using a pH meter (Model 420A Watson Victor LTD, Auckland, New Zealand). Water activity of each of the *Rihaakuru* samples (n = 28) was analyzed using a water activity meter (DECAGON CX-2, Formula Foods Corporation Ltd, Christchurch, New Zealand).

3.3.3 Amino acid, fatty acid, minerals and composition analysis

Two samples of *Rihaakuru* were randomly (by picking 2 out of 28 samples coded with numbers) selected and sent to the Nutrition Commercial Laboratory, Massey University, New Zealand for amino acid, fatty acid, minerals, composition, salt, vitamin and energy analysis. Following paragraphs describe the method of analysis used by the laboratory.

Fatty acids were analyzed by gas chromatography using a Shimadzu GC-17A Gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector (FID), fitted with a Supelco™-2560 Capillary Column (Shimadzu Corporation, Kyoto, Japan) 100 m x 0.25 mm x 0.2 µm film thickness. The oven temperature was programmed to hold at 140°C for 5 min then to increase to 240°C at the rate of 4°C/min, and hold for 38 min. Injector temperature was 250°C, and detector temperature was 255°C. Standards were purchased from Sigma-Aldrich Co Ltd (Auckland, New Zealand).

Protein was analyzed by the total combustion method (AOAC 968.06), fat by soxtec extraction (AOAC 991.36), moisture by convection oven at 105 °C (AOAC 930.15, 925.10), ash by Furnace at 550 °C (AOAC 942.05) and carbohydrate by difference.

Mineral content was determined by plasma emission spectrometry (Fetcher et al., 2001), chloride by potentiometric titration, iodine and selenium by Inductively Coupled Plasma-Mass Spectrometry using Tetramethylammonium Hydroxide (TMAH) digestion for milk and milk proteins.

Dietary fibre was determined by an enzymatic gravimetric method (AOAC 991.43), amino acids by hydrochloric acid extraction followed by HPLC separation (AOAC 994.12), tryptophan by alkaline hydrolysis, followed by HPLC separation (AOAC 988.15).

Gross energy was analyzed by bomb calorimetry using Leco AC 350 (Leco Corporation, St Joseph, MI, USA). Vitamin A (as retinol) was determined by HPLC (AOAC methods 974.29(4), 971.30, and 948.26), vitamin E (total α-tocopherols) by HPLC (AOAC methods 971.30, 948.26, and 974.29) and vitamin D3 by HPLC (AOAC method 981.17) (Nielsen, 1994).

3.3.4 Total aerobic and anaerobic count

To determine the total aerobic and anaerobic count associated with the *Rihaakuru* (n = 28), sub-samples were serially diluted with peptone and plated (pour plates) in triplicate on trypticase soy agar (TSA) which were incubated at 30 °C for 48 h, under aerobic or anaerobic conditions. The anaerobic conditions were obtained by growing the bacteria in an anaerobic jar containing anaerobic sachet. Colonies were counted using a colony counter (model 560; A SUNTEX, Taipei, Taiwan).

3.3.5 Natural microflora isolation and identification

Two samples of *Rihaakuru* were randomly (by picking 2 out of 28 samples coded with numbers) selected for microflora isolation and identification. All the bacteria isolated were identified by motility tests, Gram staining, and 16S rDNA sequencing. The DNA from isolates grown in Trypticase Soy Broth (TSB) for 48 h at 30 °C was extracted by boiling culture for 10 min, centrifuging (15 min, 3000 x g), and re-suspending the pellet in 500 µL of sterile water. A standard PCR protocol was followed based on Manachini et al. (2002).

To 75 µL PCR mixture (containing 10 µL Tfl polymerase, 40 µL 20x buffer, 80 µL 25 mM $MgCl_2$, 160 µL of 1.25 mM DNTP mix, 5 µL of each conserved 10 µM primer [1/5 diluted], 450 µL sterile distilled water) 5 µL DNA [25 µg] extracted culture was added. The forward primer of Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and the reverse primer of Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3') were used, chosen based on the method of J. P. W. Young, Downer, and Eardly (1991). In total 80 µL reaction was performed in a PCR (Techne PHC-3 Thermal Cycler; John Morris Scientific Ltd, Auckland, New Zealand) programmed to perform a denaturation step (94 °C for 5 min), followed by an annealing step (40 cycles of 94 °C for 30 s, 50 °C for 45 s, 72 °C for 45 s) and an extension step (72 °C for 7 min). The DNA band size and concentration was approximated by agarose gel electrophoresis.

Electrophoresis was conducted for 1.5 h at 80 V with 5 µL of PCR product, a negative control (sterile water), and a 1 kb DNA reference ladder (Gene Ruler ™ 1 kb Plus DNA, Fermentas) loaded onto 2% agarose gel containing 10 µl of ethidium bromide in Tris EDTA (TE) buffer. The gel was viewed under UV illuminator, for DNA fragment identification.

The PCR products were purified using the DNA clean and Concentrator™ kit (Zymo Research, Orange, California, USA). The purified PCR products were analyzed by the Genome Sequence Service, at Massey University, New Zealand. The sequence results were analyzed using Sequence scanner V.1.0 and blast searched using internet (http://bioinfo.unice.fr/blast/) for bacterial identification.

3.4 Results and Discussion

3.4.1 Physicochemical analysis

The intrinsic properties of *Rihaakuru* varied between samples giving water activities between 0.55 and 0.80 (n = 28), and pH between 5.6and 6.1 (n = 28), and salt content between 1.4 and 1.6 g/100g (n = 2).

Among the twenty eight samples of *Rihaakuru* analyzed, twenty four samples had pH between 5.6 and 5.9and four samples had pH between 6.0 and 6.1. The pH of *Rihaakuru* is not sufficiently low to prevent fungal growth (Bergquist & Pogosian, 2000), (personal observation through experience). Compared to the literature the pH of four samples of fish paste agree with the pH of marine fish (6.1) (Mizutani et al., 1992), *post mortem* fish (> 6.0) (Gram & Huss, 1996) and tuna dumplings (6.0 - 6.4) (Chen et al., 2008). However, none of the fish paste sample pHs agrees with that of shrimp paste (7.5) (Mizutani et al., 1992). The shrimp paste pH differs and this maybe because of the difference in raw material; fresh shrimp pH is 7 (Shamshad et al., 1990) and fresh tuna pH is 5.8 (Ben-Gigirey et al., 1999).

The water activity of most *Rihaakuru* samples was below 0.7 (15 out of 28), ten samples at 0.7 and only one sample was at 0.8. The salt content in the two randomly selected samples was between 1.4 to 1.6% (Table 5).

The salt content of *Rihaakuru* is insufficient to prevent microbial growth; however, the water activity is too low to expect any bacterial growth. Hence the bacteria present in the samples must reflect bacterial growth in the raw material or in the early stages of production which survive but do not grow on the product, and post-process contamination by environmental organisms.

3.4.2 Amino acid, composition analysis, minerals and fatty acid of *Rihaakuru*

Table 5 through Table 9 summarize the amino acid, essential amino acid, composition, minerals and fatty acids of two randomly selected samples of *Rihaakuru*.

Tuna are well known for their high protein and essential amino acids, minerals (micro and macroelements), vitamins, and unsaturated fatty acids (García-Arias et al., 1994). The following subsections will describe each of the listed nutritional information of *Rihaakuru*, in detail.

3.4.3 Compositional analysis of *Rihaakuru*

Table 5 presents the results of compositional analysis of *Rihaakuru* on a wet weight basis (n = 2). Protein content was between 56 and 59% (g/100g), total dietary fibre between 1.9 and 2% (g/100g), and fat was 4% (g/100g). Vitamin A and E were found and no carbohydrate was found.

Table 5: Composition analysis of *Rihaakuru* (n=2)

Composition	**Sample code**	
	C	**H**
Dry matter % (w/w)	68.8	68.2
Ash % (w/w)	8.6	7.9
Moisture % (w/w)	31.2	31.8
Fat % (w/w)	4.3	4
Protein % (w/w)	56.8	59
Total dietary fibre (TDF) % (w/w)	2	1.9
Carbohydrate (CHO) % (w/w)	ND	ND
Energy (GE) kJ/g	13.8	13.8
Vitamin A (ug/kg)	470.1	779.7
Vitamin E (mg/kg)	4.6	2.7
Vitamin D (ug/kg)	<0.5	<0.5
Salt (g/100g) [a]	1.6	1.4
pH	5.84	5.81
Water activity	0.74	0.74

ND: none detected

Nitrogen-protein conversion factor = 6.25

[a] salt = Clx1.66

When compared with raw tuna, the protein content of *Rihaakuru* is somewhat lower. The protein content on a dry basis from raw, steamed and canned tuna are 88.55, 86.48, and 80.99 (g/100g), respectively. The protein content on a wet basis of raw, steamed and canned tuna are 27.33, 31.41, and 31.23 (g/100g), respectively (Castrillón et al., 1996). The low protein content in *Rihaakuru* (n = 2) maybe due to the processing conditions (Castrillón et al., 1996), the types of fish species used and the time of fish harvest (Kristinsson & Rasco, 2000). Tuna cooking juice (the condensed water from tuna steaming) was reported to contain 4% of protein (K. C. Hsu et al., 2009). This indicates that cooking tuna may not leach all of the protein from the loins, resulting in less protein content in the soup than the loin; this could also be why *Rihaakuru* contains less protein compared with tuna.

Rihaakuru fat content is lower compared to tunas. For example, raw white tuna fat content is 1.9% (g/100g) on a wet basis and canned white tuna fat content is 4.7% (g/100g) on a wet basis showing the increase in fat content when processed (García-Arias et al., 1994). Reasons for the low fat content in *Rihaakuru* are likely to be the same as low protein content- that is, not all the fat is released during cooking. The low fat content may also be due to the skimming carried out during boiling as the foam being removed may have a high fat content.

In *Rihaakuru* (n = 2), no carbohydrate was found, because no ingredients such as glucose or sugar are added. Contrarily, fermented fish pastes such as ‘Chikuwa’ and ‘Kamaboko’ contain (fructose 0.6 mg/g, glucose 6.6 mg/g and sucrose 49.8mg/g) carbohydrates (Mizobuchi et al., 1986).

There was a low amount of fibre present in *Rihaakuru* (n = 2). It was not expected to find fibre in the product since the raw material used was tuna, which has been reported to not contain fibre (USDA, 2009). The reason for a low amount of fibre in *Rihaakuru* is unknown.

3.4.4 Amino acid profile

In the human body, 18 out of 20 available amino acids are nutritiously important. There are 8 essential amino acids (lysine, methionine, phenylalanine, valine, isoleucine, leucine, threonine, and tryptophan) that cannot be synthesized in humans therefore are required in the diet. Semi-essential amino acids (serine, arginine and histidine) are needed in stressful conditions. Non-essential amino acids include glutamic acid, tyrosine, proline, aspartic acid, glycine, cystine and alanine, are synthesized in the human body. These amino acids are important building blocks for protein synthesis (Usydus et al., 2009).

The *Rihaakuru* samples (n = 2) tested in this study contained 18 free amino acids. Histidine and glutamic acid were the predominant amino acids in the samples, with tryptophan being the least prevalent (Table 6).

Table 6: Amino acid profile of Rihaakuru (n=2)

Amino acids	**Sample code**	
	C	**H**
	mg/100g	**mg/100g**
Aspartic acid	2.8	3.15
Threonine	1.41	1.56
Serine	1.01	1.14
Glutamic acid	3.7	4.15
Proline	1.47	1.55
Glycine	2.59	2.57
Alanine	2.46	2.64
Valine	1.57	1.76
Methionine	0.87	0.95
Isoleucine	1.34	1.51
Leucine	2.37	2.65
Tyrosine	0.89	0.99
Phenylalanine	1.12	1.25
Histidine	8.76	7.34
Lysine	2.77	3.04
Arginine	1.8	1.94
Taurine	1.24	1.21
Tryptophan	0.3	0.33
Cysteine	-	-

The total essential amino acids required for an adult man with 70 kg of body weight is 5.59 g (Usydus et al., 2009). The consumption of 100 g *Rihaakuru* will only provide between 12.64 and 14.04 mg of essential amino acids (Table 7). A typical serving of *Rihaakuru* is approximately 100 g which may be consumed daily (through personal experience) and provides about 0.2 % of recommended daily intake of amino acids per 70 kg body weight and 16 % for per one kg body weight.

Table 7: Essential amino acids of Rihaakuru (n=2) in comparison to recommended level

Essential amino acids			Sample code		Reference
	RDI mg/kg	RDI g/70kg	C mg/100g	H mg/100g	
lysine	9.4	0.66	2.77	3.04	
Methionine+cysteine	12.1	0.85	0.87	0.95	
Phenylalanine+tyrosine	12.1	0.85	2.01	2.24	
valine	11.4	0.8	1.57	1.76	(Usydus et al., 2009)
isoleucine	15.7	1.1	1.34	1.51	
leucine	9.5	0.67	2.37	2.65	
threonine	6.5	0.46	1.41	1.56	
tryptophan	2.9	0.2	0.3	0.33	
Total	**79.6**	**5.59**	**12.64**	**14.04**	

RDI: recommended daily intake

When compared with skipjack tuna viscera, the total 19 amino acids (same types as in *Rihaakuru*) has been recorded as 20030 mg/100g (Cha & Cadwallader, 1995), showing amino acid level in *Rihaakuru* had been reduced during production process. This suggests a low extraction of protein from the fish during the cooking process.

3.4.5 Mineral profile

The mineral composition of *Rihaakuru* is given in Table 8. Potassiumand phosphorous were present in the highest concentrations.

Sodium content in *Rihaakuru* ranged between 640 and 710 mg/100g. The recommended dietary intake per day is 1500 mg. The maximum level that could be consumed daily is 2500 mg (Karppanen et al., 2005). The content of sodium in *Rihaakuru* is low compared with that of recommended daily intake.

Table 8: Mineral content of Rihaakuru (n=2)

Mineral	**Sample code**	
	C	**H**
	mg/100g	**mg/100g**
Calcium	60	70
Magnesium	270	250
Potassium	2600	2300
Sodium	710	640
Phosphorous	1400	1300
Aluminium	4.4	3.6
Cadmium	0.14	0.17
Copper	4	3.9
Lead	0.13	0.09
Manganese	0.63	0.65
Zinc	26	30
Iodine	0.69	0.69
Selenium	6.4	6.4
Chloride	1000	900

Potassium protects against stroke (Karppanen et al., 2005) and its content in *Rihaakuru* (n = 2) was found to be between 2300 and 2600 mg/100g. The daily recommended level of potassium is 4700 mg/day, for adolescents (Karppanen et al., 2005), showing that *Rihaakuru* provide a low level of potassium compared with the recommended level.

Calcium in *Rihaakuru* (n = 2) ranged between 60 and 70 mg/100g. Calcium may prevent osteoporosis and lessen obesity (Karppanen et al., 2005). The recommended daily intake is between 1000 and 1300mg (Karppanen et al., 2005). Therefore, *Rihaakuru* may not provide the recommended amount of daily calcium intake.

The intake of sufficient magnesium may prevent heart disease (Karppanen et al., 2005), and the recommended daily intake for women is given as 300 mg/day (Karppanen et al., 2005). The *Rihaakuru* samples contained magnesium between 250 and 270 mg/100g, showing slightly lower than the recommended level.

Copper, which is involved in haemoglobin production and iron absorption (Biego et al., 1998; Hart et al., 2002) was found between 3.9 and 4 mg/100g in *Rihaakuru* (n = 2).

Hence, the level is slightly higher than the recommended range of 1 to 3 mg/day (Biego et al., 1998; WHO, 1985).

Manganese maintains glucose homeostasis (Biego et al., 1998; Korc, 1988), and was found in *Rihaakuru* (n = 2) between 0.63 and 0.65 mg/100g. The recommended daily intake is between 2 and 9 mg/day, for adults (WHO, 1985). This shows the manganese content of tested samples is below the recommended level.

Zinc an important metal for enzymatic function (Vallee & Falchuk, 1993), ranged between 26 and 30 mg/100g in *Rihaakuru* (n = 2). Compared to the recommended daily intake, 15 mg/day (National Research Council, 1989), *Rihaakuru* contains almost twice this level. The toxicity level of zinc in humans is low and only occurs after consumption of at least 2 g of zinc (Prasad, 1976). Thus the zinc level in *Rihaakuru* is unlikely to be harmful.

The aluminium level in *Rihaakuru* (n = 2) was found to be between 3.6 and 4.4 mg/100g, and is in the range for recommended daily intake range is 2 and 10 mg/day (Greger, 1985; Pennington, 1988). Some of the aluminium in the samples may come from aluminium utensils (Biego et al., 1998; Gartrell et al., 1986), which are generally used in *Rihaakuru* cooking.

Rihaakuru (n = 2) contained between 0.14 and 0.17 mg/100g cadmium, which is two times higher than the safe limit of cadmium, 68 μg (0.068 mg) (WHO, 1985). Cadmium is known as an environmental toxicant, and may damage liver and decrease fertility in men (Siu et al., 2009). However, cadmium toxicity may be reduced in foods such as *Rihaakuru* due to the high levels of protein, and minerals (Asagba, 2009).

3.4.6 Fatty acid profile

Twenty two different fatty acids were present in *Rihaakuru* (n = 2) (Table 9).

Table 9: Fatty acid profile of Rihaakuru (n=2)

Fatty acids	**Sample code**	
	C	**H**
	g/100g	**g/100g**
C6:0 Caproic	ND	ND
C 8:0 Caprylic	ND	ND
C 10:0 Capric	ND	ND
C11:0 Undecanoic	ND	ND
C 12:0 Lauric	ND	ND
C13:0 Tridecanoic	ND	ND
C 14:0 Myristic	0.11	0.11
C 14:1-cis9 Myristoleic	ND	ND
C15:1 - cis10 Pentadecenoic	ND	ND
C 16:0 Palmitic	0.88	0.84
C 16:1-cis9 Palmitoleic	0.15	0.14
C17:0 Margaric	0.08	0.08
C17:1 - cis10 Heptadecenoic	ND	ND
C 18:0 Stearic	0.37	0.37
C18:1 -trans9 Elaidic	0.01	0.01
C18:1 -trans11 Vaccenic	ND	ND
C 18:1-cis9 Oleic	0.47	0.45
C18:1 -cis11 Vaccenic	0.08	0.07
C 18:2-trans9,12 Linolelaidic	ND	ND
C 20:0 Arachidic	0.02	0.02
C20:1 -cis11 Eicosenoic	0.03	0.03
C21:0 Heneicosanoic	ND	ND
C 22:0 Behenic	0.01	0.01
C20:3n6-cis8,11,14 Eicosatrienoic	ND	ND
C 22:1-cis13 Erucic	ND	ND
C20:4n6-cis5,8,11,14 Arachidonic	ND	ND
C20:4n6-cis5,8,11,14 Arachidonic	ND	ND
C23:0 Tricosanoic	0.07	0.07
C 24:0 Lignoceric	0.01	0.01
C 24:1-cis15 Nervonic	0.02	0.03
Omega-3 fatty acids		
C22:6n3-cis4,7,10,13,16,19 DHA	0.45	0.44
C22:5-cis7,10,13,16,19 DPA	0.02	0.02
C20:5n3-cis5,8,11,14,17 EPA	0.1	0.09
C20:3n3-cis11,14,17 Eicosatrienoic	0.01	ND
C 18:3 -cis9,12,15 linolenic	0.01	0.01

Fatty acids	Sample code	
	C	H
	g/100g	g/100g
Omega-6 fatty acids		
C22:2 -cis13,16 Docosadienoic	0.01	0.01
C18:2 -cis9,12 Linoleic	0.05	0.05
C20:2 -cis11,14 Eicosadienoic	0.02	0.02

ND: none detected.

Among this, total unsaturated fatty acid was 63%, and total saturated fatty acid was 36%. Of the 63% of the unsaturated fatty acids, 36% was omega fatty acids. Of the saturated fatty acid, palmitic acid was predominant in *Rihaakuru*, between 0.84 and 0.88% (g/100g). This fatty acid is known to enhance memory and learning (Hosseinzadeh et al., 2007). Among the omega fatty acids, docosahexaenoic acid [DHA] (omega-3) was predominant; the level was between 0.44 and 0.45% (g/100g). Tuna species have been reported to have similar fatty acid composition. However, the fatty acid ratio in tuna differs due to factors such as internal, external and metabolic requirements (Aubourg et al., 1996). Since among fatty acids omega fatty acids are the beneficial to health, these will be discussed in more detail.

3.4.7 Omega fatty acids

Omega fatty acids are essential fatty acids (polyunsaturated [PUFA]) which in food are lost during cooking and other food manufacturing steps (Das, 2006). Essential fatty acids are important to maintain cell membrane functions in both humans and animals. There are two types of omega fatty acids, omega-3 and omega-6. The recommended dietary proportion of omega-3 to omega-6 is 1:1 (Kris-Etherton et al., 2002). Omega-3 suppresses cancer and cardiovascular diseases (Wheeler & Morrissey, 2003). For example, breast cancer in women was reported to decrease with the consumption of an omega-3 rich diet (Simopoulos, 2002). The consumption of omega-3 fatty acids and fish are believed to be associated with a reduced risk of coronary heart disease (CHD) in men and women (Hu et al., 2002).

Rihaakuru (n = 2) contained omega-3 fatty acids DHA, eicosapentaenoic acid [EPA], docosapentaenoic acid [DPA], eicosatrienoic [ETE], and linolenic [ALA]) and omega-6 fatty acids (docosadienoic, linoleic, and eicosadienoic) (Table 9). The total omega-3 fatty acids of *Rihaakuru* (n = 2) was between 0.56 to 0.59% (g/100g), and total omega-6 fatty acid was 0.08% (g/100g). The recommended daily intake of omega-3 (DHA plus EPA) is 0.3 to 0.5 g/day and 0.8 to 1.1 g/day of alpha linolenic acid. Canned tuna contain omega-3 fatty acids (DHA plus EPA) between 0.31 to 0.85 g/100g fish (Kris-Etherton et al., 2002). *Rihaakuru* contained omega-3 fatty acids (DHA plus EPA) between 0.53 to 0.55 g/100g, thus falls within the range of canned tuna. The omega-3 fatty acid level was compared in raw (58.3%) and cooked (49.1%) albacore (Aubourg et al., 1996), with the 9% reduction explained by the effect of heat during cooking. We assume a similar loss of omega-3 fatty acids during the cooking of tuna soup. The fatty acid content in tuna also varies according to season with omega-3 fatty acid being high in winter (35.56%) and low in summer (13%) (Soriguer et al., 1997).

The omega-6 to omega-3 ratio of *Rihaakuru* (n = 2) was between 0.13 and 0.14, showing omega-3 content was seven times higher than the omega-6. The ratio in little tunny, skipjack, frigate, big eye, and yellowfin was 4.4, 5.0, 6.6, 6.5, and 8.1, respectively (Aubourg et al., 1996).

3.4.8 Total aerobic and anaerobic count

The mean aerobic and anaerobic counts of *Rihaakuru* (n = 28) were 2.31 ± 0.98 and 1.54 ± 1.20 log_{10}cfu/g, respectively. The highest microbial counts observed were aerobic 4.3 and anaerobic 4.1 log_{10} cfu/g. The microbial contamination is likely to reflect heat resistant species that survived the cooking process or post process contamination.

3.4.9 Natural microflora isolation and identification

The microflora of *Rihaakuru* (n = 2) isolated and identified. *Bacillaceae* (Genus *Clostridium, and Bacillu*s), Streptococcaceae (Genus *Streptococcus*), Micrococcaceae (*Genus Staphylococcus*), and *Corynebacterium* were the groups of bacteria identified. One of the two samples contained 25 *Bacillus* spp. (1 *Bacillus polyfermenticus*, 1 *Bacillus malacitensis*, 2 *Bacillus flexus*, 1 *Lysinibacillus boronitolerans*, 1 *Bacillus cibi*, 15 *Bacillus thuringiensis*, 1 *Bacillus megaterium*,1 *Bacillus oleronius*, 1 *Bacillus* *subtilis*, 1 *Bacillus massiliensis*)out of 27 isolates with *Acinetobacter lwoffii* and *Enterococcus Saccharolyticus* being the 2 remaining bacteria. The second sample contained 12 *Enterococci spp*. (7 *Enterococcus faecalis*, 2 *Enterococcus casseliflavus*, 2 *Enterococcus gallinarum*,1 *Enterococcus saccharolyticus*), and 6 *Bacillus spp.* (1 *Virgibacillus halodenitrificans*, 3 *Bacillus firmus*, 2 *Bacillus thuringiensis*) out of 21 isolates with *Brevibacterium casei*, *Staphylococcus hominis* and *Clostridium irregulare* being the remaining bacteria.

The large variety of heat-resistant bacteria, especially spore formers, presumably reflects the poor quality of the raw tuna used in the manufacture of *Rihaakuru*. Other bacteria are likely to have entered the product after manufacture. The variations in bacteria between the two samples may be due to the geographical locations reflecting the source of raw material (fish) or subtle variations in the methods used in the production. However, it is difficult to draw conclusions from two samples and these results should therefore be interpreted as examples of the types of bacteria that are found in *Rihaakuru*.

The microflora of *Rihaakuru* appears to be similar to the microflora reported in tuna and other fish products. The isolates from *Rihaakuru* are similar to those reported from tuna obtained from tropical waters (Indian Ocean) where higher numbers of Gram positive and enteric bacteria have been reported compared with the microflora of temperate fish (Gram & Huss, 1996). Lightly cured (smoked, salted and dried) fish products have similar microflora consisting primarily of Gram positive cocci, and spore forming *Bacillus* and *Clostridium* species, although Gram negative psychrotrophic *Enterobacteriaceae* have also been reported in cured products (ICMSF, 2005).

In summary, the flora isolated in *Rihaakuru* is what might be expected in a heat treated product made from a tropical fish, excluding the non-spore formers introduced due to post process contamination. Variation of the types of bacteria may be explained by the raw material and different locations of manufacture.

3.5 Conclusion

Rihaakuru as appears to be a rich source of protein, minerals, amino acids and omega -3 fatty acids. Larger sample sizes are needed to confirm the reproducibility of these findings. The low water activity of the product makes this a shelf stable product, with the microbes present in the product, likely to have survived the manufacturing process or contaminated the product after manufacture.

3.6 Acknowledgement

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3.7 References

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DRC 16

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Name of Published Research Output and full reference:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., & Meerdink, G. (2011). Chemistry and microbiology of traditional Rihaakuru (fish paste) from the Maldives. International Journal of Food Sciences and Nutrition, 62(2), 139-147.

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Chapter 4

Biogenic Amines in *Rihaakuru*

The work presented in this chapter represents the following peer reviewed publication

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., & Meerdink, G. (2011a). Biogenic amines and potential histamine – Forming bacteria in Rihaakuru (a cooked fish paste). *Food Chemistry, 128*(2), 479-484.

4.1 Abstract

The biogenic amine concentration in *Rihaakuru* (fish paste) (n = 28), obtained from different parts of the Maldives (North, South, and Central), was determined by HPLC. Ten biogenic amines were detected; agmatine, not detected (ND)-161 ppm; cadaverine, ND-387 ppm; histamine, ND-5487 ppm; putrescine, ND-290 ppm; phenylethylamine, ND-23 ppm; serotonin, ND-91 ppm; spermine, ND-329 ppm; spermidine, ND-79 ppm; tryptamine, ND-<5 ppm; and tyramine, ND-50 ppm. Nine biogenic amines were found in three samples, eight in ten samples, seven in six samples, six in three samples, four in five samples, and one found in one sample. Histamine was detected at levels that are regarded as a risk to human health. Among the microbial profiles of two randomly selected samples out of the twenty eight samples, isolates that have not been reported as potential histamine producers were mainly screened for histamine production: *Bacillus cibi* Nai10, *B. firmus* Nai17a, *B. flexus* Nai8a, *B. malacitensis* Nai11, *B. massiliensis* Nai5, *B. oleronius* Nai7, *B. polyfermenticus* Nai4, *B. thuringiensis* Nai2i, *Clostridium irregular* Nai9, *Enteroccus gallinarum* Nai20a, *E. saccharolyticus* Nai9b, *E. faecalis* Nai13f, *Lysinibacillus boronitolerans* Nai3, and *Virgibacillus halodenitrificans* Nai18. Among these the highest histamine producer was found to be *B. massiliensis* Nai5 (6.65 ppm) and *Bacillus polyfermenticus* (5.58 ppm) while *Bacillus malacitensis* was the least (<0.5 ppm) and two isolates did not produce histamine [*E. gallinarum* Nai20a and *E. saccharolyticus* Nai9b]. Of all the species that we isolated from *Rihaakuru*, *Staphylococcus homini* is the only one ever reported to produce high levels of histamine.

Key words: Maldives, *Rihaakuru*, biogenic amines, histamine, scombroid poisoning, bacteria

4.2 Introduction

The fish paste *Rihaakuru*, which is an important condiment in the Maldives, could contain high concentrations of biogenic amines, due to raw tuna, from which the product is made from, being subjected to temperature abuse (Naila et al., 2010) . Biogenic amines could cause scombroid poisoning, with symptoms including rash, facial flushing, itching, lip swelling, headache, vomiting and fever (Motil & Scrimshaw, 1979; Russell & Maretic, 1986). Biogenic amines such as histamine have been reported to occur in fish and fish products such as fermented fish paste (Fardiaz & Markakis, 1979). While there are a limited number of reports (Fardiaz & Markakis, 1979; Tsai et al., 2006) of fermented fish paste products causing histamine poisoning this may possibly reflect underreporting rather than absence of cases (Tsai et al., 2006).

The tuna used in the production of *Rihaakuru* include skipjack (*Katsuwonus pelamis*), yellowfin (*Thunnus albacores),* big eye (*Thunnus obesus)*, frigate (*Auxis thazard*) and little tunny (*Euthynnus affinis*) or a mixture of these tunas(Naila et al., 2011b). These may be held without chilling for up to 18 h before processing, which could promote the growth and activity of a range of histamine producing bacteria (Arnold et al., 1980; Behling & Taylor, 1982; Taylor et al., 1978). The formation and diffusion of histamine in fish depend upon the growth of histamine forming bacteria that varies depending on which of these bacteria are present (Tao et al., 2009). When fish are held without chilling for 16 h, high histamine levels have been found (Personal Communication, Mohamed Sobah). This supports the work of Taylor and Speckhard (1983), where histamine producing bacteria were reported to grow and multiply enough to produce a toxic concentration of histamine within 24 h.

Live fish contains bacteria on the skin and gills but once the fish is dead the loins become the main locus for bacterial proliferation. Tuna loins contain high concentrations of free histidine which is decarboxylated into histamine by histamine producing bacteria (Yoshinaga & Frank, 1982). Histamine producing bacteria found in Skipjack tuna (*Katsuwonus pelamis*) include: *Clostridium perfringens*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Vibrio alginolyticus*(Arnold et al., 1980). Fish has been reported to contain 10 different biogenic amines: agmatine, cadaverine, histamine, putrescine, phenylethylamine, spermine, spermidine, serotonin, tyramine and tryptamine (Yen & Hsieh, 1991).

To make *Rihaakuru*, tuna is boiled in water (1:1, fish:water) containing approximately 1% salt, for about 45 min, the cooked fish is removed (for further processing into Maldive dried fish) and the remaining liquid is filtered through layers of cheese cloth or a metal/plastic sieve. The filtered liquor is simmered for approximately 4-8 h until it turns into a thick brown paste. The viscosity of the soup is estimated using a spatula to judge the drip flow (Naila et al., 2011b) .

In this paper, the biogenic amine concentration in *Rihaakuru* (28 samples) was determined and some bacteria that produce histamine were isolated.

4.3 Materials and Methods

4.3.1 Sampling

Twenty-eight samples of *Rihaakuru* were obtained from different parts of the Maldives (North, South and Central) and quantitatively analysed for 10 biogenic amines (agmatine, cadaverine, histamine, putrescine, phenylethylamine, spermine, spermidine, serotonin, tyramine and tryptamine). Two samples were randomly (by picking 2 out of 28 samples coded with numbers) selected from the 28 samples of *Rihaakuru*. Isolates from these randomly selected samples (B and T; refer Table 10) were identified by motility test, Gram staining and 16S rDNA sequencing(Naila et al., 2011b).

Twelve out of the 48 isolates previously identified (Naila et al., 2011b) were selected for their ability to produce histamine by incubating in trypticase soy broth containing 2% histidine (TSBH). These isolates were selected as they have not previously been reported as histamine producers. Therefore, it was felt important to determine if any of these isolates could contribute to the level of histamine in the product. The isolates were *Bacillus cibi* Nai10, *B. firmus* Nai17a, *B. flexus* Nai8a, *B. malacitensis* Nai11, *B. massiliensis* Nai5, *B. oleronius* Nai7, *B. polyfermenticus* Nai4, *Clostridium irregular* Nai9, *Enteroccus gallinarum* Nai20, *E. saccharolyticus* Nai9b, *Lysinibacillus boronitolerans* Nai3 and *Virgibacillus halodenitrificans* Nai18. All the other isolates represented species for which strains have the potential to produce histamine and therefore represent a potential risk to the safety of the product. Two of these (*B. thuringiensis* Nai2i and *E. faecalis* Nai13f) were also tested for histamine production to confirm the potential of histamine production of such species.

4.3.2 Standards and reagents

The following biogenic amine standards were obtained from Sigma-Aldrich, New Zealand Ltd.: agmatine, cadaverine, histamine, putrescine, phenylethylamine, spermine, spermidine, serotonin, tyramine and tryptamine. HPLC grade methanol was obtained from Merck Ltd., New Zealand. Diethyl ether, benzoyl chloride, sodium chloride, sodium hydroxide pellets and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich, New Zealand Ltd. MilliQ water was used after vacuum filtration through a 0.45 µm membrane filter.

4.3.3 Biogenic amines standards preparation

Stock solutions of individual biogenic amines were prepared by dissolving 1 mg of amine in 0.1 M HCl. Serial dilutions (0-1 mg/ml) of the stock solutions were used to make calibration curves of each amine.

4.3.4 Sample extraction

Rihaakuru samples were extracted according to the procedure of Hwang, et al. (1997) and Kung et al. (2008) with few modifications, which involved weighing 5 g of sample dissolving in 20 ml of 6% trichloroacetic acid (TCA) and centrifuging for 20 min at 3000 x g, and vacuum filtering through Whatman No. 2 filter paper and make up to 50 ml with 6% TCA.

4.3.5 Derivatization reaction

Biogenic amines standards and fish paste samples (2 ml) were derivatized according to the method of Hwang et al. (1997) and Kung et al. (2008) with a few modifications. This involved the addition of 1 ml of 2 M sodium hydroxide and 10 µl benzoyl chloride, mixing in a flask shaker at high speed for 30 s (Griffin flask shaker, Kumar Group, Delhi, India) and incubating at 30 °C for 40 min. Next, 2 ml of saturated sodium chloride was added followed by 3 ml of diethyl ether. Then, this was mixed for 5 min in a flask shaker at maximum speed (Griffin flask shaker, Kumar Group, Delhi, India). The samples were then centrifuged at 4400 rpm for 15 min (Eppendorf Centrifuge 5702, Bio-Strategy Ltd., Auckland, New Zealand). Two millilitres of upper layer was transferred into new 15 ml centrifuge tubes and evaporated to dryness with nitrogen gas. Residues were dissolved with 1.5 ml of HPLC grade methanol and filtered through a 0.2 µm filter (single use syringe filter, Startorius Stedium Biotch GmbH, Germany) into HPLC vials. Twenty microlitres aliquots were injected into the HPLC for analysis.

4.3.6 HPLC analysis

Biogenic amine levels were determined using Liquid Chromatography with a LiChrospher 100 RP-18 reversed-phase column (5 µm, 125 x 4.6 mm, Merck, Ltd., New Zealand), Waters 2487 Dual λ Absorbance detector (Global Science) at 254 nm, and Alliane Waters 2690 separation model (Global Science). The gradient elution programme was set at a flow rate of 0.8 ml/min and the column oven temperature was set at 25 °C, throughout the analysis. The gradient programme started at 50:50 (methanol/water) for 0.5 min, linearly increasing to 85:15 for the next 6.5 min, held constant at 85:15 for 5 min and decreased back to 50:50 over the next 2 min. Each sample was run for 20 min (Hwang et al., 1997).

4.3.7 Histamine production by bacteria

Fourteen out of 48 strains of bacteria previously isolated from *Rihaakuru* (Table 10) by Naila et al. (2011b) (*Bacillus cibi* Nai10, *B. firmus* Nai17a, *B. flexus* Nai8a, *B. malacitensis* Nai11, *B. massiliensis* Nai5, *B. oleronius* Nai7, *B. polyfermenticus* Nai4, *Clostridium irregular* Nai9, *E. gallinarum* Nai20, *E. saccharolyticus* Nai9b, *E. faecalis* Nai13f, *Lysinibacillus boronitolerans* Nai3, and *V. halodenitrificans* Nai18, *B. thuringiensis* Nai2i), were tested for their ability to produce histamine in trypticase soy broth containing 2% histidine (TSBH). One millilitre of a 48 h culture was transferred into 9 ml of TSBH and incubated at 30 ºC for 48 h. Two millilitres of each bacterial culture broth were derivatized using the same procedures as for *Rihaakuru* sample extract (Section 2.6) and were used (20 µl) for HPLC injection.

Table 10: Identified bacteria isolated from Rihaakuru by 16S rDNA, using internet blast server (http://bioinfo.unice.fr/blast/)

		Nucleotide sequence identification details		
***Rihaakuru* code**	**Strain**	**Closest relative**	**Bioinfo accession number**	**Sequence homology (%)**
B	Nai6	*Acinetobacter lwoffii*	Z93441	99
B	Nai10	*Bacillus cibi*	EU624419	99
B	Nai8a	*B. flexus*	FJ948078	94
B	Nai8b	*B. flexus*	DQ837542	95
B	Nai1	*B. megaterium*	DQ870736	97
B	Nai11	*B. malacitensis*	DQ993673	93
B	Nai5	*B. massiliensis*	EU434513	97
B	Nai7	*B. oleronius*	X82492.1	99
B	Nai4	*B. polyfermenticus*	EF178464	96
B	Nai2a	*B. thuringiensis*	Z84594.1	98
B	Nai2b	*B. thuringiensis*	Z84594.1	92
B	Nai2c	*B. thuringiensis*	Z84594.1	99
B	Nai2d	*B. thuringiensis*	Z84594.1	100
B	Nai2e	*B. thuringiensis*	Z84594.1	94
B	Nai2f	*B. thuringiensis*	Z84594.1	98
B	Nai2g	*B. thuringiensis*	Z84593.1	96
B	Nai2h	*B. thuringiensis*	Z84594.1	96
B	Nai2i	*B. thuringiensis*	Z84594.1	97
B	Nai2j	*B. thuringiensis*	Z84594.1	94
B	Nai2k	*B. thuringiensis*	Z84594.1	98
B	Nai2l	*B. thuringiensis*	Z84594.1	98
B	Nai2m	*B. thuringiensis*	Z84594.1	98
B	Nai2n	*B. thuringiensis*	Z84594.1	96

***Rihaakuru* code**	**Strain**	**Nucleotide sequence identification details**		
		Closest relative	**Bioinfo accession number**	**Sequence homology (%)**
B	Nai2o	*B. thuringiensis*	Z84594.1	98
B	Nai12	*B. subtilis*	Z82044.1	98
B	Nai9a	*Enterococcus saccharolyticus*	Y18357	100
B	Nai3	*Lysinibacillus boronitolerans*	FJ237498	96
T	Nai17a	*B. firmus*	DQ290000	100
T	Nai17b	*B. firmus*	EU707377	100
T	Nai17c	*B. firmus*	EU624421	97
T	Nai2p	*B. thuringiensis*	Z84594.1	99
T	Nai2q	*B. thuringiensis*	Z84594.1	99
T	Nai15	*Brevibacterium casei*	EU652099	98
T	Nai19	*Clostridium irregulare*	FJ384372	96
T	Nai16a	*E. casseliflavus*	Y18295	100
T	Nai16b	*E. casseliflavus*	Y18295	99
T	Nai13a	*E. faecalis*	Y18293	99
T	Nai13b	*E. faecalis*	Y18293	99
T	Nai13c	*E. faecalis*	Y18293	100
T	Nai13d	*E. faecalis*	Y18293	99
T	Nai13e	*E. faecalis*	Y18293	100
T	Nai13f	*E. faecalis*	Y18293	99
T	Nai13g	*E. faecalis*	Y18293	100
T	Nai20a	*E. gallinarum*	Y18160	100
T	Nai20b	*E. gallinarum*	Y18160	100
T	Nai9b	*E. saccharolyticus*	U30931	98
T	Nai14	*Staphylococcus hominis*	L37601	100
T	Nai18	*Virgibacillus halodenitrificans*	AY543168	100

Rihaakuru code:

B: from south of Maldives

T: from central of Maldives

4.4 Results and Discussion

4.4.1 Analysis of biogenic amines

The range of concentrations of biogenic amines found in the 28 *Rihaakuru* samples is outlined in Table 11.

Histamine was detected at the highest concentration (5487 ppm) and, with the exception of sample U which had very low biogenic amine levels, either histamine or spermine were the dominant biogenic amines. Tryptamine was not detected in most of the samples (only 3 samples contained < 5 ppm) and phenylethylamine only occurred at low levels (<25 ppm).

Among the tested samples of *Rihaakuru*, 9 biogenic amines were found in 3 samples, 8 in 10 samples, 7 in 6 samples, 6 in 3 samples, 4 in 5 samples, and 1 biogenic amine was found in 1 sample.

Studies on the biogenic amine content in similar products such as fermented fish paste, report the presence of cadaverine, histamine and tyramine (Fardiaz & Markakis, 1979). Putrescine, spermidine, spermine and serotonin have been reported in chilled catfish fillets (Özogul et al., 2009). The oral toxicity levels for putrescine (2000 ppm), cadaverine (2000 ppm), tryptamine (believed to be similar to tyramine at 2000 ppm), spermidine (600 ppm) and spermine (600 ppm) are all greater than the levels detected in *Rihaakuru* (n = 28) (Table 11), suggesting that these biogenic amines in *Rihaakuru* may not be a safety hazard (Chang et al., 1985; Til et al., 1997).

Fifteen *Rihaakuru* samples (out of 28) exceeded the allowable histamine limit (>50 ppm) (FDA, 2001) and seven samples exceeded the level that is associated with histamine poisoning (>500 ppm) (Gonzaga et al., 2009). Therefore, a food safety hazard may be associated with the histamine levels found in *Rihaakuru*. There is also the possibility of synergistic effects with the presence of putrescine and cadaverine which are believed to enhance the effect of histamine toxicity (Stratton et al., 1991).

Table 11: Contents of biogenic amines in tested Rihaakuru samples (n=28)

Samples	Content of biogenic amines (ppm)									
	Put[a]	**Cad**	**Try**	**Spd**	**Phe**	**Spm**	**His**	**Ser**	**Tyr**	**Agm**
AA	<5	<5	<5	<5	<5	170.61 ± 31.44[b]	ND[c]	<5	<5	ND
BB	7.34 ± 0.16	<5	<5	<5	ND	137.17 ± 6.80	ND	<5	<5	ND
A	ND	<5	ND	14.24 ± 0.33	ND	64.46 ± 50.78	ND	ND	<5	ND
B	31.44 ± 0.01	50.66 ± 0.00	<5	<5	<5	5.60 ± 0.98	731.61 ± 31.97	ND	ND	<5
C	<5	6.25 ± 0.04	ND	<5	<5	6.21 ± 0.45	142.04 ± 6.52	ND	<5	102.58 ± 20.07
D	171.82 ± 19.63	248.98 ± 2.59	ND	23.08 ± 0.06	22.35 ± 9.99	58.64 ± 13.80	2834.64 ± 172.32	33.91 ± 17.85	ND	161.73 ± 4.12
E	<5	5.61 ± 0.00	ND	<5	<5	6.74 ± 0.12	230.56 ± 1.14	ND	ND	8.25 ± 0.87
F	72.83 ± 0.62	151.21 ± 0.63	ND	27.05 ± 0.50	23.94 ± 1.33	44.12 ± 0.24	225.54 ± 0.01	21.52 ± 0.30	ND	61.13 ± 6.67
G	6.12 ± 0.07	7.18 ± 0.02	ND	<5	<5	6.85 ± 0.05	129.76 ± 0.67	ND	<5	8.46 ± 0.19
H	6.15 ± 0.06	6.86 ± 0.04	ND	<5	<5	ND	160.11 ± 0.52	ND	ND	8.40 ± 1.39
I	114.53 ± 0.33	280.51 ± 0.21	ND	79.93 ± 0.12	18.48 ± 0.27	329.32 ± 63.5	5487.14 ± 301.99	55.24 ± 33.63	<5	130.59 ± 1.40
J	201.95 ± 0.74	251.70 ± 0.97	ND	10.26 ± 0.34	13.68 ± 0.16	7.75 ± 1.39	2567.935 ± 14.61	24.69 ± 0.03	ND	63.09 ± 2.66
K	ND	<5	ND	8.72 ± 0.64	ND	118.91 ± 2.51	ND	22.39 ± 3.27	ND	ND
L	ND	<5	ND	9.50 ± 0.06	ND	ND	ND	<5	22.82 ± 0.08	ND
M	121.18 ± 1.85	85.96 ± 0.22	ND	30.65 ± 0.07	<5	ND	146.39 ± 3.31	17.67 ± 2.18	50.76 ± 0.71	48.74 ± 0.96
N	<5	<5	ND	ND	<5	115.70 ± 0.12	6.44 ± 0.80	<5	<5	ND
O	ND	<5	ND	ND	<5	60.97 ± 52.33	ND	ND	<5	ND
P	7.46 ± 1.25	291.10 ± 0.96	ND	8.45 ± 0.35	10.52 ± 0.61	33.75 ± 2.24	1994.23 ± 19.86	42.06 ± 0.087	ND	59.80 ± 1.99
Q	98.27 ± 0.47	113.17 ± 0.40	ND	24.17 ± 0.11	ND	58.73 ± 1.74	642.11 ± 21.16	10.76 ± 0.83	<5	134.93 ± 1.34

Samples	Content of biogenic amines (ppm)									
	Put[a]	**Cad**	**Try**	**Spd**	**Phe**	**Spm**	**His**	**Ser**	**Tyr**	**Agm**
R	290.09 ± 0.68	387.05 ± 0.19	<5	14.57 ± 0.19	14.11 ± 0.52	15.85 ± 0.10	5080.03 ± 16.65	ND	5.13 ± 0.09	ND
S	<5	<5	ND	ND	<5	53.87 ± 0.01	<5	<5	<5	ND
T	7.17 ± 6.18	17.40 ± 0.10	<5	<5	5.74 ± 3.59	15.09 ± 0.41	256.89 ± 2.83	6.15 ± 0.06	ND	8.53 ± 0.21
U	ND	ND	ND	10.33 ± 0.33	ND	ND	ND	ND	ND	ND
V	ND	5.95 ± 5.76	ND	ND	<5	17.66 ± 0.56	9.04 ± 0.68	9.58 ± 0.14	<5	ND
W	<5	<5	ND	ND	<5	38.49 ± 0.55	ND	10.01 ± 0.05	<5	ND
X	66.16 ± 0.10	17.13 ± 16.84	<5	67.33 ± 0.05	19.82 ± 0.89	379.62 ± 13.19	85.22 ± 2.83	91.28 ± 1.59	25.71 ± 4.24	ND
Y	ND	ND	ND	ND	<5	19.94 ± 12.08	15.63 ± 1.79	ND	<5	ND
Z	<5	ND	ND	<5	<5	118.36 ± 0.73	ND	<5	<5	16.80 ± 23.77

[a] Put: putrescine; Cad: cadaverine; Try: tryptamine; Spd: spermidine; Phe: phenylethylamine; Spm: spermine; His: histamine; Ser: serotonin; Tyr: tyramine; and Agm: agmatine.

[b] Mean ± Standard deviation (n=4).

[c] ND: not detected.

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4.4.2 Bacteria isolated from Rihaakuru

In our previous work we isolated and identified 48 (Table 10), mostly Gram positive, bacterial strains as part of the microflora of *Rihaakuru* (Naila et al., 2011b). Eight of these species had previously been reported as producing histamine: *Acinetobacter lwoffii*, *Bacillus megaterium*, *B. subtilis*, *B. thuringiensis*, *Brevibacterium casei*, *Enterococcus casseliflavus*, *E. faecalis*, and *Staphylococcus hominis*. Isolates of species that are known to have the potential to produce histamine are clearly a concern in this product (even though we acknowledge that not all strains produce histamine) and therefore a focus in controlling histamine in *Rihaakuru*. Among the eight species, two species, *B. thuringiensis* and *E. faecalis* were found abundant in the tested samples (Table 10). Therefore one strain from each of these species was blindly selected to examine for histamine production: *B. thuringiensis* Nai2i and *E. faecalis* Nai13f. These two strains produced low concentrations of histamine (Table 12) agreeing with other researchers (Burdychova & Komprda, 2007; H. H. Hsu et al., 2009;S. H. Kim et al., 2004). The results confirmed that these strains are a potential concern in the safety of the product, therefore no further isolates of these species were tested.

Isolates of species for which there are no records of histamine production were then screened to determine if they were potential risks in this product. However, we acknowledge that determining histamine forming ability of all 48 isolates identified from *Rihaakuru* would be ideal-due to budget constraints we chose to focus on the key information.

Table 12: Histamine production by selective bacterial strains isolated from Rihaakuru

Bacteria	**Histamine (ppm)**
Bacillus cibi Nai10	2.38 ± 0.19 [a]
B. flexus Nai 8a	1.10 ± 0.18
B. firmus Nai17a	1.73 ± 0.90
B. malacitensis Nai11	0.36 ± 0.07
B. massiliensis Nai5	6.65 ± 0.67
B. oleronius Nai7	1.73 ± 0.57
B. polyfermenticus Nai4	5.87 ± 1.82
B. thuringiensis Nai2i	0.28 ± 0.12
Clostridium irregulare Nai19	4.64 ± 1.45
Enterococcus faecalis Nai13f	0.79 ± 0.20
E. gallinarum Nai20a	ND[b]
E. saccharolyticus Nai9b	ND
Lysinibacillus boronitolerans Nai3	4.28 ± 0.29
Virgibacillus halodenitrificans Nai18	3.66 ± 1.39

[a] Mean ± Standard deviation (n=4)

[b] ND, not detected.

The twelve that have not been reported as potential histamine producers were screened for histamine production: *B. cibi* Nai10, *B. firmus* Nai17a, *B. flexus* Nai8a, *B. malacitensis* Nai11, *B. massiliensis* Nai5, *B. oleronius* Nai7, *B. polyfermenticus* Nai4, *Clostridium irregular* Nai19, *E. gallinarum* Nai20a, *E. saccharolyticus* Nai9b, *Lysinibacillus boronitolerans* Nai3, and *V. halodenitrificans* Nai18. Among these the highest histamine producer was found to be *B. massiliensis* Nai5 (6.65 ppm) and *B. polyfermenticus* Nai4 (5.87 ppm), while *B. malacitensis* produced the least (<0.5 ppm); and two isolates did not produce detectable levels of histamine (*E. gallinarum* Nai20 and *E. saccharolyticus* Nai9b) (Table 12). Of all the species that we isolated from *Rihaakuru*, *Staphylococcus homini* is the only one ever reported to produce high levels of histamine (Table 13).

While the focus of our study has been histamine production by the microflora isolated from the product, the histamine found in *Rihaakuru* is most likely to have originated from Gram negative bacteria growing in the fish before processing or within the fish

Table 13: Identified potential histamine forming bacterial species from Rihaakuru basing on other researches

Bacteria	Literature reported histamine	Reference
Acinetobacter lwoffii	Present	(Hoštacká & Klokočníková, 2002)
	38.8 - 151 ppm	(Kim et al., 2002)
	0-42 ppm	(López-Sabater et al., 1996)
Bacillus megaterium	8.1 ppm	(Tsai et al., 2006)
	1.08 ppm	(Lee et al., 2005)
	12.6-16.5 ppm	(H. F. Kung et al., 2007)
B. subtilis	11.7-32.4 ppm	(Tsai et al., 2007b)
	20.4-39.4 ppm	(H. F. Kung et al., 2007)
	13.4-17.5 ppm	(Tsai et al., 2007a)
	13.3-13.4 ppm	(H. F. Kung et al., 2007)
B. thuringiensis	10.0 – 19.1 ppm	(H. H. Hsu et al., 2009)
	3.79 ppm	(S. H. Kim et al., 2004)
Brevibacterium casei	0.07 ppm	(Koei, 1996)
Enterococcus casseliflavus	1 isolate out of 5 is histamine former	(Roig-Sagués et al., 2002)
E. faecalis	Detected histamine gene (HDC-histidine decarboxylase) 15 ppm	(Burdychova & Komprda, 2007) (Economou et al., 2007)
Staphylococcus hominis	1.0 - 3050 ppm	(Economou et al., 2007)
	3.97 - 4.88 ppm	(S. H. Kim et al., 2004)

soup early in the manufacture of this product. Most common histamine producers are reported as Gram negative bacteria such as *Morganella morgani*(Kim et al., 2000) and *Photobacterium phosphorium*(Kanki et al., 2004). These heat sensitive bacteria would be inactivated during the cooking of the tuna fish and therefore would not be recovered from the final product.

Controlling histamine in *Rihaakuru* is likely to be difficult. Recommended chilled handling of fish to prevent the growth of histamine producing bacteria is difficult in the Maldives where refrigeration is too costly for the community. Alternative methods of control, such as degrading the histamine once it has been formed, will be difficult as histamine is stable to heat and chemical treatment. Histamine reducing bacteria possessing the enzyme diamine oxidase (DAO) that reduces histamine or purified DAO enzyme itself may be used as an alternative control method (Naila et al., 2010).

4.5 Conclusions

Ten biogenic amines were present in *Rihaakuru* among which histamine was found in the highest concentration that may harm human health. Most of the isolated bacteria from *Rihaakuru* that were tested for histamine production were weak histamine formers. Therefore, the high concentration of histamine found in *Rihaakuru* most likely originated during the early steps of manufacture.

4.6 Acknowledgement

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Aishath Naila

Name/Title of Principal Supervisor: Associate Professor Steve Flint

Name of Published Research Output and full reference:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., & Meerdink, G. (2011). Biogenic amines and potential histamine - forming bacteria in Rihaakuru (a cooked fish paste). Food Chemistry, 128(2), 479-484.

In which Chapter is the Published Work: Chapter 4

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Chapter 5

Histamine Stability in *Rihaakuru* at -80°C, 4°C and 30°C

The work presented in this chapter has been submitted for peer review:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., Meerdink, G., (Submitted). Histamine stability in *Rihaakuru* at -80°C, 4°C and 30°C. *Food Chemistry*.

5.1 Abstract

Rihaakuru is a shelf stable fish paste product formed from a fish soup prepared from tuna. Histamine contamination is a food safety issue with this product that is manufactured from tuna fish that has been temperature abused. Histamine concentrations decreased between 31-73% in *Rihaakuru* stored for 10 months at either -80°C, 4°C or 30°C. This appears to be a property of the product as histamine solutions are reported to be stable, at least under frozen storage. The risk of histamine food poisoning due to *Rihaakuru* may reduce during the storage of the product.

Keywords:*Rihaakuru*, histidine, histamine, enzymes, bacteria.

5.2 Introduction

Histamine is a biogenic amine formed by bacterial decarboxylation of the amino acid, histidine. Histamine at high concentration (500 ppm) causes histamine poisoning which typically lasts up to 24 h producing allergy-like symptoms such as facial flushing, nausea and headache. Histamine poisoning can be diagnosed by testing food or the patient's plasma for the presence of histamine. In the USA, 5% of food borne diseases are from histamine poisoning and this is believed to be under-reported (Jantschitsch et al., 2011). Effective control measures are needed for preventing and eliminating high concentrations of histamine in food (Jantschitsch et al., 2011).

A variety of foods naturally contain histamine. These include fish and fish sauce, cheese, wine (Naila et al., 2010; Shalaby, 1996) and marine macroalgae (de Alencar et al., 2011). *Rihaakuru* is a shelf-stable cooked tuna paste that also contains histamine(Naila et al., 2011a, 2011b). Histamine is reported to be stable during cooking, hot smoking or frying (Lehane & Olley, 2000), cold storage, and sterilization (Marwaha & Johnson, 1986).

Studies on histamine levels during the storage of raw food products such as fish (Chotimarkorn, in press), grapes for wine production (Cecchini & Morassut, 2010) or ready-made products such as wine (Kim et al., 2011) and tuna dumpling (Lee et al., 2011) have shown that histamine production is delayed under low temperature storage (4°C and -20°C) and elevated under high temperature storage (Lee et al., 2011). Few studies have focused on histamine stability in shelf stable ready-to-serve products. Fish meal was stored at 4-8°C for 40 months and a 26% reduction was recorded after one year, 28% after two years and 50% after three years from an initial value of 5.1g/kg. Histamine concentration remained stable when the same samples were transferred to 24°C and exposed to natural light for 3 months (Macana et al., 2006). Histamine concentration in Asian fish sauce was stable after one year storage at 4-8°C, and for 6 months at 18-22°C (Schröder et al., 2011). These studies indicate that the histamine concentration is stable or decreases only slightly during storage and that stability increases at low temperatures (<5°C). However, the degree of stability may depend on the type of food. For example, the fish meal showed a reduction in histamine after one year but no reduction in histamine was shown in the fish sauce, with both products stored at 4-8°C.

Rihaakuru is often stored for longer than a year at ambient temperatures (30°C in the Maldives) and is stable in terms of microbial growth due to the water activity and pH(Naila et al., 2011b) . The stability of histamine in *Rihaakuru* at this temperature is unknown. Therefore, the focus of this study was to determine the stability of histamine in *Rihaakuru* at three different temperatures, -80°C, 4°C or 30°C for 10 months. *Rihaakuru* is typically stored at ambient temperatures of around 30°C although a few individuals store the product at 4°C (personnel experience). A lower temperature -80°C was included as this is a typical temperature used for the storage of samples for research.

5.3 Materials and Methods

5.3.1 Rihaakuru

Rihaakuru samples AN-1, AN-2 and AN-3, were randomly selected (n=3), divided into 3 equal portions (approximately 100 g) and transferred into sterile plastic bottles with plastic caps, covered with aluminium foil. Each batch of 3 samples were then stored at either -80°C, 4°C, or 30°C for 10 months. Samples were taken (1 g) at 0 and 10 months.

5.3.2 Chemical and microbial and analysis

Histamine was analysed in 1g of *Rihaakuru* according to the previously described method(Naila et al., 2011a).

The total aerobic and anaerobic counts of AN-1, AN-2 and AN-3 were determined as described previously (Naila et al., 2011b).

The pH of the samples was determined using Orion pH meter (Model 420A) fitted with Orion Model 915BN probe (Orion Research Inc., Boston, USA). Water activity was measured using a water activity meter (Decagon CX-2; Formular Foods Corporation Ltd, Christchurch, New Zealand).

Samples of *Rihaakuru* AN-1, were randomly selected to measure the amino acid and protein concentration of the sample, at the start of the trial and after 10 months storage at 30°C. Samples were analyzed by the Nutrition Laboratory, Massey University, New Zealand.

5.3.3 Statistical analysis

All data were analyzed using paired t-test and One-way analysis of variance (ANOVA). The former was used to test significant difference for each sample between the initial (0 months) and final (10 months) histamine concentration during storage and the latter was used to find the significance difference of histamine between temperatures. Statistical analysis was performed using PASW statistics 18 (SPSS Inc., IBM Company, Chicago, Illinois).

5.4 Results and Discussion

The initial histamine values for AN-1, AN-2, and AN-3 were 348.20 ppm, 23.22ppm, and 193.93ppm, respectively. AN-1, AN-2 and AN-3 had total aerobic counts of 1.36, 1.87 and 2.13 $\log_{10}$ cfu/g and total anaerobic counts of 0.83, 1.36 and 1.91 $\log_{10}$ cfu/g, respectively. Water activity readings for AN-1, AN-2 and AN-3 at the start of the storage trial were 0.58, 0.80, and 0.68, and after 10 months were 0.57, 0.80, and 0.67 respectively, showing stability in water activity over 10 months' storage. The initial pH readings of AN-1, AN-2 and AN-3 were 5.79, 5.76, and 6.00 and after 10 months 5.75, 5.64, and 5.80, respectively, showing reasonable stability in the pH throughout the 10 month storage period. The protein and amino acid content of AN-1 (Table 14) were also relatively stable during the 10 month storage period.

Histamine levels reduced significantly ($p<0.05$) in *Rihaakuru* samples stored at -80°C, 4°C or 30°C for 10 months, irrespective of the storage temperature (Table 15).

Table 14: Amino acid and protein content in Rihaakuru AN-1 before and after storage at 30°C

Protein and amino acids	AN-1	AN-1

	Initial mg/100mg	**10th month** mg/100mg
Aspartic acid	2.51	2.52
Threonine	1.28	1.31
Serine	1.10	1.13
Glutamic acid	4.37	4.31
Proline	2.58	5.01
Glycine	5.13	4.95
Alanine	3.32	2.71
Valine	1.35	1.39
Isoleucine	0.95	0.97
Leucine	2.00	1.97
Tyrosine	0.65	0.64
Phenylalanine	0.97	0.99
Histidine	8.15	8.61
Lysine	2.36	2.18
Arginine	2.31	2.26
Taurine	1.35	1.36
Protein %	*67.3*	*66.8*

Rihaakuru stored at -80°C for 10 months showed a reduction in histamine of 73.68%, 31.57%, and 60.58% for samples AN-1, AN-2, and AN-3, respectively. This is the first report identifying significant changes in histamine concentration in food stored at -80°C (Table 15). The histamine reduction seemed to be less pronounced when the concentration was low (AN-2) and more when the concentration was high (AN-1 and AN-3). Marshik, Siyawosh, Tebbett and Hendeles (1999) exposed histamine diphosphate solution stored at 4°C to room light at 20°C until seven days; first day exposed to 6 h, and returned the solution back to 4°C and repeated as before after 2 days and then after 4 days. Histamine reduction varied depending on the concentration of the solution; low concentrations of histamine (the lowest tested was 125 ppm) were more stable over time than the relatively high concentrations (> 125 ppm) in which reduction varied and was rapid. Histamine diphosphate solution stored at -20C for one year did not decrease in histamine concentration (Marshik et al., 1999; Marwaha & Johnson, 1986) and also we observed that histamine dihydrochloride stored at -80°C for 1 month was stable (data not shown). Therefore, histamine decreases in *Rihaakuru* in frozen storage likely reflect some properties in the product. Y. C. Lee et al.

(2011)demonstrated that histamine was stable in tuna dumplings stored at -20°C, contrasting with this study. However, the products are different.

Histamine reduction at -80°C is difficult to explain. Literature support on this finding is not available. Therefore, further investigation is required to clarify the mechanism of histamine reduction at -80°C, which may include investigating the structure of histamine before and after storage of the product at -80°C to see if any adsorption or changes occurred. It appears unlikely that histamine degrading enzymes are very active at this temperature although this has not been tested. The adsorption of histamine to another compound in the product may bind the histamine so it is no longer able to be extracted and quantified. Histamine adsorbs to fuller's earth or magnesium trisilicate under acidic conditions (Gaddum, 1948) and histamine may adsorb to histidine between pH 4.5 and 6.5 (Phelps & Peters, 1929). Another possible explanation for histamine reduction may be chemical oxidation (Kuda et al., 2012). Alternatively, histamine may be diffusing into protein as was postulated in salted anchovies during ripening (Hernández-Herrero et al., 1999). *Rihaakuru* was found to contain 0.004% copper (Naila et al., 2011b) that may enhance histamine degradation as the activity of histamine oxidizing enzyme is enhanced in the presence of metal ions such as copper (Buffoni, 1966).

At 4°C over 10 months histamine in *Rihaakuru* (AN-1, AN-2 and AN-3) reduced significantly ($p<0.05$) between 35.87-63.79% (Table 15), Macana et al. (2006) documented that histamine in fish meal reduced by 26% at 4-8°C of histamine over a year, in general agreement with our results although we found higher reductions. In contrast, histamine was stable in Asian fish sauces stored at 4-8°C for 1 year (Schröder et al., 2011). N. H. Nielsen et al. (1988) demonstrated that histamine dihydrochloride was stable for 6 months at 4°C and -18°C.

Table 15: Histamine stability in Rihaakuru stored for 10 months

Sample	**Temperature (°C)**	**Initial histamine concentration[b]**	**Final histamine concentration (ppm and % reduction)**	**p-value[c]**	**Water activity (aw)**	**pH**
AN-1	-80	348.20 ± 13.45	91.64 ± 6.35[a] (73.68)	< 0.0001	[e]0.58-0.59	[e]5.79-5.76
	4		126.07 ± 3.62[b] (63.79)	< 0.0001	0.58-0.60	5.79-5.75
	30		224.91 ± 10.21[c] (35.41)	< 0.0001	0.58-0.51	5.79-5.74
AN-2	-80	23.22 ± 1.98	15.89 ± 0.28[d] (31.57)	0.007	0.80-0.82	5.76-5.66
	4		14.89 ± 0.83[d] (35.87)	0.008	0.80-0.80	5.76-5.62
	30		15.41 ± 0.22[d](33.63)	0.004	0.80-0.79	5.76-5.63
AN-3	-80	193.93 ± 2.07	76.45 ± 2.87[e](60.58)	< 0.0001	0.68-0.68	6.00-5.81
	4		111.31 ± 4.73[f](42.60)	< 0.0001	0.68-0.69	6.00-5.81
	30		88.21 ± 4.01[g](54.51)	< 0.0001	0.68-0.63	6.00-5.79

[a] Mean ± standard deviation (n = 3)

[b] Mean with different superscript letter within a column are significantly different at $p<0.05$;

[c] P-value for differences between initial and final concentrations

[d] Significantly not different between temperatures ($p>0.05$)

[e] initial (0 months)- final (10 months) data

Rihaakuru stored at 30°C for 10 months showed a reduction in histamine of 35.41%, 33.63%, and 54.51% for samples AN-1, AN-2, and AN-3, respectively (Table 15). Pratter, Marwaha, Irwin, Johnson and Curley (1985) reported that histamine diphosphate solution stored at 12°C was stable for up to 4 months while the following year Marwaha and Johnson (1986) found that it was stable for 8 months at 12°C under normal exposure to fluorescent light. When histamine diphosphate was stored at 60°C for 5 days histamine only reduced by 6% (Marwaha et al., 1985) and no reduction was observed at 20°C stored for one week (Marshik et al., 1999). We observed that histamine dihydrochloride solution at 30°C for 1 month was stable.

The different results from the different products suggest that the reduction of histamine in *Rihaakuru* is due to some components of the product. That histamine in *Rihaakuru* at ambient temperatures (about 30°C in the Maldives) appears to reduce during long term (10 month) storage; it appears that the product is safer after keeping it for an extended period of time (> 6 months).

The histamine decrease in all the samples at 30°C and 4°C may be due to the presence of histamine oxidizing enzymes (Cecchini & Morassut, 2010). Our results differ from those of Schröder et al (2011) on fish sauce where at 18-22°C, histamine was found to be stable for 6 months.

The AN-1 and AN-3 histamine reduction was significant ($p<0.05$) between temperatures -80°C, 4°C and 30°C but AN-2 histamine reduction was not dependent on the temperature of storage ($p = 0.068$) with the same amount of reduction occurring at all temperatures tested. In AN-1 most histamine reduction occurred at -80°C (73%) and least at 30°C (35%) and in AN-3 most at -80°C (60%) and least at 4°C (42%).

5.5 Conclusion

Histamine in *Rihaakuru* reduced over time and this reduction occurred at all the temperatures tested. It appears that a particular property of this product is responsible for this reduction. The risk of histamine food poisoning due to *Rihaakuru* may reduce as the product is stored.

5.6 Acknowledgement

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Name of Candidate: Aishath Naila

Name/Title of Principal Supervisor: Associate Professor Steve Flint

Name of Published Research Output and full reference:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., & Meerdink, G. (submitted). Histamine stability in Rihaakuru at -80°C, 4°C and 30°C. Food Chemistry.

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Chapter 6

Histamine Degradation by Diamine Oxidase, *Lactobacillus* and *Vergibacillus halodonitrificans* Nai18

The work presented in this chapter has been submitted for peer review:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., Meerdink, G., (Submitted). Histamine degradation by diamine oxidase*, Lactobacillus* and *Vergibacillus halodonitrificans* Nai18. *Food Chemistry.*

6.1 Abstract

The aim of this work was to explore suitable methods (bacteria/enzyme) to degrade histamine as an alternative method for the control of histamine in food. Histamine degradation by bacteria was studied in trypticase soy broth containing 500 ppm of histamine at 30°C for 9 days. The bacteria studied were *Lactobacillus sakei* (AGR 37, AGR 46, Lb 706), *Arthrobacter crystallopoietes* DSM 20117 and *Vergibacillus halodonitrificans* Nai18. All *L. sakei* strains reduced histamine to about 50% while *V. halodonitrificans* Nai18 degraded histamine by 30%. *A. crystallopoietes* DSM 20117 did not degrade histamine. The enzyme diamine oxidase was studied for histamine degradation in 0.5 M phosphate buffer containing 500 ppm of histamine for 10 h, and in tuna soup under salt concentration of 1% and pH 6.0. The enzyme completely degraded histamine in both buffer and soup. The enzyme is the most suitable method for histamine degradation and has potential to reduce histamine content in food. DAO reduction of histamine can be applied to a food system to prevent histamine food poisoning.

Keywords: *Vergibacillus halodonitrificans* Nai18, DAO, histamine degradation, *Lactobacillus sakei*, *Arthrobacter crystallopoietes*, HPLC

6.2 Introduction

Scombroid poisoning is caused mainly by the consumption of fish, fish products and fermented foods that are processed under poor hygiene and/or stored at elevated temperatures. This allows the growth of histamine producing bacteria that may result in dangerous histamine levels (> 500 ppm) (Shalaby, 1996). The symptoms of scombroid poisoning are urticaria, flushing, itching, vomiting, diarrhoea and nausea (Arnold & Brown, 1978; Silva et al., 2011). Histamine poisoning is increasing internationally in countries including the USA, Great Britain, Japan (Tao et al., 2011b) and Taiwan (Hwang et al., 1995). Many incidents may be unreported as official records are not kept by all countries and many people do not report this illness. Traditional foods such as fish sauce from Korea and Malaysia (Moon et al., 2010; Zaman et al., 2010), fermented fish paste from Taiwan (Fardiaz & Markakis, 1979; Tsai et al., 2006), and a cooked fish paste (*Rihaakuru*) from the Maldives (Naila et al., 2011a) have been reported to contain high levels of histamine. While *Rihaakuru* is generally stable in terms of bacterial deterioration (Naila et al., 2011b), bacterial growth in the fish used to manufacture this product, has been speculated to be responsible for the high histamine levels in the product (Naila et al., 2011a) as refrigeration is not widely available in the Maldives. In the absence of effective temperature control alternative methods are needed to control histamine levels in *Rihaakuru,* which continues to be a popular and widely consumed stable food. Two alternative control methods (Naila et al., 2010) that have the potential to be applied to the raw material extract (tuna soup) used to manufacture *Rihaakuru*, are the addition of microorganisms or enzymes that degrade histamine. The by-products of the histamine degradation are aldehyhyde, ammonia and hydrogen peroxide (Blaschko, 1974), as shown in reaction equation **4**.

$$\mathrm{R.CH_2.NH_2 + H_2O + O_2 \rightarrow R.CHO + NH_3 + H_2O_2} \qquad \text{........(4)}$$

Some bacteria have been reported to reduce histamine, including *Staphylococcus carnosus* FS19 and *Bacillus amyloliquefaciens* FS05 (Zaman et al., 2011), *Lactobacillus sakei*(Dapkevicius et al., 2000), and *Vergibacillus sp.* SK33 (Yongsawatdigul et al., 2007). Diamine oxidase (DAO) has been found to degrade histamine from fish slurry more effectively than histamine reducing bacteria (Dapkevicius et al., 2000). The research reported in this paper aims to identify possible histamine reducing bacteria and to test the ability of DAO to reduce histamine in a model system (phosphate buffer system) as well as the cooked tuna soup used in the manufacture of *Rihaakuru*.

6.3 Materials and Methods

6.3.1 Materials

All the bacterial species chosen for this study were based on the literature reports of their potential histamine degradation. *Lactobacillus sakei* strains (AGR 37, AGR 46, Lb 706) were isolates from dairy products kindly provided by Fonterra^{TM} (Palmerston North, New Zealand). *Arthrobacter crystallopoietes* DSM 20117 was purchased from DSMZ (GmbH, Germany). *Vergibacillus halodonitrificans* Nai18 was isolated from *Rihaakuru* as described in our previous work (Naila et al., 2011a). Histamine dihydrochloride ($C_5H_9N_3.2HCl$) as the source of histamine and 99% pure benzoyl chloride (C_7H_5ClO) were obtained from Acros Organics (New Jersey, USA). Salt (NaCl, Labserv) and hydrochloric acid (HCl, Labserv) were used to adjust the salt and pH of the tuna soup and were purchased from Biolab (Biolab (Aust) Ltd, Victoria, Australia). Sodium hydroxide (NaOH) pellets were obtained from BDH Limited Poole England (Great Britain). Distilled water (H_2O) was used to prepare all the solutions. Diamine oxidase (DAO: EC 1.4.3.6) from porcine liver (0.18 unit/mg) was obtained from Sigma-Aldrich (ST. Louis, USA).

Chilled yellowfin tuna (*Thunnus albacares*) loins (4.84 kg) were purchased from Ocean Fisheries (Palmerston North, New Zealand) and transferred to the Food Technology Pilot Plant freezer (-18°C) of Massey University until required to produce the tuna soup base for *Rihaakuru*. To obtain tuna soup from yellowfin tuna, the tuna were cooked in salt as described in our previous work (Naila et al., 2011b).

6.3.2 Histamine degradation by bacteria

Five bacteria [*Lactobacillus sakei* (AGR46, AGR37, Lb706), *Arthrobacter crystallopoietes* DSM 20117, *Vergibacillus halodonitrificans* Nai18] were grown in trypticase soy broth (TSB) for 24 h at 30ºC, then 10% of the cultures were inoculated into TSB containing histamine dihydrochloride (500 ppm). The samples were incubated at 30ºC for 216 h. Samples were taken at 0, 24, 50, 96 and 216 h. Two millilitres of samples were stored at -80°C until histamine analysis.

6.3.3 Histamine degradation by DAO

DAO activity test

The DAO activity was tested at optimum conditions (pH 7.0 and 37°C) based on Dapkevicius et al. 2000, with little modification. Briefly, 633.5 units/L enzymes (units calculated based on the manufacture's label; 0.18 unit/mg) were added into 0.5 M phosphate buffer containing 0.05 g/L (50 ppm) histamine dihydrochloride. The samples were incubated at 37°C and agitated at 100 rpm for 5 h (Model Amper Chart Multitron II, Infors HT, Total Lab Systems Ltd, Christchurch, New Zealand). Samples (2 mL) were taken at 0, 0.5, 1, 3, and 5 h and immediately boiled for 30 min. The samples were stored at -80°C until histamine analysis.

Selection of DAO concentration

The ability of DAO to degrade histamine was tested at several levels (between 633.5 to 2534 units/L) to obtain a suitable concentration to degrade 500 ppm of histamine by at least 50%. A 0.5 M phosphate buffer model system was prepared by adding 500 ppm of histamine at pH 6 and salt 1% and this was incubated for 10 h at 37°C and agitated at 100 rpm (Model Amper Chart Multitron II, Infors HT, Total Lab Systems Ltd, Christchurch, New Zealand) after adding the enzyme. The pH and salt concentrations used were selected based on the value of these parameters present in the tuna soup used in the manufacture of *Rihaakuru* since the authors are interested in applying this to the manufacture of *Rihaakuru*. The initial enzyme concentration and the experimental procedure were selected based on the work of Dapkevicius et al. (2000), with a few modifications. Samples were taken at 0, 0.5, 1, 3, 5 and 10 h and immediately boiled for 30 min to inactivate the enzyme. The samples were transferred to a -80°C freezer until histamine analysis.

Histamine degradation in buffer and tuna soup

Based on the result of the selection of DAO concentration experiment where 2534 units/L of DAO were found to degrade histamine by 50%; this concentration was used to run the experiment in the buffer system and in the tuna soup. The experimental procedures were similar those in the above section.

6.3.4 Histamine analysis

Histamine analysis was carried out by the methods of D. F. Hwang et al. (1997) and Kung et al. (2008) with slight modifications as described in our previous work(Naila et al., 2011a). Histamine degradation was monitored by measuring the percentage of histamine reduction in the samples over time.

Preparation of histamine standards

A series of histamine standards (0-500 ppm) were prepared by weighing appropriate amounts of histamine dihydrochloride and dissolving in 0.1 M hydrochloric acid. The standards were derivatized using benzoyl chloride and analyzed using high performance liquid chromatography (HPLC). The data were used to plot a calibration curve for histamine quantification.

Derivatization

Samples and histamine standards (2 ml) were derivatized by adding 1 ml of 2 M sodium hydroxide followed by 10 µl benzoyl chloride, and stored at 30ºC for 40 min. Benzoylation was stopped by mixing with 2 ml of saturated sodium chloride, and histamine was extracted with the addition of 3 ml of diethyl ether followed by mixing in a mixer (Griffin flask shaker, Kumar Group, Delhi, India) for 5 min, and centrifugation at 3000 x g for 15 min (Eppendorf Centrifuge 5702, (Eppendorf, Hamburg, Germany). The organic layer was then dried by passing through a nitrogen stream and the residue dissolved in 1.5 ml of HPLC grade methanol. The samples were mixed using the mixer

(Griffin flask shaker, Kumar Group, Delhi, India) for 30 s followed by centrifugation for 5 min at 3000 x g. The samples and the standards were filtered through a nylon membrane filter (0.2 µm, Startorium Stedim Biotch, Germany) into HPLC vials. A 20 µl volume was injected into the HPLC system described below, run for 28 min, and monitored at a 254 nm. Each assay was carried out in triplicate.

HPLC system

Histamine was analyzed by an HPLC system that consisted of a Waters 2487 Dual λ Absorbance detector (Global Sience) at 254 nm, Alliane Waters 2690 separation model (Global Science), using a LiChrospher 100 RP-18 reversed-phase column (5 µm, 125x 4.6 mm, Merck, Ltd., New Zealand). The gradient elution program was set at a flow rate of 0.8 ml/min and the column oven temperature was set at 25°C throughout the analysis. The gradient program started at 50:50 (methanol/water) for 0.5 min, linearly increasing to 85:15 for the next 6.5 min, held constant at 85:15 for 5 min and decreased back to 50:50 over the next 2 min (Hwang et al., 1997).

6.4 Results and Discussion

6.4.1 Histamine degradation by bacteria

The *L. sakei* strains (AGR46, AGR37, Lb706) (Figure 2) and *V. halodonitrificans* Nai18 (Figure 3) degraded histamine in TSB containing 500 ppm of histamine by approximately 40-50%, and 30% respectively. Most of the reduction in histamine occurred within the first 50 h. After 50 h the bacteria may have reached stationary phase and viable cells may have started to die. Interestingly, the results using *V.*

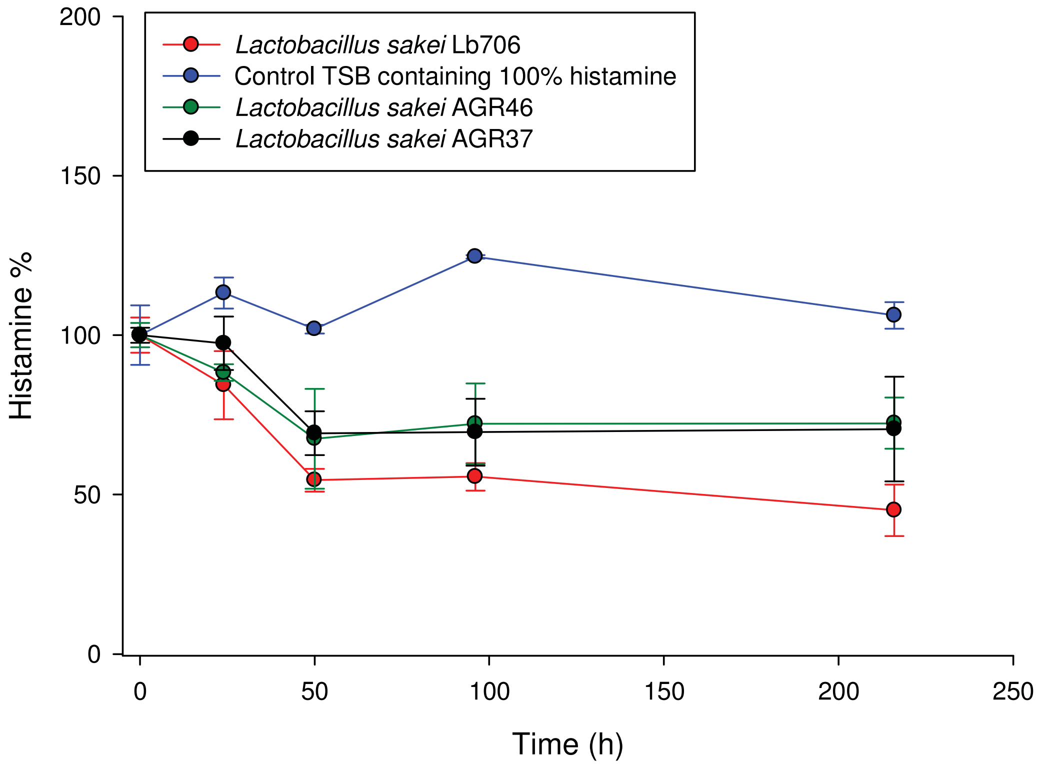

Figure 2: *Lactobacillus* species degradation of histamine in TSB containing 500 ppm of histamine. Error bars representing mean standard deviation of n = 3.

halodonitrificans Nai18 isolated from *Rihaakuru* (Naila et al., 2011b) are similar to those of Yongsawatdigul et al. (2007) using *V. halodonitrificans* SK33 isolated from Thai fish sauce which showed a 50% degradation. The *Lactobacillus* strains were previously tested for histamine production prior to the histamine degradation tests and found negative for histamine formation (data not shown).

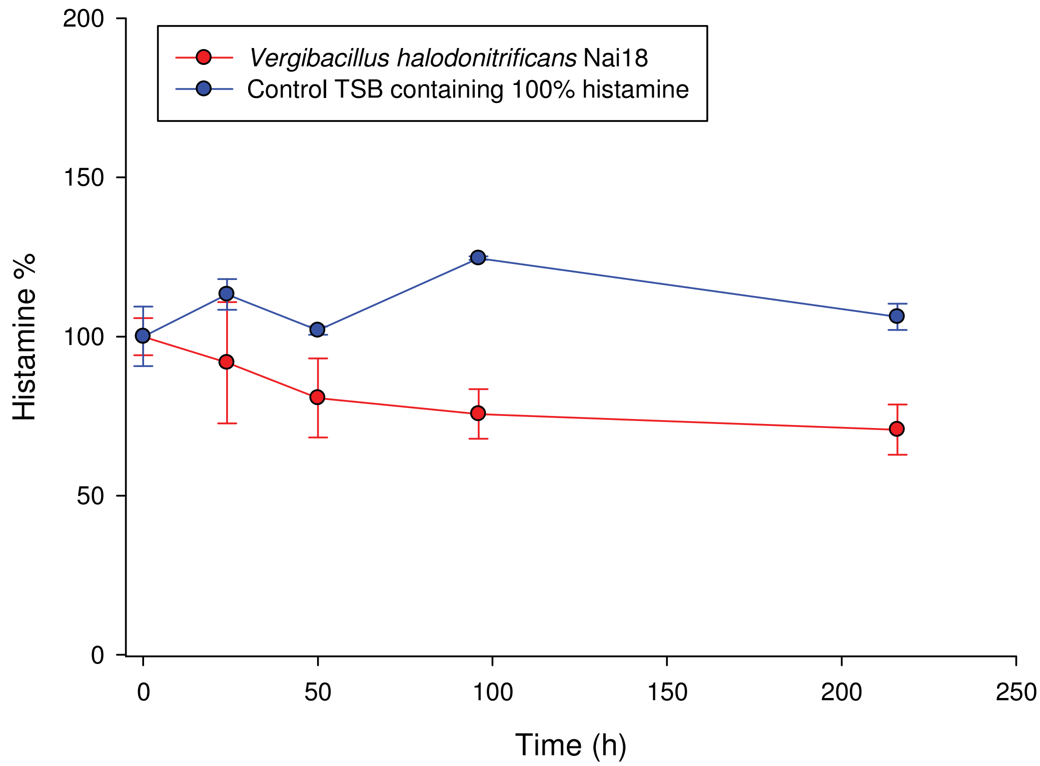

Figure 3: *Vergibacillus halodonitrificans* Nai18 degradation of histamine in TSB containing 500 ppm histamine. Error bars represent mean standard deviation of n = 3.

Arthrobacter crystallopoietes DSM 20117 did not degrade histamine (Figure 4). This was interesting as DAO isolated from *A. crystallopoietes* KAIT-B-007 had previously been reported to degrade histamine by 100% (Sekiguchi et al., 2004). Possibly *A. crystallopoietes* DSM 20117 may not possess the DAO enzyme or the enzyme may be inactive. Though some bacterial species have the ability to degrade histamine, not all the strains of the same species are able to degrade histamine (Zaman et al., 2010). It would be interesting to test the ability of *A. crystallopoietes* KAIT-B-007 rather than using the isolated DAO enzyme. Unfortunately, we could not obtain this bacterium and therefore selected another strain for testing.

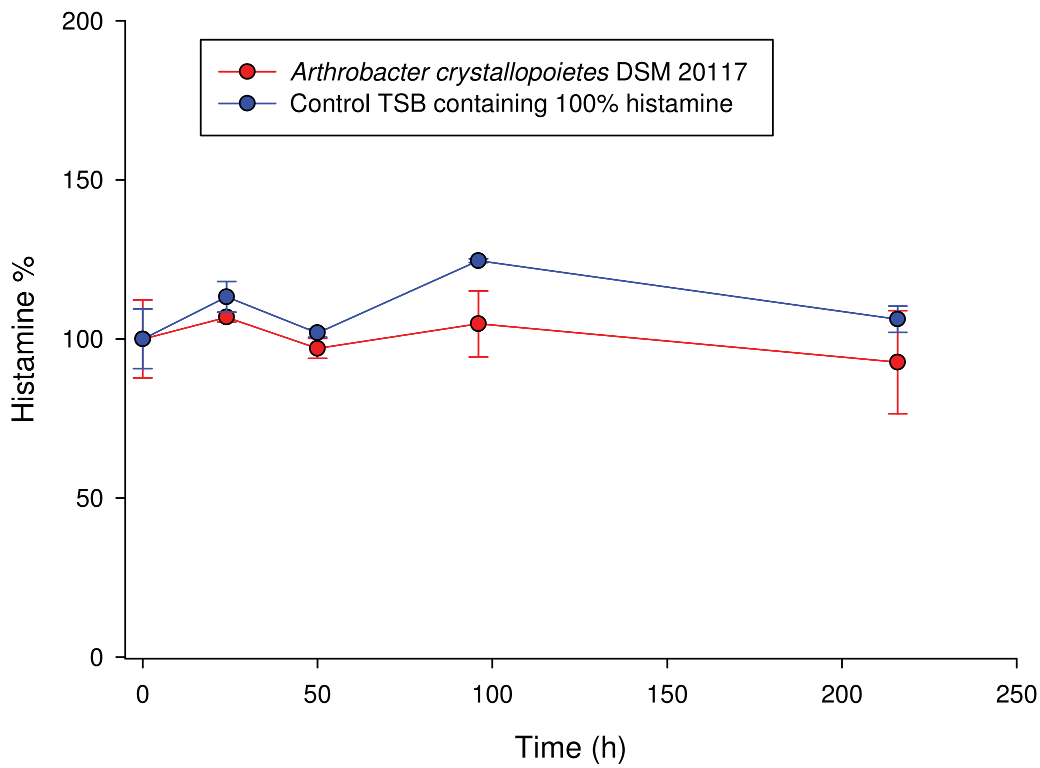

Figure 4: *Arthrobacter crystallopoietes* DSM 20117 degradation histamine in TSB containing 500ppm histamine. Error bars represent mean standard deviation of n = 3.

6.4.2 Histamine degradation by DAO

DAO activity test

DAO enzyme (633.5 units/L) degraded histamine dihydrochloride (0.050 g/L) to an undetectable level (< 0. 1 ppm) in 0.5 M phosphate buffer (pH 7.0) when incubated at 37°C with agitation at 100 rpm for 5 h. The DAO degraded 100 % of the histamine within 1 h of incubation.

Selection of DAO concentration

DAO at 2534 units/L degraded 500 ppm of histamine in 0.5 M phosphate buffer (pH 6) containing 1% salt to an undetectable level (<0.1 ppm) when incubated for 10 h at 37°C and agitating at 100 rpm. For subsequent experiments, a concentration of 2534 units/L DAO was used.

Degradation of histamine in buffer and tuna soup using DAO

Histamine (500 ppm) was degraded to undetectable levels (<0.1 ppm) by DAO in both the buffer and tuna soup at pH 6 and salt 1%, when incubated for 10 h at 37°C and agitated at 100 rpm (Figure 5). Therefore, DAO activity was almost as affective in tuna soup as in the buffer although up to the first 5 h of incubation, the rate of degradation was slightly slower in the tuna soup than in the buffer. Tuna soup without added DAO showed slight auto-degradation of histamine (approximately 25%) after 5 h of incubation. Dapkevicious et al. (2000) also found similar results in that histamine auto-degraded in fish slurry used for the manufacture of animal feed.

To provide better information on the effectiveness of DAO under variable pH and salt concentrations found in tuna soup, a statistically designed experiment, such as central composite design (CCD) is required. Initially such a study could use a controlled buffer system and then be repeated in the tuna soup. While a previous study has reported the use of DAO to degrade histamine in fish slurry used to manufacture animal feed

(Dapkevicius et al., 2000), this is the first study on the use of DAO to degrade histamine in tuna soup used to manufacture food (*Rihaakuru*) for human consumption.

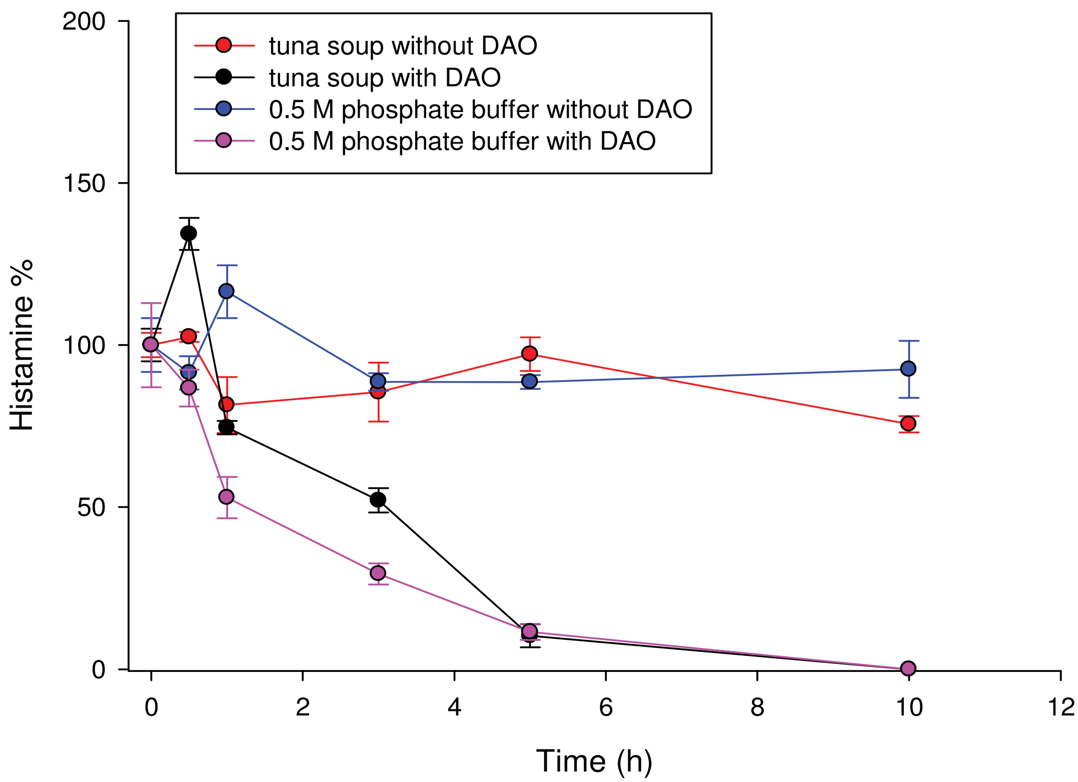

Figure 5: Histamine degradation (500 ppm) by DAO (2534 units/L) in 0.5 M phosphate buffer and tuna soup under pH 6 and salt 1% incubated at 37°C and agitated at 100 rpm for 10 h. Error bars represent mean standard deviation of n = 3.

6.5 Conclusion

Bacterial strains were shown to be able to degrade histamine by 30-50% in TSB containing 500 ppm of histamine. DAO enzyme can degrade histamine in tuna soup to below detectable concentrations (<0.1 ppm). DAO degradation of histamine is more effective than histamine degrading bacteria. DAO degradation of histamine in food is a potential alternative to standard temperature control methods to prevent histamine food poisoning.

6.6 Acknowledgement

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Name of Candidate: Aishath Naila

Name/Title of Principal Supervisor: Associate Professor Steve Flint

Name of Published Research Output and full reference:

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Chapter 7

Prediction of the Amount and Rate of Histamine Degradation by Diamine Oxidase (DAO)

The work presented in this chapter has been submitted for peer review:

Naila, A., Flint, S., Fletcher, G. C., Bremer, P. J., Meerdink, G., (Submitted). Prediction of the amount and rate of histamine degradation by diamine oxidase (DAO). *Food Chemistry*.

7.1 Abstract

Histamine is a biogenic amine that forms in a variety of foods and can cause food poisoning at high concentrations (> 500 ppm). In situations where the formation of histamine in food cannot be prevented through refrigeration, diamine oxidase (DAO) enzyme may be used to degrade histamine to safe levels. The aims of this work were to apply DAO in model (buffer) and real (cooked tuna soup used in the manufacture of a fish paste product, Rihaakuru) systems, in order to obtain predictions for the rates and amounts of histamine degradation. The two systems were set up with a constant concentration of histamine (500 mg/L) and the DAO enzyme (2534 units/L) at a temperature of 37°C, agitation at 100 rpm, and an incubation time of 10 h with variable pH (5-7) and salt concentrations (1-5%). A total of 15 experiments were designed for each system using central composite design (CCD). The data from these experiments were fitted into regression models; initially the data were used to generate an exponential decline model and then the data from this were fitted into a secondary response surface model (RSM) to predict the rate and amount of histamine degradation by DAO. The model system results indicated that DAO activity was not significantly affected by salt (p>0.05), and that activity reached a maximum within the pH range of 6-6.5 with an optimum at pH 6.3. However, the results obtained with the tuna soup model showed that the optimum oxidation of histamine using DAO occurred between pH 6-7 and salt 1-3%. This study defined the conditions for the use of DAO to degrade 500 mg/L of histamine in tuna soup used to manufacture Rihaakuru. The models generated could also be used to predict the rate and amount of histamine degradation in other foods that have similar characteristics to tuna soup.

Keywords: Diamine oxidase enzyme, histamine, tuna soup, *Rihaakuru*, Maldives, fish paste.

7.2 Introduction

Histamine forms in a variety of foods such as wine, sauerkraut, cheese, fish and fish products, and fermented meats. Histamine at high concentration (> 500 ppm) can cause histamine poisoning. Elevated levels of histamine in food result from mishandling, in particular from temperature abuse and a lack of good manufacturing practice. For example, histamine concentrations increased to unsafe levels when tuna was exposed to high temperatures, due to the growth of bacteria containing the histidine decarboxylase that converts histidine into histamine (Arnold et al., 1980). Any food that contains free histidine is susceptible to the production of histamine if conditions for bacterial growth are favourable(Shalaby, 1996).

Histamine poisoning symptoms are similar to allergy reactions including facial flushing, itching, hypotension, diarrhoea and nausea (Arnold & Brown, 1978). Healthy people have an effective histamine detoxification system in the body so unless an excess amount of histamine is taken, the system can efficiently degrade histamine into aldehydes, ammonia and hydrogen peroxide (Koutsoumanis et al., 2010) . For individuals on medication such as that used for depression, drugs can inhibit oxidase enzymes such as mono-, di- and polyamine oxidases in the detoxification system (Koutsoumanis et al., 2010).

Histamine poisoning can be prevented by preventing histamine formation or by degrading histamine in foods. Histamine formation is most commonly prevented through the control of temperature. Cold storage of food in combination with other methods such as packaging methods, preservatives, and irradiation have been shown to reduce histamine production in food (Naila et al., 2010) . However, control of the cold chain alone or combined with other methods may not be sufficient to control histamine. Some bacteria were found to produce histamine at low temperatures (<4°C) (Dalgaard et al., 2006), therefore alternative methods need to be explored to reduce histamine in food.

Histamine can be degraded by bacteria or enzymes (Naila et al., 2010). Bacteria that have been reported as histamine degraders are: *Micrococcus varians*(Leuschner et al., 1998) , *Natrinema gari*(Tapingkae et al., 2010b), *Brevibacterium linen*(Leuschner & Hammes, 1998a), *Vergibacillus sp* SK33 (Yongsawatdigul et al., 2007), *Lactobacillus* *curvatus*, *L. Sakei*(Dapkevicius et al., 2000), and *Staphylococcus xylosus*(Mah & Hwang, 2009b). Similarly *Arthrobacter crystallopoietes* KAIT-B-007 is a potential histamine degrader through the activity of diamine oxidase (DAO) that degrades histamine (Sekiguchi et al., 2004). These bacteria can be added to fermented foods, contributing to the flavor as well as the safety of the final product. However, for food such as *Rihaakuru*, which is not a fermented product, the use of bacteria may not be a useful solution to the problem, as they are likely to change the nature of the product. In addition, the bacteria reported as histamine degraders only reduce but do not eliminate histamine completely (Dapkevicius et al., 2000; Leuschner & Hammes, 1998a, 1998b; Naila et al., 2010; Tapingkae et al., 2010b; Yongsawatdigul et al., 2007).

The application of diamine oxidase (DAO, EC 1.4.3.6) or the bacteria containing this enzyme are emerging approaches to degrade histamine in food (Dapkevicius et al., 2000; Mondovi et al., 1971; Naila et al., 2010). DAO has been isolated from many sources such as the organs of pigs (liver, kidney), human placenta and blood plasma, and from microorganisms including *Microbacterium lacticum* (Voigt & Eitenmiller, 1978) and *Arthrobacter crystallopoietes* KAIT-B-007 (Sekiguchi et al., 2004). Voigt and Eitenmiller (1978) found that cheese had both DAO and amino acid decarboxylase activity. Low amine content was found in cheese which had more DAO activity and a high amine concentration was found in cheese that had more amino acid decarboxylase activity suggesting that amine concentration in foods is dependent on the ratio of amine producing and degrading enzymes. Leuschner and Hammes (1998b) reported tyramine degradation in fermented sausages inoculated with one bacterial strain containing tyramine decarboxylase activity and another containing tyramine oxidizing activity. In the presence of tyramine oxidizing bacteria, the total amount of the tyramine formed was reduced to 40% of the concentration found in the sausage inoculated with the tyramine decarboxylating bacteria. DAO from porcine liver has been shown to lower histamine accumulation in ensiled fish slurry (animal feed) during the early stages of fermentation when the product had a high pH (initial pH 6.4) and oxygen concentration (Dapkevicius et al., 2000). The research by Dapkevicius et al (2000) focused on degrading histamine in animal feed as histamine can cause liver damage to animals eating feed with elevated histamine concentrations (Křížek, 1991). Since the potential of DAO to degrade histamine has been shown to work in animal feed, its application in other foods needs to be investigated. It has previously been reported that DAO activity is optimum at pH 7 and 37°C, in the presence of oxygen (Beutling, 1992). Enzyme activity is likely to be affected by the various conditions found in different environments or foods. Many foods vary in pH and salt concentrations and this may affect the degradation of histamine. In order to determine the influence of these factors on enzyme activity, experimentation and modelling is required.

Rihaakuru is a fish paste produced from a tuna soup. This product generally has low microbial numbers (1-2 $\log_{10}$ colony forming units/g) and water activity (0.55-0.8). The pH and the salt concentration of the product ranges between 5.6-6.1 and 1.4-1.6%, respectively (Naila et al., 2011b). Histamine contamination is a food safety issue with this product as it is often manufactured from tuna fish that has been temperature abused(Naila et al., 2011a). For details on *Rihaakuru* characterization and biogenic amines levels, refer to our previous publications(Naila et al., 2011a, 2011b).

The best approach to prevent histamine in *Rihaakuru* is by preventing bacterial growth in the raw tuna through immediate refrigeration and freezing after catch. However, this is not always practically possible as *Rihaakuru* is usually made from poor quality fish. Degrading already formed histamine in what will become a microbiologically stable food such as *Rihaakuru* is justified as the preferred approach of chilled storage is not possible (Naila et al., 2010). In the manufacture of *Rihaakuru*, tuna is cooked for 45 min in 1% salt, the cooked fish are removed and the remaining soup is filtered and further cooked until it becomes a thick brown *Rihaakuru*(Naila et al., 2011b). Addition of the DAO into the filtered soup and then further boiling to produce *Rihaakuru* will kill any pathogenic micro-organisms that may have grown during the treatment (Figure 6).

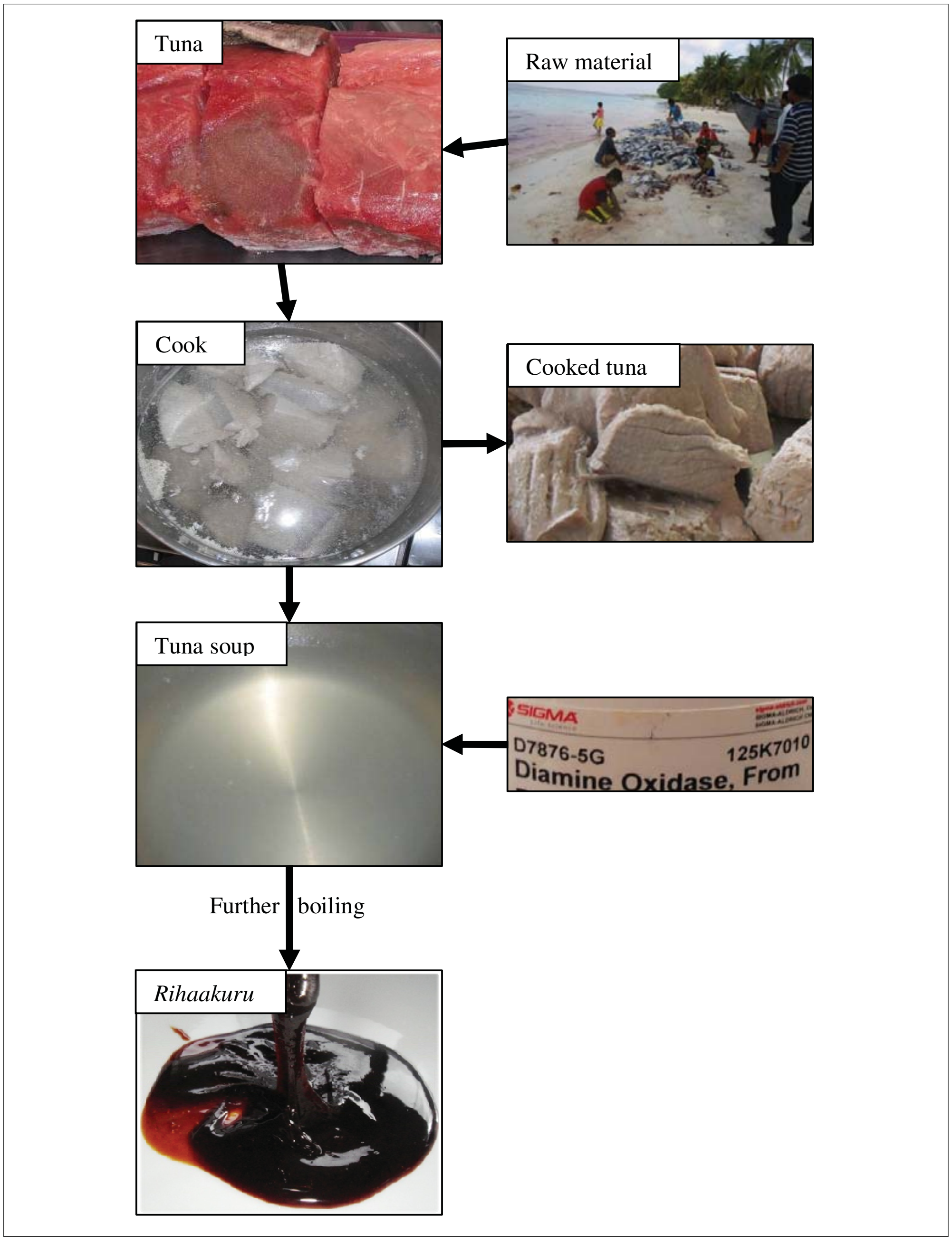

Figure 6: *Rihaakuru* manufacturing steps, including the tuna soup step where DAO can be added after which the soup is further boiled until it becomes *Rihaakuru*. The boiling will kill all the pathogenic microorganisms that may have grown during DAO treatment.

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The pH and salt concentrations in the tuna soup can be altered to account for the range of conditions naturally found during the manufacture of the *Rihaakuru* product. The salt concentration in the tuna soup will be approximately half that in the final product. Tuna soup was selected as a medium instead of *Rihaakuru* for DAO treatment because *Rihaakuru* is a thick paste with low water activity, due to which the enzyme activity may be slow and uniform distribution may not be achieved.

Mathematical modelling has been used to optimize the conditions for bacterial growth in foods such as minced tuna (Koseki et al., 2011). Response surface methodology (RSM) is a statistical tool used for the optimization of multiple variables to predict optimum conditions with the least number of experiments. It is preferred over traditional experimental designs that are achieved by varying one factor at a time (Myers et al., 2009).

The aims of this study were to optimize the rate and the amount of histamine degradation by DAO in a model (buffer) and in the tuna soup used to manufacture *Rihaakuru*, by varying the factors of pH and salt concentrations found in the tuna soup used to manufacture *Rihaakuru*, using a RSM technique. Regression models were used to predict the rate and amount of histamine degradation by this enzyme.

7.3 Materials and Methods

7.3.1 Materials

Analytical grades of anhydrous sodium dihydrogen orthophosphate (NaH_2PO_4), anhydrous di-sodium hydrogen orthophosphate (Na_2HPO_4), salt (NaCl), hydrochloric acid (HCl), and sodium hydroxide (NaOH) pellets were used. Histamine dihydrochloride ($C_5H_9N_3.2HCl$) and 99% pure benzoyl chloride (C_7H_5ClO) were obtained from Acros Organics (New Jersey, USA). DAO (EC 1.4.3.6, 0.18 units/mg) from porcine liver was obtained from Sigma-Adrich (ST. Louis, USA). Distilled water (H_2O) was used to prepare all the solutions.

Chilled yellowfin tuna (*Thunnus albacares*) loins (4.84 kg) to produce the tuna soup base for *Rihaakuru* were purchased from Ocean Fisheries (Palmerston North, New

Zealand) and transferred to the Food Technology Pilot Plant freezer (-18°C) at Massey University until required.

7.3.2 Experimental design

The experiment was designed using Minitab 15.0 statistical software (Minitab Private Ltd, Sydney NSW, Australia). A factorial design consisting of 15 experiments was designed using central composite design (CCD). The design had pH and salt concentrations as 2 factors with 1 centre point representative of the conditions found in the tuna soup used in the manufacture of *Rihaakuru*. Each factor had 5 levels: pH 5-7 and salt 1-5% (Table 16).

7.3.3 Preparation of phosphate buffer

For the model system, 0.5 M phosphate buffer at pH 7.0 was prepared by mixing monosodium phosphate (34.79 g/L) and disodium phosphate (28.82 g/L) in distilled water. The buffer pH was then adjusted to various pH levels with 6 M hydrochloric acid (HCl) and/or 0.1 M sodium hydroxide (NaOH). The pH was measured using an Orion Research Inc. pH meter (Model 420A) fitted with an Orion probe (Model 915BN).

7.3.4 Preparation of tuna soup

Frozen yellowfin tuna was thawed overnight at 4°C, cut into approximately 8 cm cubes and washed with potable water. The tuna cubes were added to boiling salted (1%) water and boiled for 45 min while continuously removing the scum. The cooked tuna cubes were removed, the soup was filtered using cheese cloth and sterilized by UHT treatment (143°C for 10 s). The UHT treated soup was transferred aseptically into glass bottles and kept at 4°C until use.

7.3.5 DAO Experimental procedure

The DAO experiment was based on the method of Dapkevicius et al. (2000). A summary of the experimental procedure is described in Figure 7. The pH and salt concentration of 60 mL volumes each of 0.5 M phosphate buffer and tuna soup were adjusted for each experiment as per Table 16 and two 30 mL sub-samples from each were dispended into 50 mL glass bottles. The pH was measured using the Orion pH meter and probe.

Histamine dihydrochloride (500 mg/L) and DAO concentration (2,534 units/L), temperature (37°C), agitation (100 rpm) and time (10 h) were kept constant of all experiments. Histamine dihydrochloride was dissolved into one sample of each buffer and tuna soup, and DAO (0.56 g of DAO/60mL of buffer; based on the manufactures' label of 0.18 units/mg) into the others. The buffer and tuna soup containing histamine or DAO were then placed in a shaker incubator (Model Amper Chart Multitron II, Infors HT, Total Lab Systems Ltd, Christchurch, New Zealand) for 10 min at 37ºC and 100 rpm, to increase their temperature to 37°C. Buffer and tuna soup containing histamine was transferred into a series of 15 mL centrifuge tubes, 1 mL in triplicate for each sampling time. Buffer and tuna soup containing enzyme (1 mL) was added into the same tubes. Control samples (DAO free) were taken for each sampling time: 1 mL aliquots of buffer and tuna soup containing histamine were transferred into 15 mL centrifuge tubes containing 1 mL of distilled water (1:1). Tubes were mixed in a vortex mixture. The 0 h samples were immediately placed in a boiling water bath for 30 min to prevent any action of the DAO. The remaining samples for each sampling time (0.5, 1, 3, 5 and 10 h) were placed into labelled plastic beakers that were then placed inside the shaker incubator (37 °C, 100 rpm). At their respective times, all samples were placed in the boiling water bath for 30 minutes, then cooled to ambient temperature and transferred to -80°C until analyzed for histamine.

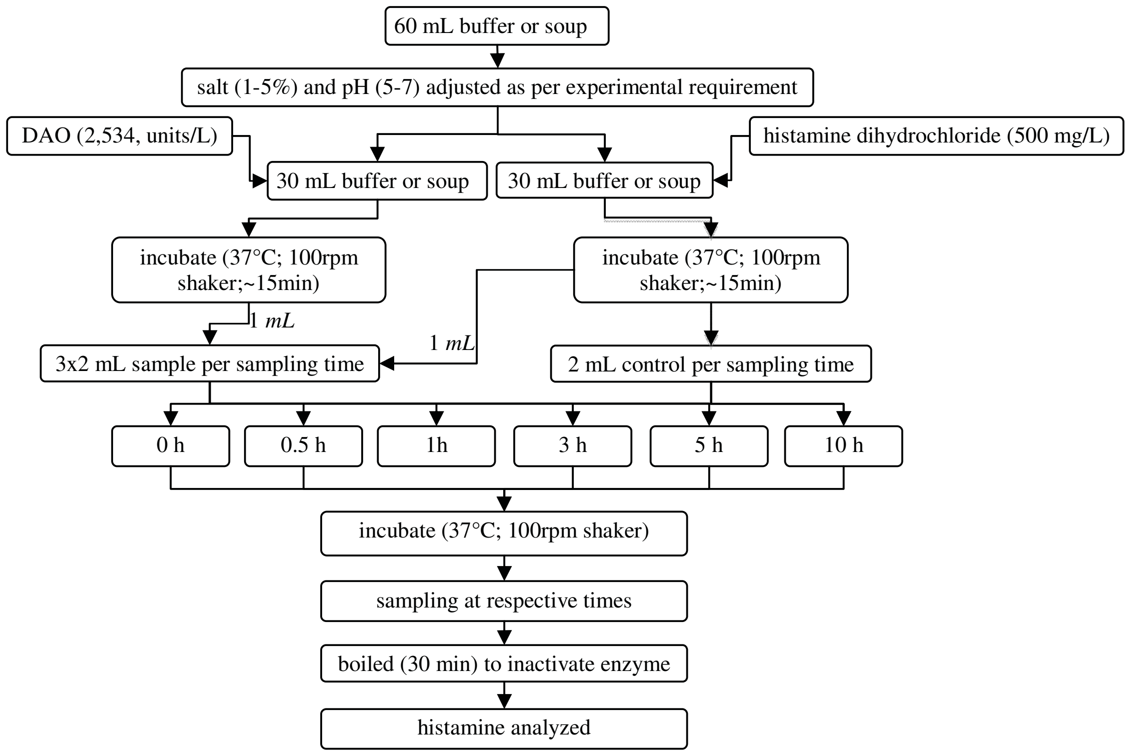

Figure 7: Summary of experimental procedure for DAO activity in buffer and tuna soup.

7.3.6 Analysis

Samples stored at -80°C were thawed at 4°C overnight. Histamine analysis followed the methods of Hwang et al. (1997) with the modifications described in our previous work (Naila et al., 2011a).

7.3.7 Statistical analysis

Fifteen experimental data points obtained for buffer and tuna soup were fitted into an exponential decline model (1) using nonlinear regression (PASW statistics 18, SPSS Inc., IBM Company, Chicago, Illinois) to obtain the parameters L, a, and r in equation 5. The target optimizations were the rate and asymptote of the reaction (r and L in equation 5).

$$Y = L + a * e^{(-r*t)} \quad \textbf{(5)}$$

L = ultimate limits to the decline in mg/L; a = initial histamine amplitude above L in mg/L; r = exponential rate constants for the decline over time; t = time; and Y = amount of histamine in mg/L. The L value was constrained: $L \geq 0$.

The parameters r and L were fitted into RSM model with salt (y) and pH (x) as independent variables using linear regression (PASW statistics 18, SPSS Inc., IBM Company, Chicago, Illinois). A backward elimination method was utilized for the RSM fits.

Analysis of variance (ANOVA) was used to test the significance of the regression models. Significance was accepted for $p<0.05$. Contour diagrams were plotted for L-fit and r-fit with the factors of pH and salt for both buffer, and tuna soup. The models were validated by interpolation.

7.4 Results and Discussion

Control samples (DAO free) for buffer showed no degradation of histamine throughout the treatments. However, the control samples (DAO free) of tuna soup showed some autodegradation (approximately 25%) irrespective of the pH and salt concentrations.

7.4.1 Exponential decline model

Buffer

The exponential model developed from equation 5 showed a good fit between experimental (observed) and predicted data (the coefficient of determination (R^2) ranged between 0.857-0.998) except for experiment 10, where histamine was not reproducibly degraded which resulted in a negligible R^2 value (Table 16).

Table 16: Comparison of primary and secondary model values for r and L in the 2- factor 15-run central composite design and validated experiments

			buffer			tuna soup		
exp	**pH**	**salt**	**r/r-fit**	**L/L-fit**	**R^2**	**r/r-fit**	**L/L-fit**	**R^2**
1[a]	5.5	2	0.138/0.140	0/59.93	0.945	0.205/0.19	0/26.94	0.965
2	6.5	2	0.687/0.584	1.871/-15.40	0.985	0.401/0.34	0/-21.05	0.923
3	5.5	4	0.106/0.140	0/59.93	0.857	0.117/0.17	57.65/78.071	0.914
4	6.5	4	0.575/0.584	0/-15.40	0.995	0.192/0.32	0/30.09	0.968
5	5.5	2	0.251/0.140	44.402/59.93	0.935	0.177/0.19	0/26.94	0.978
6	6.5	2	0.500/0.584	0/-15.40	0.975	0.427/0.34	0/-21.05	0.974
7	5.5	4	0.151/0.140	74.095/59.93	0.939	0.129/0.17	24.65/78.07	0.985
8	6.5	4	0.703/0.584	0/-15.40	0.998	0.413/0.32	0/30.09	0.998
9	6	3	0.169/0.353	0/-8.41	0.935	0.207/0.15	46.58/24.25	0.972
10	5	3	0/-0.054	243/189.63	0	0.278/0.20	150.78/72.23	0.982
11	7	3	0.843/0.834	0/38.98	0.998	0.460/0.49	0/-23.73	0.985
12	6	1	0.417/0.353	1.146/-8.412	0.964	0.336/0.36	0/-9.84	0.946
13	6	5	0.212/0.353	0/-8.412	0.905	0.467/0.36	152.29/92.43	0.982
14	6	3	0.215/0.353	0/-8.412	0.894	0.145/0.15	0/24.25	0.95
15	6	3	0.353/0.353	0/-8.412	0.994	0.157/0.15	0/24.25	0.97
16[b]	5.6	1	0.205/0.181	0/41.35	0.979	0.116/0.37	0/9.36	0.888
17	5.9	1.5	0.215/0.309	0/0.35	0.989	0.343/0.28	71.36/0.29	0.993
18	6.1	2	0.328/0.398	0/-14.71	0.972	0.277/0.23	0/-1.85	0.998

[a] experiment 1-15 a standard 2-factor (pH and salt) 15-run central composite design

[b] experiment 16-18 experiments conducted to validate the model

r/r-fit comparison of observed and fitted r values

L/L-fit comparison of observed and fitted L values

The pH and salt concentration used in experiment 10 was 5.0 and 3%, respectively, which may have partially or fully inactivated the enzyme activity and caused the poor reproducibility. The results of our study are in line with that of Mondovì et al. (1967) in that they observed rapid denaturation of DAO between pH 5 to 5.5. Figure 8 shows an example (experiment 11, Table 16) of the good fit between the experimental data and primary model. According to this the rate of histamine degradation was at 284.8 ppm/h and 499.9 ppm/10h (replacing the r value of 0.843 into equation 5 to obtain the rate of the reaction).

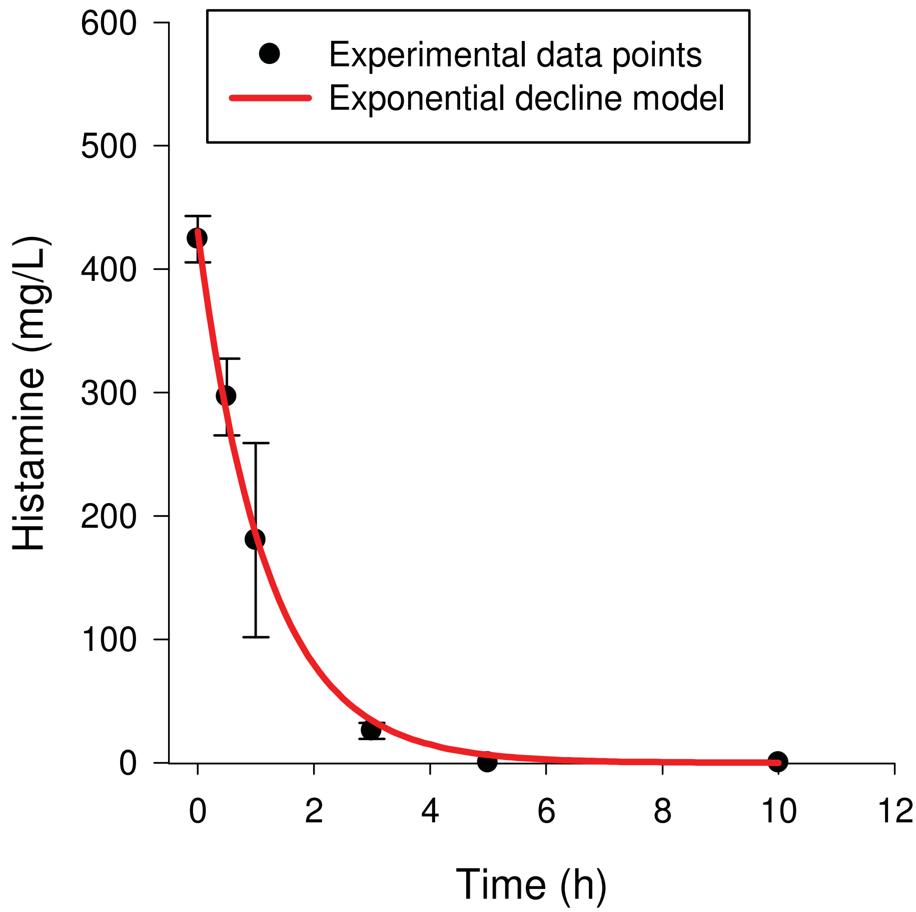

Figure 8: Experiment 11 run at pH 7.0 and salt 3% in 0.5 M phosphate buffer containing 500 mg/L histamine dihydrochloride, and 2534 units/L DAO, shows that the observed and predicted values are in strong agreement ($R^2 = 0.998$).

Tuna soup

The exponential decline model fitted over time to each of the 15 runs showed R^2 between 0.914-0.998 for observed and predicted data (Table 16). This demonstrated that the experimental values fitted well with the primary model (e.g. Figure 9).

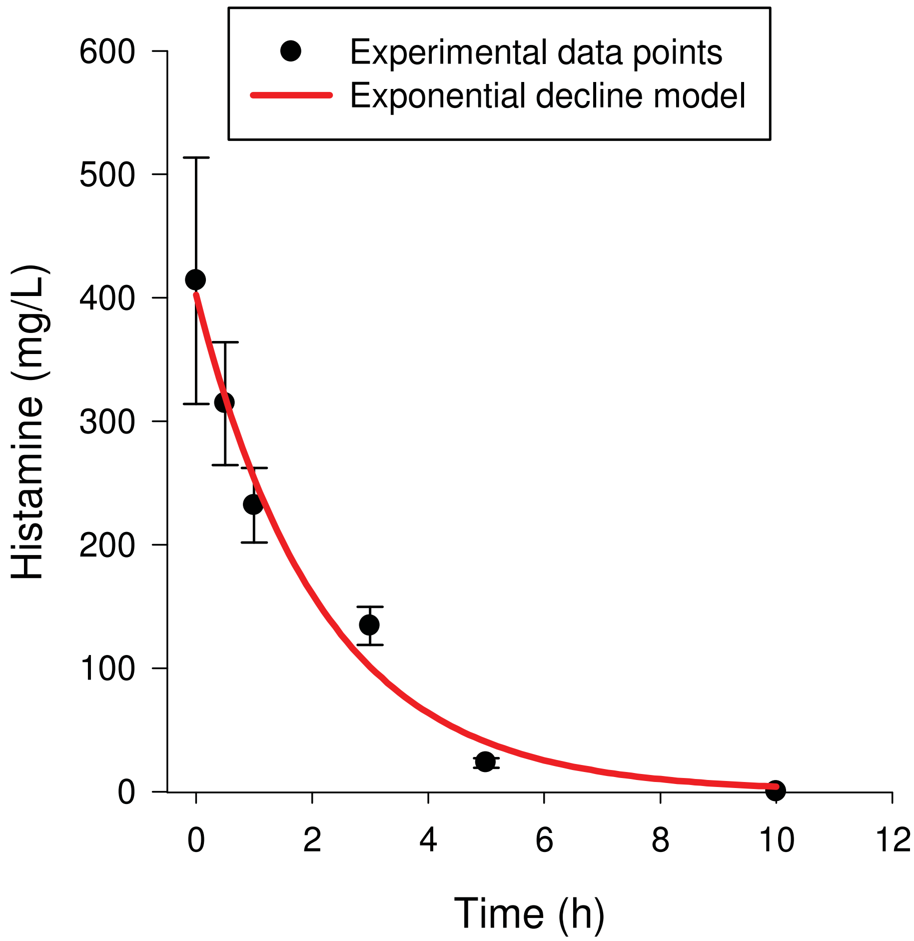

Figure 9: Experiment run at pH 7.0 and salt 3% (exp 11) in tuna soup containing 500 mg/L histamine dihydrochloride, and 2534 units/L DAO, shows that the observed and predicted values are in strong agreement ($R^2 = 0.985$).

According to the Figure 9, the relative rate of histamine degradation was at 184 ppm/h (r = 0.46, Table 16) and 495 ppm histamine was degraded within 10 h, by DAO.

7.4.2 Response surface model

Buffer

The developed RSM models (equations 6 and 7) with pH (x) and salt (y) as independent variables, showed that salt was not significant at the 5% level for histamine degradation by DAO therefore was excluded from the final model (Table 17). This finding suggests that the DAO activity is not dependent on salt between 1 to 5%. The RSM model coefficients were found significant ($p < 0.05$, Table 17).

$$\mathrm{L-fit} = 4861.206 - 1547.881\mathrm{x} + 122.713\mathrm{x}^2 \qquad \textbf{(6)}$$

$$\mathrm{r-fit} \ = 0.037\mathrm{x}^2 - 0.979 \qquad \textbf{(7)}$$

L-fit = predicted ultimate limit to the decline (mg/L), x = pH, and r-fit = predicted exponential rate constant for the decline over time.

The r-fit and L-fit values can be replaced with r and L in the primary model (equation 5) to obtain the predicted amount and rate of histamine degradation.

Analysis of variance (ANOVA) (Table 18) showed that model predictors were highly statistically significant for r-fit ($p < 0.0001$) and L-fit ($p < 0.0001$) indicating the suitability of the model to represent the actual relationship within the experimental regions of pH (linear), and pH^2 (quadratic) terms. These linear and quadratic terms of pH also have a strong correlation with the predicted DAO ability to degrade histamine.

Table 17: Significance of the models coefficients

		Model	Unstandardized Coefficients		t	p
			B	Std. Error		
buffer	L-fit	(Constant)	4861.206	1001.557	4.854	0.000
		x	-1547.881	335.061	-4.620	0.001
		x^2	122.713	27.887	4.400	0.001
	r-fit	(Constant)	-0.979	0.150	-6.539	0.000
		x^2	0.037	0.004	9.038	0.000
tuna soup	L-fit	(Constant)	273.801	122.526	2.235	0.045
		x	-47.983	20.159	-2.380	0.035
		y^2	4.261	1.647	2.586	0.024
	r-fit	(Constant)	7.067	2.712	2.606	0.026
		x	-2.268	0.887	-2.556	0.029
		x^2	0.201	0.074	2.724	0.021
		y	-0.357	0.113	-3.166	0.010
		y^2	0.058	0.018	3.164	0.010

x = pH, y = salt, B = unstandardized coefficients, t = t-statistics, p = significance

Table 18: ANOVA results of buffer and soup models

		Model	Sum of Squares	df	Mean Square	F	p
buffer	L-fit	Regression	44281.113	2	22140.556	19.863	0.000
		Residual	13376.277	12	1114.690		
		Total	57657.390	14			
	r-fit	Regression	0.781	1	0.781	81.691	0.000
		Residual	0.124	13	0.010		
		Total	0.905	14			
tuna soup	L-fit	Regression	20083.577	2	10041.789	6.178	0.014
		Residual	19505.627	12	1625.469		
		Total	39589.204	14			
	r-fit	Regression	0.169	4	0.042	6.405	0.008
		Residual	0.066	10	0.007		
		Total	0.235	14			

Df = degrees of freedom, F = F-statistics, p = significance

The R^2 for L-fit and r-fit were 0.77 and 0.86, respectively, indicating the models' goodness of fit and success in predicting the exponential rate constant and the amount of histamine degradation, by DAO (Table 19).

Table 19: Model summary of buffer and tuna soup

	Model	R	R^2	Adjusted R^2	Std. error of the estimate
buffer	L-fit	0.876	0.768	0.729	33.387
	r-fit	0.929	0.863	0.852	0.098
tuna soup	r-fit	0.848	0.719	0.607	0.081
	L-fit	0.712	0.507	0.425	40.317

The contour diagrams described by the RSM model of L-fit and r-fit of buffer are shown in Figure 10 and Figure 11.

Figure 10 represents the predicted amount of histamine remaining after the experiment (ultimate limit) in buffer. The observed L (primary model) and predicted L-fit were in satisfactory agreement (Table 16). Some of the predicted values are negative values because of the use of the equation (theoretical) but were considered as zero since in practice the values would not be negative (Table 16). The predicted amount of histamine remaining after the experiment decreased as the pH increased and the maximum degradation occurred between pH 6 - 6.5 with the optimum at pH 6.3. This result is in agreement with Mondovi et al. (1964) in that histamine oxidation by DAO was optimum at pH 6.3. The predicted amount of histamine remaining increased slightly as the pH rose towards 7.0. This observation may be due to the limitation of the secondary model with just one experiment above pH 6.0 (exp 2) giving an L of slightly above zero in the primary model. Since the DAO activity is reported to be optimum at pH 7.0 (Schwelberger & Bodner, 1997) the predicted amount of histamine remaining after degradation by DAO at this pH was expected to be zero and agreed with the experimental data at pH 7.0 (Figure 8). It is difficult to draw conclusions from a single experimental data point, whereas the model was developed from 15 experimental data points, providing a sounder picture of the histamine degradation by the enzyme. This finding supports that conclusion of Mondovi et al. (1964) that the optimum pH for the rate of enzyme activity (pH 7) and the optimum pH for histamine oxidation (6.3) may be different.

The validation of the L-fit model showed (Table 16) a satisfactory correlation between the observed and predicted values. The model is more accurate at or above pH 5.9. Inaccuracy below pH 5.9 may be because the enzyme is rapidly denatured at pH range of 5 to 5.5 (Mondovì et al., 1967).

Figure 11 shows the effect of pH and salt as function of the predicted exponential rate constants for histamine degradation (r-fit), by DAO in buffer. The observed and predicted values of the model were in close agreement (Table 16). The contour diagram shows that the predicted exponential rate constant for histamine degradation is highly dependent on pH; as the pH increases the rate of the reaction also increases. The validation data showed that (Table 16) the observed and predicted data were in close agreement.

When the r-fit and L-fit models were overlapped, the common optimum conditions were found between pH 6-6.5 and salt 1-5%, within which the rate of histamine degradation was between 148-282 ppm/h.

In summary, the buffer model reflected the published DAO kinetics with varied pH and salt concentrations applied in a controlled environment. The DAO activity was not significantly affected by the salt levels. The optimum pH for histamine oxidation (L-fit) by DAO was 6.3 and the maximum degradation continued within the range of 6-6.5 though optimum rate of reaction occurred at pH 7. DAO denaturation may have started below pH 6 and been completely inactivated at pH 5. These findings therefore confirm results published 40-50 years ago (Blaschko et al., 1959; Mondovì et al., 1967; 1964).

The next section describes the results of the DAO activity when applied in the tuna soup used to manufacture *Rihaakuru* (real system).

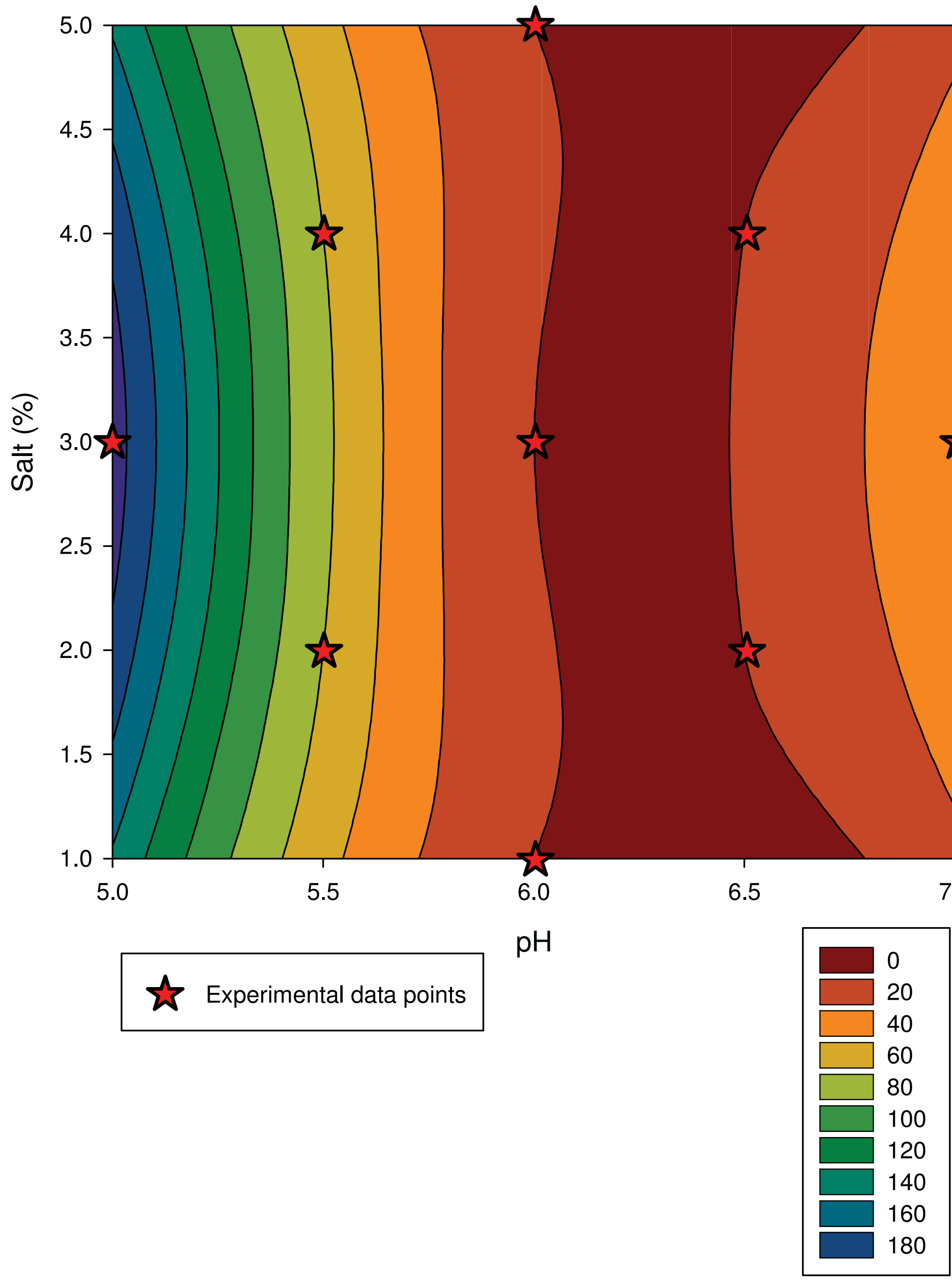

Figure 10: pH (x) and salt (y) as a function of the L-fit- amount of histamine remained in buffer (mg/L) [see key] after degradation by DAO. Salt was not significant at the 5% level.

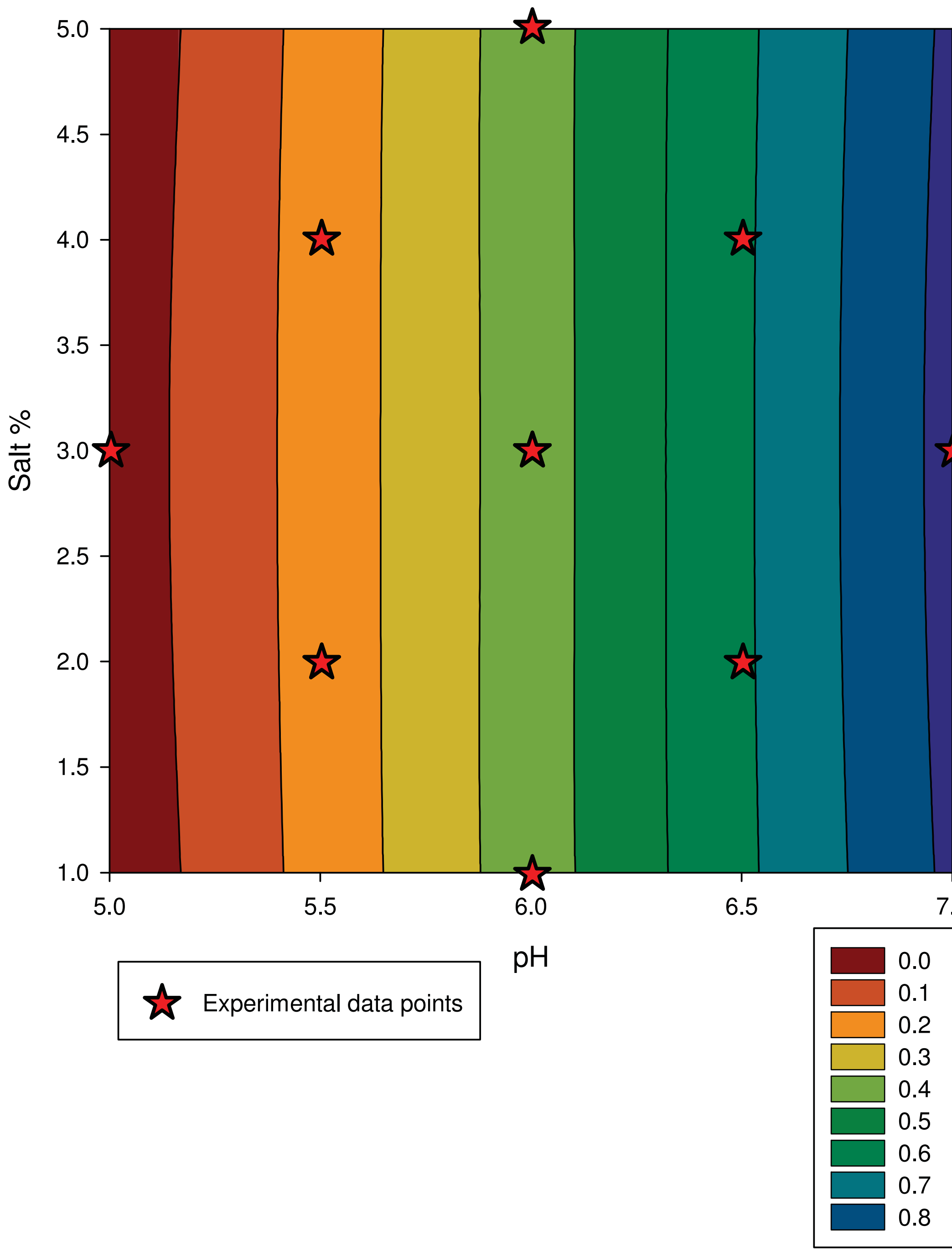

Figure 11: pH (x) and salt (y) as a function of the predicted exponential rate constant (r-fit) of histamine degradation in buffer, by DAO. Salt was not significant at the 5% level.

Tuna soup

The RSM model was developed using salt (y) and pH (x) as independent variables, and L and r as dependent variables, in equations (8) and (9).

$$\text{L-fit} = 273.801 - 47.983x + 4.261y^2 \quad \textbf{(8)}$$

$$\text{r-fit} = 7.067 - 2.268x + 0.201x^2 - 0.357y + 0.058y^2 \quad \textbf{(9)}$$

Where L-fit = ultimate limits to the decline in mg/L, x = pH, y = salt in %, r-fit = predicted exponential rate constants for the decline over time.

The coefficients of the r-fit and L-fit equations were significant at $p<0.05$ (Table 17). Unlike the buffer models, the soup models showed a significant effect due to salt concentration. The buffer model showed evidence that the salt concentration between 1-5% did not inhibit the enzyme, however other components of the soup are likely to have interacted with the salt to inhibit DAO.

ANOVA showed (Table 17) that the predictors of the r-fit (constant, pH, salt, pH x pH, salt x salt) and L-fit (constant, pH, salt x salt) model equations were all statistically significant at $p<0.05$, indicating the goodness of the model. The models are also suitable to represent a real relationship between pH and salt concentration. The R^2 values for L-fit and r-fit were 0.507 and 0.719, respectively. The R^2 indicated that using r-fit and L-fit model equations, a satisfactory prediction can be made on histamine degradation by DAO. There is likely to be degrees of natural variation as the enzyme is a biological entity in which activity can be expected to vary slightly within duplicate runs or experiments conducted on different days and this may explain why the L-fit coefficient of determination was low. The R^2 of the r-fit values in the soup was low compared with that in the buffer. This may be due to the components in the soup binding to the DAO and lowering the rate of the reaction and affecting the R^2 value.

Figure 12 describes the predicted amount of histamine remaining after degradation (mg/L) by DAO in tuna soup at given pH values and salt concentrations (L-fit). The predicted amount of histamine degradation increased with the increase in pH. The optimum degradation of histamine (L-fit reached to zero) occurred between pH 6-7 at salt 1%, pH 6.5-7 at salt 2%, pH 7 and salt 3%. Within this range the relative rate of the histamine oxidation was between 151-193 mg/L per hour. The amount of histamine degradation reduced as the pH decreased with minimal degradation at pH 5. Moreover, as the salt level increased the amount of histamine degradation reduced particularly between pH 5-5.5. This effect diminished as the pH increased, but salt beyond 3.5% slowed the amount of histamine degradation. It has been previously shown that salt ions such as sodium and chloride play an inhibitory role and/or are involved in regulating the activity of DAO (Floris, 1980; Quinet et al., 2010).

The L-fit model validation data (Table 16) suggested that the observed data at pH 6.1 is in strong agreement with that of the predicted value. This suggests that the model validity is more accurate above pH 5.9 as observed in the buffer model.

The validation of the r-fit model for tuna soup (Figure 13) was satisfactory showing the observed and predicted r-fit values agreed more as the pH is increased, again showing a similar pattern to that for the buffer model validation.

From the contour diagram (Figure 13), the predicted exponential rate constant (r-fit) of histamine degradation by DAO gets weaker as the pH decreases but this decrease depends on salt concentration. However, at pH 5 and salt concentration 3%, the r-fit value was higher compared to that of pH 5.3 and salt 3%. This may be an artifect of the model. It is an interesting observation to note that around pH 5-6.2 and salt concentrations between 2% to 4%, the predicted exponential rate constant was lowest (r =0.2) indicating that low pH and certain salt concentrations have an inhibitory effect on the DAO activity. This inhibitory effect diminished as the pH (6.2-7.0) and salt concentrations (4-5%) increase. The DAO activity may decrease if the components in the soup are reacting with pyridoxal – the cofactor of the enzyme, if metal ions in the soup are interfering with copper ions of DAO at substrate binding site, or if other compounds in the soup are acting as histamine analogues (Zeller, 1963). Guerrieri, Finazzi-Agrò, Costa, Rotilio and Mondovì (1976) found similar observations in that DAO activity was lowered by high salt concentration. The mechanism for the inhibition may be same as the 'primary kinetic salt effect' that Bardsley (1973) found with substrate inhibition resulting when the ionic strength of the tested medium was low but once ionic strength increased, the inhibition disappeared. Although the rate of the reaction increased up to a salt level of 4-5% the optimum amount of histamine degradation (L-fit reached zero) occurred up to a salt concentration of 3.5%. This is evidence that increasing the rate of the enzyme activity does not guarantee maximum histamine oxidation.

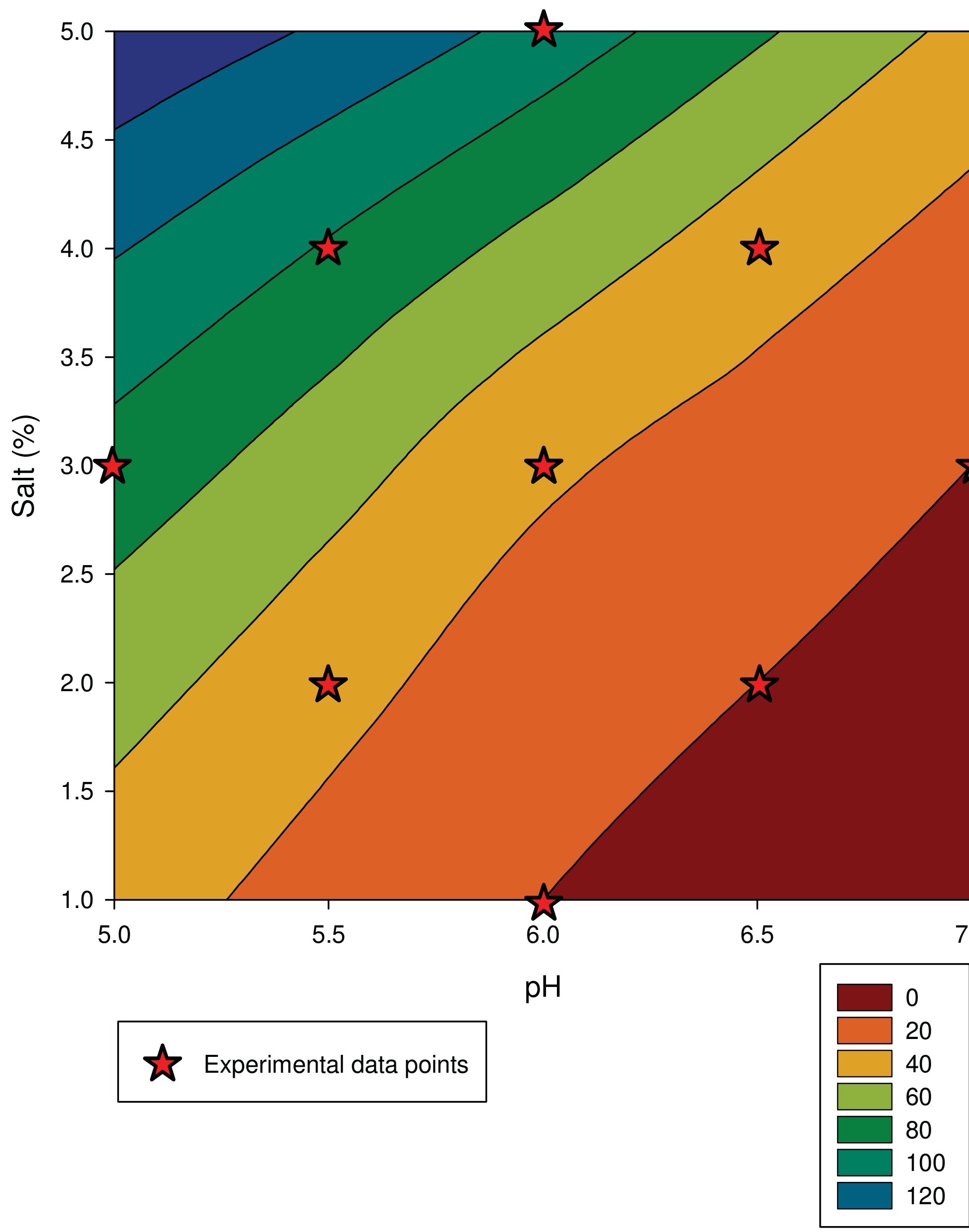

Figure 12: pH (x) and salt concentration (%) (y) as a function of L-fit- predicted amount of histamine remained (mg/L) [see key] in tuna soup after degradation by DAO.

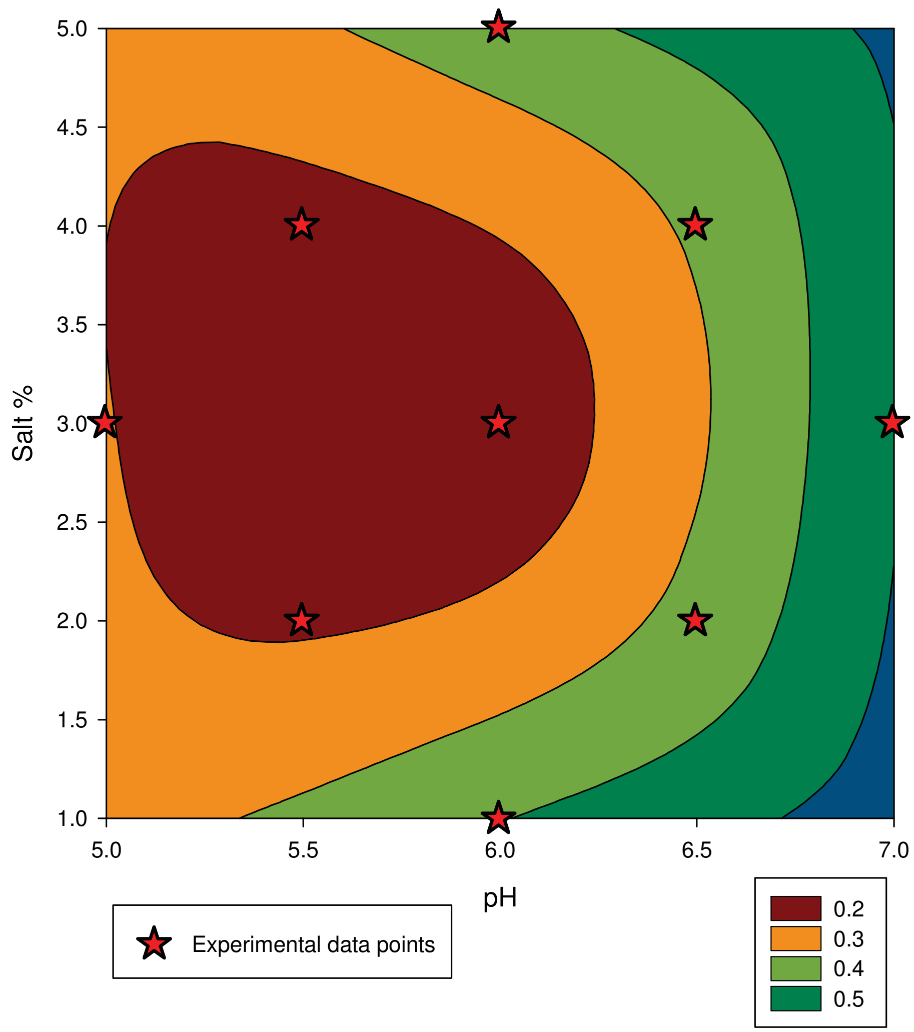

Figure 13: pH (x) and salt concentration (%) (y) as a function of the r-fit- predicted exponential rate constant of histamine degradation in tuna soup, by DAO.

When the L-fit and the r-fit models were overlapped the optimum condition of histamine degradation (L-fit reached zero) by DAO was between pH 6-7 salt 1%, and pH 7 and salt 3%. In this region the relative rate of histamine oxidation was between 151-193 mg/L per hour. This suggests that the tuna soup pH can be varied between pH 6-7 and salt 1-1.5% to degrade 500 mg/L of histamine by DAO, and likewise can be set at pH to 7 and salt level between 1-3%. Since the DAO is added into tuna soup and not to the *Rihaakuru*, it is not critical if the pH below 6 did not degrade histamine because the pH below 6 found in *Rihaakuru* does not have to reflect the pH in the tuna soup.

7.4.3 Comparison of buffer and tuna soup models

The main similarity between buffer and soup model was that the increase in pH increased the rate and amount of the histamine degradation and the decrease in pH decreased these dependent variables. There were several differences in the models for the buffer and soup. First, the latter was significant to a salt level of 5% while the salt level in the phosphate buffer did not affect the amount of histamine oxidation by DAO in either our work or that of Guerrieri et al. (1976). However, Guerrieri et al. demonstrated that DAO was sensitive to the nature of anions in the medium and this could be why salt concentration was significant parameter in the tuna soup. Secondly, each media generated different model equations. This difference may be related to the significance of DAO activity in tuna soup based on salt and the rate of enzyme activity being higher in the buffer compared to soup.

Thirdly, histamine was degraded in experiment 10 (pH 5 and salt 3%) of tuna soup while in the buffer it did not degrade or the degradation was very slow. This shows that although the enzyme maybe inactivated at pH 5 due to some other components in the soup, the enzyme may be protected and retain activity and/or some auto-degradation of histamine may have occurred. The least histamine degradation in tuna soup occurred in the experiment 10 confirming that the enzyme activity was very slow at pH 5.0. The presence of metal ions such as copper may enhance the DAO (Buffoni, 1966). *Rihaakuru* was found to contain 0.004% copper (Naila et al., 2011b) and therefore this is likely to be present, albeit at a lower concentration, in the tuna soup.

Finally, the r-fit of the soup and buffer differed. In general the rate of the reaction in buffer was higher than the soup (Figure 11, and Figure 13). This is expected, since the DAO activity within food matrices may be slower to that of the controlled environment due to the interaction of DAO with soup components, particularly ions. This may also be why the R^2 in r-fit of the soup was lower compared with that in buffer.

That the models in buffer and soup differ illustrates the importance of characterising enzyme reactions in each food system rather than depending on model systems developed in simple buffers.

7.4.4 Conclusion

Here we report a technique using DAO to reduce histamine from 500 mg/L to undetectable levels (< 0.5 ppm) in tuna soup used to manufacture *Rihaakuru* (Figure 6).

Tuna soup containing 500 mg/L of histamine can be degraded at pH 6-7 and salt 1%, pH 6.5-7 and salt 1.5%, and pH 7 and salt 3%, using 2534 units/L DAO enzyme at 37°C and 100 rpm for 10 h. There are no guidelines available on the accepted level of ingredients to be added into the *Rihaakuru*. Therefore, we suggest keeping the salt level low in the tuna soup at maximum range of 1-2% and pH 6-7. However, as pH 7 is outside the normal range of the final product, the sensory characteristics of the product may vary at this pH.

7.5 Future work

As the DAO enzyme is expensive (NZ $ 800/5g) if this were to be used as a practical control measure, a cheaper source of the enzyme would be needed or a culture of the enzyme producing bacteria would need to be used.

A cheaper option to remove histamine from *Rihaakuru* may be worth exploring until the DAO cost drops.

Once the histamine is degraded in the soup using DAO, the soup would have to be further processed into *Rihaakuru* without delay to avoid the possible bacterial growth and histamine production in the soup following the DAO treatment. The significance of any delay in further processing and the overall safety of the process need to be determined. The effect of this DAO treatment of the soup may have some effect on the taste and functionality of the final *Rihaakuru* product and this need to be determined. To determine if the DAO technique for histamine control can be used without affecting the quality of the *Rihaakuru*, tuna soup treated with DAO under the conditions described will need to be taken through the final manufacturing steps to produce *Rihaakuru*. Once the histamine in tuna soup is degraded by the DAO, a system to remove this enzyme from the soup may need to be developed if DAO affects the flavor and functionality of *Rihaakuru*.

Studying *Rihaakuru* production with the application of pH variation from 5 to 7 and salt concentration from 1 to 5% while simultaneously testing the sensory characteristics of the product may be important. If the sensory characteristics do not change or are improved then the present model can be applied across broader ranges or fit with the most desirable characteristics shown in the sensory evaluation.

It would be interesting to trial the activity of DAO in tuna soup at different water activity levels to determine the feasibility of treating tuna soup later in the manufacturing process. If effective, this may reduce the risk of microbial growth in the soup during DAO treatment.

The ability of DAO to degrade histamine at more than 500 mg/L (e.g. 5000, and 50,000 mg/L) is worth determining since these levels will readily cause histamine poisoning. However, the optimum concentration of histamine that is required for the optimum activity of DAO is reported to be 1 mM, below which activity is inhibited (Bouvrette et al., 1997). Guerrieri et al. (1976) had demonstrated 3 mM histamine oxidation by DAO at pH 6.3. In this study we demonstrated DAO oxidation of 4.5 mM histamine.

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Chapter 8

General Discussion and Conclusion

8.1 Reminder of thesis hypothesis and objectives

This thesis aimed to support the hypothesis that histamine in *Rihaakuru* can be reduced to a safe level (< 100 ppm). Specifically this work had the following objectives:

1. To characterize *Rihaakuru* in terms of compositional, physiochemical analysis and isolation of bacterial flora (Chapter 3).
2. To characterize biogenic amines from *Rihaakuru* and identify potential histamine forming bacteria (Chapter 4).
3. To determine histamine stability in *Rihaakuru* over time (Chapter 5).
4. To investigate histamine degradation by bacteria and enzyme (diamine oxidase, DAO) in order to select the most effective method to degrade histamine in *Rihaakuru* (Chapter 6).
5. To examine the ability of DAO to degrade histamine in *Rihaakuru* (based on Chapter 6) using model (phosphate buffer) and real (tuna soup used to manufacture *Rihaakuru*) systems and find the predicted optimum rate and amount of histamine degradation using regression modelling (Chapter 7).

8.2 Major contributions and findings of this thesis

This research aimed to benefit the people of the Maldives, by addressing a problem with a specific Maldive product; however the findings will also be of interest to those wanting to control histamine in other foods.

This work has contributed a great deal to the Maldivian community. This research has already been appreciated by the Maldives through the publication of the summary of this study extracted from our published article (Chapter 3) in the local news paper 'Haveeru' (Saeed, 2011). This study has been greatly acknowledged and valued by the Maldivians through personal communications to the author. Such communications include comments that the public has been waiting for this information and that the authors have contributed a great deal to the Maldivian community. This work has also influenced *Rihaakuru* consumption as some people have stopped eating it. Unfortunately, some people have complained that their children will not eat their meals if it does not include *Rihaakuru*. Therefore, *Rihaakuru* lovers are waiting for the outcome of this thesis, wanting to know if they can now produce *Rihaakuru* that is safe from histamine contamination.

Rihaakuru was found to be a nutritious and shelf stable product. The product and the manufacturing process were found to be unique. The product has been used traditionally as a daily side dish yet there were no reports on its nutritional value or its basic attributes such as water activity, salt content, pH or total bacterial count. This study demonstrated that *Rihaakuru* is stable once produced as the water activity, microbial count and pH were low. *Rihaakuru* is a source of protein and minerals. The protein content of raw tuna is soluble and leaches to the broth during cooking for 45 minutes or more, demonstrated by the protein content of *Rihaakuru* being more than 50% protein. The typical microflora of *Rihaakuru* belonged to *Bacillaceae*, *Streptococcaceae*, *Micrococcaceae* and *Corynebacterium*.

Rihaakuru was found to contain 10 types of biogenic amines: agmatine, cadaverine, histamine, putrescine, phenylethylamine, serotonin, spermine, spermidine, tryptamine and tyramine. Histamine was the dominant amine followed by cadaverine, spermine, putrescine, and agmatine. The histamine level found was high enough to cause food poisoning (>500 ppm). The presence of these biogenic amines provided some evidence that the fish used for *Rihaakuru* manufacture may be of low quality and temperature abused, and also the processing conditions may be unhygienic. This study identified some weak histamine formers among the *Rihaakuru* isolates, ten of these from the genus *Bacillus*, three belonged to the genus *Streptococcus*, and one from the genus *Clostridium*. Some of the remaining isolates have the potential to produce histamine according to literature reports, although this was not be demonstrated in this study.

The high histamine levels in the product were most likely to have originated from bacteria in the raw material, and these bacteria are unlikely to survive into the final product due to the temperature and cooking time used in the manufacture of this product. This study made the Maldivian and the regulatory authorities aware that *Rihaakuru* may be a food safety hazard due to the presence of more than 500 ppm histamine. This study may help the regulatory authorities to set an allowable limit of histamine in the *Rihaakuru* available commercially, and help the local manufacturers become aware of the need to manufacture safe *Rihaakuru* with a low histamine content.

The stability of histamine levels in *Rihaakuru* was investigated as histamine levels in samples stored for this study appeared to be declining during storage. Such a declined had not previously been reported and it appeared remarkable, given the low water activity and low storage temperature (-80°C). The possibility of simply storing product to reduce histamine levels is an attractive simple solution to ensure product safety. Histamine stability in *Rihaakuru* over 10 months when stored at -80°C, 4°C or 30°C was determined. This study confirmed that histamine was decreasing over time and that the decrease was independent of temperature. The mechanism for this decline is unknown and it is recommended that this receive future investigation. However, this result provides confidence to the Maldivians that *Rihaakuru* can continue to be stored at ambient temperatures of 30°C with some assurance that histamine levels will not increase during storage. Unfortunately, the reduction in histamine during storage was not enough to ensure the safety of *Rihaakuru* containing high amounts (>100 ppm) of histamine, therefore other potential control methods needed to be considered.

Two options were investigated for histamine control in *Rihaakuru* - the use of histamine reducing bacteria and the use of diamine oxidase enzyme to degrade histamine in food. The first option was investigated by inoculating trypticase soy broth containing 500 ppm of histamine with bacteria that have been reported to reduce histamine. The bacteria used were three *Lactobacillus sakei* (AGR 37, 46 and Lb706) strains, *Arthrobacter crystallopoietes* DSM 20117 and one isolate from the *Rihaakuru*, *Vergibacillus halodonitrificans* Nai18. All *L. sakei* strains showed 40-50% histamine reduction and *V. Halodonitrificans* Nai18 showed 30% histamine reduction. Histamine was not reduced by *A. crystallopoietes* DSM 20117. The histamine reduction by the isolate from *Rihaakuru* may explain the reduction of histamine on storage. However, the histamine reduction produced by the bacteria used in this study was not adequate to reduce histamine concentrations to safe levels.

For the second option, DAO was added (2534 units/L) to model (phosphate buffer) and real (tuna soup used to manufacture *Rihaakuru*) systems at pH 6 and 1% salt concentration (within *Rihaakuru* pH and salt levels) and incubated for 10 h at 37°C and 100 rpm. The result showed that histamine (500 ppm) could be reduced to undetectable levels in both buffer and tuna soup. This study supported the thesis hypothesis, that histamine in *Rihaakuru* can be reduced to a safe level by adding DAO into the tuna soup used to manufacture *Rihaakuru*. The potential for DAO to degrade histamine has been demonstrated in this study but the effect of varying the conditions that may exist in the product and the optimum conditions for DAO activity in this product were unknown.

Therefore, a statistically designed experiment investigated the effect of DAO activity by varying pH between 5-7 and 1-5% salt concentrations in buffer and tuna soup using 500 ppm of histamine and 2534 units/L DAO at 37°C and 100 rpm for 10 h. The experimental data were fitted into exponential decline models and a secondary RSM model was generated to predict the rate and amount of histamine degradation by DAO. The DAO activity in buffer was found not affected to a significant level at salt concentrations of up to 5%. The optimum oxidation of histamine in buffer model was at pH 6.3 which was in agreement to the work of Mondovi, Rotilio, Finazzi and Scioscia-Santoro (1964). This study also showed that in buffer at pH 5 DAO activity slowed or was inactive. This finding is in line with Mondovì et al. (1967) in that DAO denatured rapidly between pH 5 to 5.5. The current study observed that between pH 5-5.5 the DAO ability to degrade histamine in buffer was reduced and the predicted and observed value of the generated model differed during validation.

The DAO study was repeated in the tuna soup used to manufacture *Rihaakuru* and this produced a model that showed degradation of histamine to safe levels (<100 ppm). Interestingly, in the tuna soup, salt influenced DAO activity although this had not been observed in the buffer. Also in contrast to the buffer DAO was slightly active in the tuna soup at pH 5 though the least histamine degradation occurred at this pH, similar to buffer. This degradation may not solely be due to DAO but some other components in the soup may have degraded histamine or enhanced DAO activity. Histamine degradation (approximately 25%) was observed in the control without DAO. The *Rihaakuru* was found to contain 0.004% copper and therefore this is likely to be present in the tuna soup used in this experiment. The DAO activity is enhanced in the presence of metal ions such as copper (Buffoni, 1966). It was also an interesting observation to note that around pH 5 to 6.2 and salt concentrations above 2% in the soup, the predicted exponential rate constant was lowest. This was not observed in the buffer trials. Mondovì et al. (1967) observed that at pH 5 to 5.5 and low ionic strength, DAO was less soluble and rapidly denatured. A similar observation was reported by Bardsley

(1973)who found that at low ionic strength enzyme activity was low and as the ionic strength increased this effect disappeared. This was explained as 'a primary kinetic salt effect' where the substrate is inhibited by the interaction of a negatively charged enzyme and positively charged substrate (Bardsley, 1973). This may explain why enzyme activity slowed over certain salt and pH ranges. This inhibition may also be explained by an active site of the DAO being occupied by a compound (analogue of histamine) other than histamine in the soup, resulting in some change in protein structure causing a reduction in the rate of the reaction (Buffoni, 1966; Mondovì et al., 1967; Mondovi et al., 1964).

The application of DAO for controlling histamine in *Rihaakuru* is feasible but it assumes that histamine is the main biogenic amine in the product, as found in this study. The enzyme is expensive so the practical use of this treatment will be limited until a cheap source of this enzyme can be found. The DAO treatment would need to be used on the tuna soup prepared following prolonged boiling of fish that would eliminate any pathogens that would otherwise grow during the DAO treatment.

In conclusion this study has provided scientifically sound evidence to prove the hypothesis that histamine in *Rihaakuru* can be reduced to safe levels using 2534 units/L DAO in the tuna soup used to manufacture *Rihaakuru*.

8.3 Recommendations and future directions

8.3.1 *Rihaakuru*: manufacturing process, physiochemical analysis, nutritional composition and bacterial microflora

This study may help regulatory authorities in the Maldives create guidelines that will provide a more shelf-stable *Rihaakuru*. This could include standardizing the product and the manufacture of the product to inhibit bacterial growth.

It would be interesting to know the bacterial flora of the *Rihaakuru* raw material, which could be identified by extracting the DNA from the product and using cloning and PCR methods. By knowing the bacterial species, the bacteria responsible for histamine production can be identified.

8.3.2 Biogenic amines in *Rihaakuru*

It is important to regulate the maximum allowable level of the histamine in the *Rihaakuru*. It is suggested that regulatory authorities use these research findings as scientific evidence on the importance of setting a maximum allowable limit of histamine. This limit must be no more than 500 ppm although a limit of 100 ppm would be preferable as the product is consumed daily by children. Since a few of the products surveyed in this study did not contain any detectable histamine, *Rihaakuru* can be manufactured within such limits.

8.3.3 Long-term histamine stability in *Rihaakuru*

It is suggested that further studies on histamine stability in *Rihaakuru* be carried out and that the mechanism for the reduction in histamine observed in stored samples be determined. It will be useful for the regulatory authorities to know that there will be no increase histamine in *Rihaakuru* that is normally stored at 30°C.

8.3.4 Histamine degradation by bacteria and DAO

It is recommended that more bacterial isolates from *Rihaakuru* or the soup used for *Rihaakuru* manufacture be screened for histamine degradation. Histamine reduction using a bacterial inoculum would be cheaper than using DAO, although the bacteria may affect the sensory characteristics of the product.

8.3.5 Histamine degradation using DAO in a model system

It is important to determine DAO activity at histamine concentrations above 500 ppm and to identify the maximum concentration of histamine that can be inactivated by DAO. Schwelberger and Bodner (1997) observed that at concentrations greater than 0.5 mM of histamine, the degradation by DAO was inhibited. According to Mondovi et al. (1964) the optimum oxidation of histamine occurred at 1mM concentrations above which inhibition was observed though at 16.6 mM DAO was still slightly active. Guerrieri et al. (1976) have demonstrated 3mM histamine oxidation by DAO at pH 6.3.

In the current study 4.5 mM (500 ppm) histamine was successfully degraded by DAO. Histamine degradation by DAO with more than 4.5 mM histamine therefore needs to be investigated before application of this model.

Some early work to optimise histamine concentrations for DAO activity done in the early 90's should be re-visited using the more recent sensitive methods to detect histamine.

8.3.6 Histamine degradation by DAO in tuna soup

Exponential decline and RSM models are suitable to use to predict histamine degradation in tuna soup by DAO within pH 6-7 and 1-3% salt. Manufacturers could use this as a tool to remove histamine in *Rihaakuru* to ensure the safety of the product. This would involve using the two model equations to obtain the desired predicted optimum rate and amount of histamine degradation by the enzyme.

Determining the sensory characteristics of *Rihaakuru* produced using DAO at a pH variation from 6 to 7 and salt concentration from 1 to 3% is required before the model is implemented. If the sensory characteristics did not change or improved then the present model could be applied between pH 6-7 and salt 1-3%.

Once the histamine is degraded from the soup, the soup has to be further processed into *Rihaakuru* without delay; if delays occur bacteria in the soup may produce more histamine or spoil the product. The effect of this DAO treatment of the soup may have some effect on the taste and functionality of the final *Rihaakuru* product particularly of leaving the soup at 37°C for 10 h.

To determine if the DAO technique for histamine control can be used without affecting the quality of the *Rihaakuru*, tuna soup treated with DAO under the conditions described will need to be taken through the final manufacturing steps to produce *Rihaakuru*.

Once the histamine in tuna soup is degraded by the DAO enzyme, a system to remove this enzyme from the soup may be needed if the enzyme imparts any undesirable sensory attributes to the final product.

The activity of DAO could be trialled in tuna soup at different water activity levels to determine the feasibility of treating tuna soup later in the manufacturing process. This, if effective, may reduce the risk of microbial growth in the soup during DAO treatment.

The DAO enzyme is expensive (NZ $ 800/5g) therefore, if this is to be used as a practical control measure, a cheaper source of the enzyme will be needed. Alternatively a culture of the enzyme producing bacteria may be used, but this may affect the sensory characteristics of the product.

The first major impact of this research is likely to be regulatory authorities generating guidelines for the manufacture of *Rihaakuru* which ultimately will produce *Rihaakuru* containing lower histamine contents. Prior to this work, the guidelines were difficult to make due to popularity of the product and without scientific evidence to support the enforcement of guidelines.

Secondly, as consumers become more aware of the hazards they will demand safer production of the product which will pressure the manufactures to produce *Rihaakuru* in hygienic condition. Therefore due to this research, a safer *Rihaakuru* may be produced in the future. In cases where it is not possible to control temperature abuse of the fish (as low quality rejected fish are used in order not to throw away fish), this work provides an option for the manufactures of using the DAO technique to bring down the histamine to a safe level.

In summary, this work has described a unique fish paste, *Rihaakuru* from the Maldives. The nutritional composition of the product is now known and the food safety risk from histamine contamination has been confirmed. The traditional control of histamine is refrigeration to prevent its formation but this work provides an alternative control method which is to use DAO to degrade pre-formed histamine in the *Rihaakuru* under specific conditions.

8.4 References

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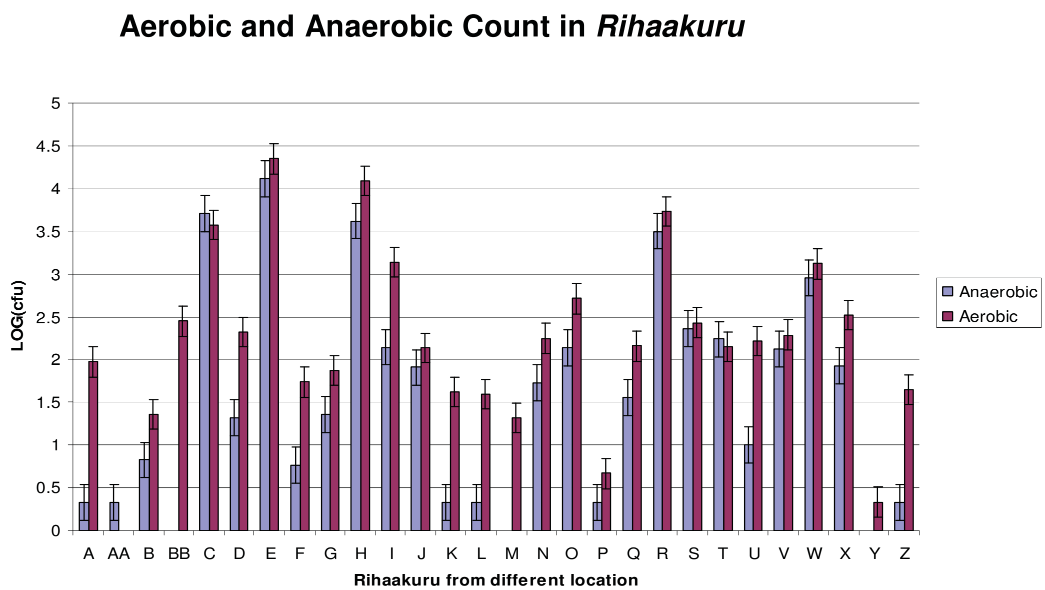
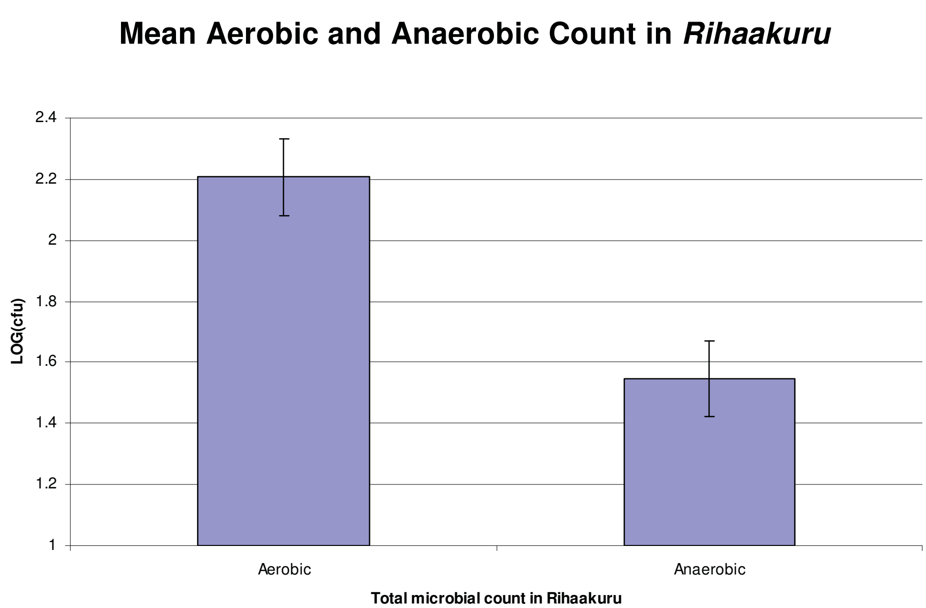
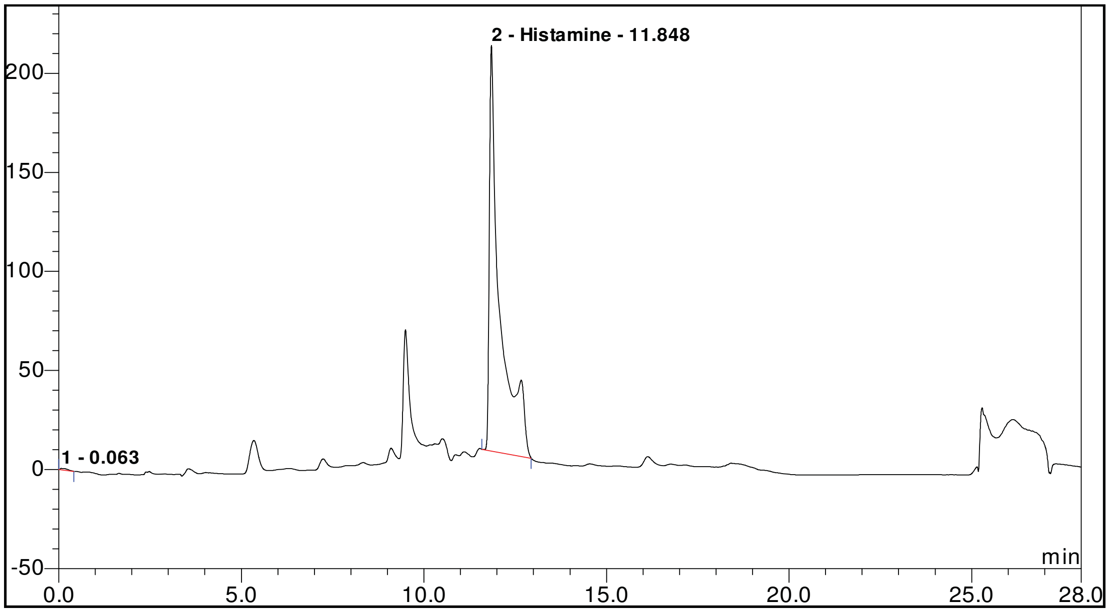
Appendix I:

Supplementary Data Relevant to Chapters 3 and 7





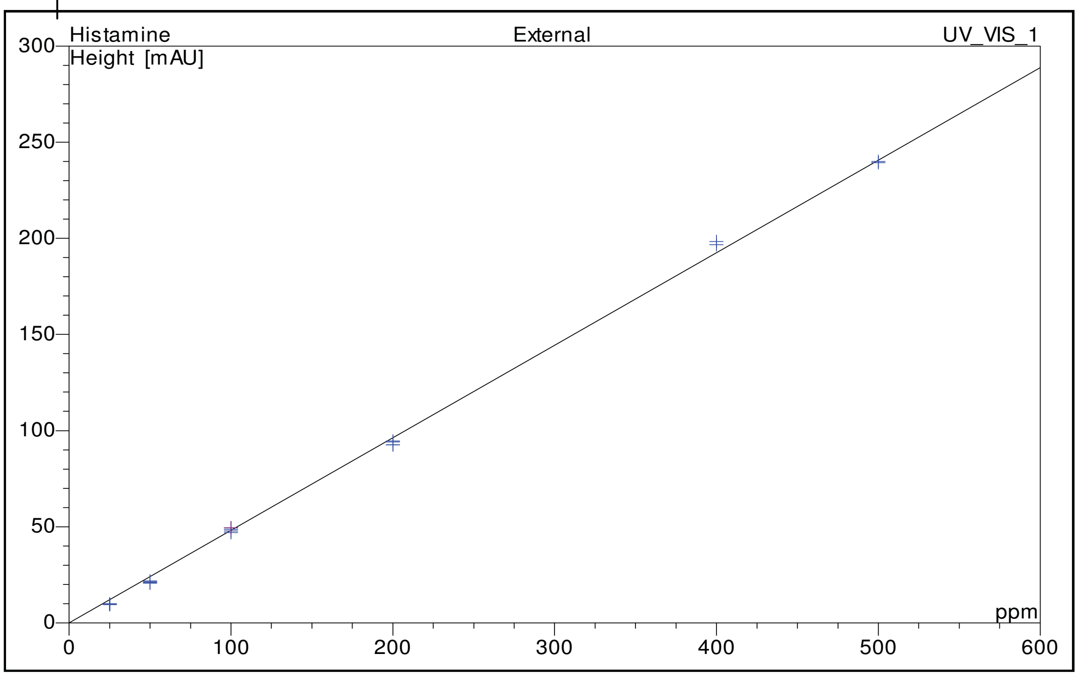
***Rihaakuru* sample code**	**water activity (Aw)**	**pH**
A	0.65	5.64
B	0.58	5.79
C	0.74	5.84
D	0.70	5.91
E	0.76	5.82
F	0.60	5.85
G	0.80	5.76
H	0.74	5.81
I	0.65	5.84
J	0.68	6.00
K	0.73	5.62
L	0.76	5.92
M	0.66	5.82
N	0.73	5.80
O	0.70	5.85
P	0.62	6.00
Q	0.66	5.81
R	0.68	5.85
S	0.66	5.83
T	0.69	6.18
U	0.66	6.09
V	0.64	6.01
W	0.70	5.99
X	0.62	5.98
Y	0.64	5.85
Z	0.60	5.88
AA	0.75	5.98
BB	0.55	5.98
Mean ±SD	0.67±0.06	5.88±0.12


2 - Histamine - 11.848
1 - 0.063
200
150
100
50
0
-50
min
0.0
5.0
10.0
15.0
20.0
25.0
28.0

Histamine peak

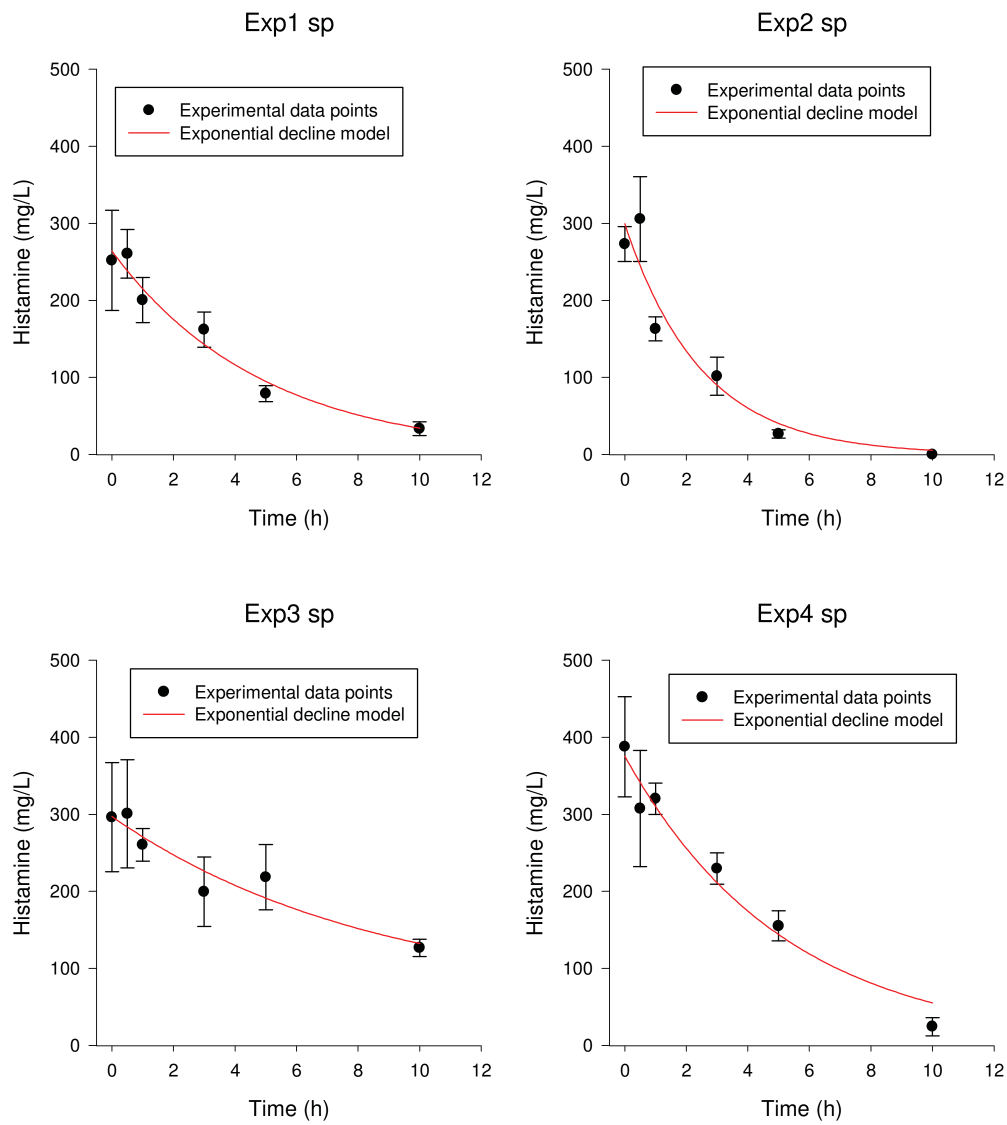
Absorbance [mAU]

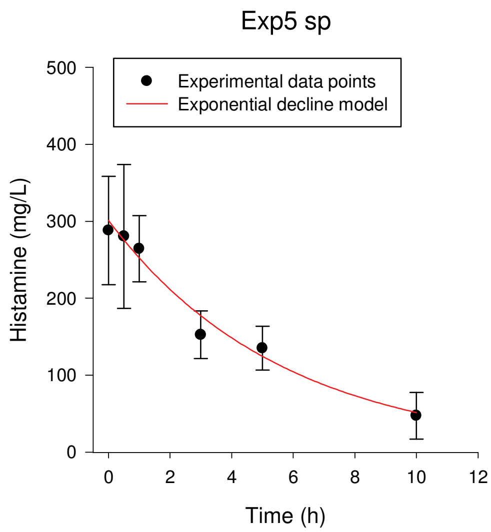
Calibration curve used to quantify histamine



No.	Ret. Time min	Peak Name	Cal. Type	Points	Corr. Coeff. %	Offset	Slope	Curve
1	11.85	Histamine	Lin	20	99.9623	0.0000	0.4812	0.0000
Average:					99.9623	0.0000	0.4812	0.0000

Experimental data of buffer (bf) and tuna soup (sp) fitted into Exponential decline model (primary model)




Exp5 sp
Experimental data points
Exponential decline model
Histamine (mg/L)
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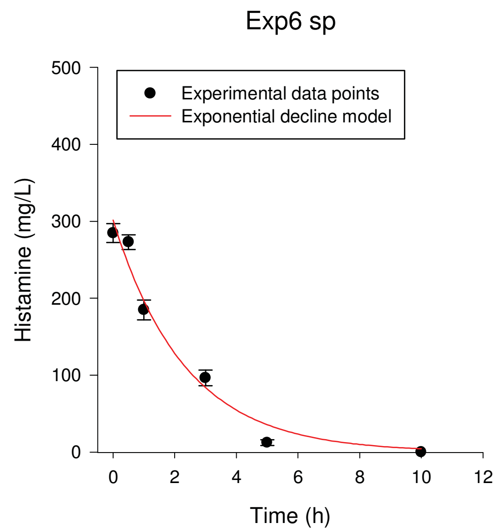
100
200
300
400
500
0
2
4
6
8
10
12
Time (h)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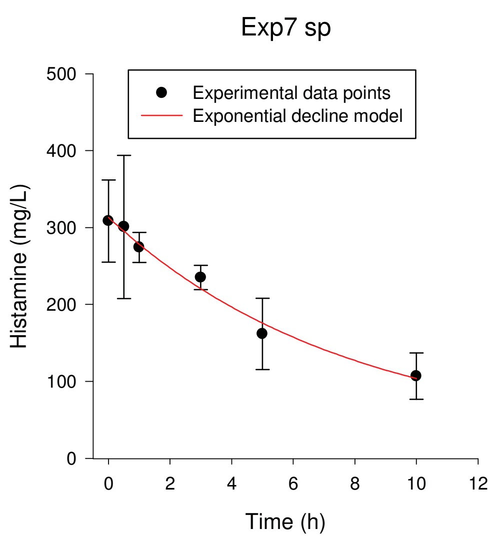
2
4
6
8
10
12
Time (h)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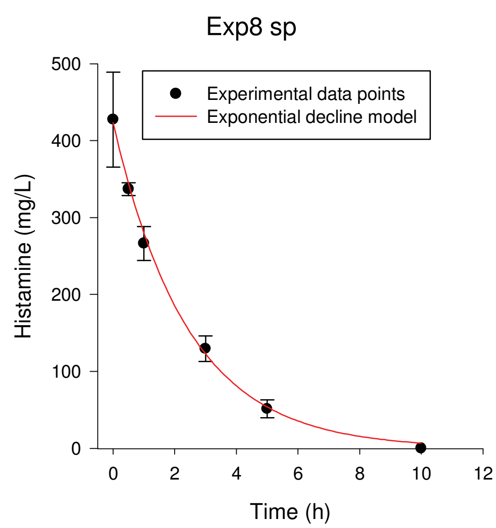
2
4
6
8
10
12
Time (h)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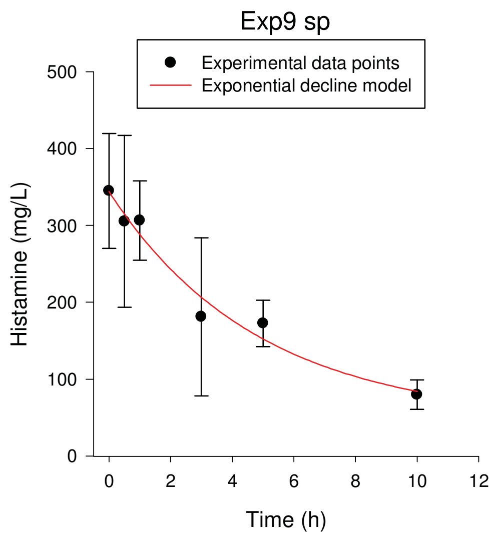
8
10
12
Time (h)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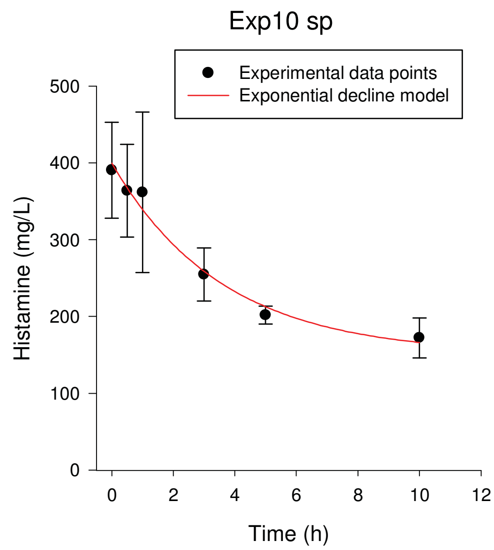
4
6
8
10
12
Time (h)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2
4
6
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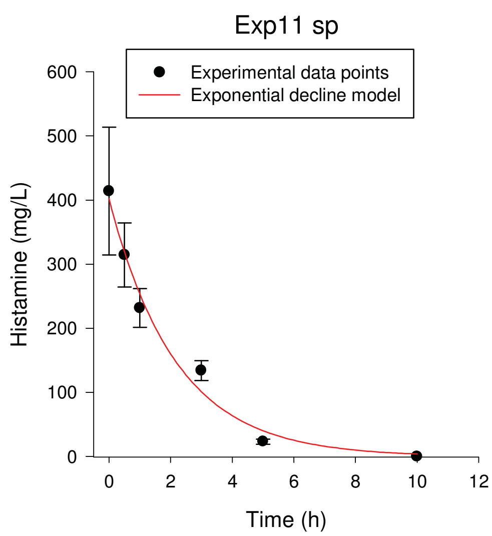
10
12
Time (h)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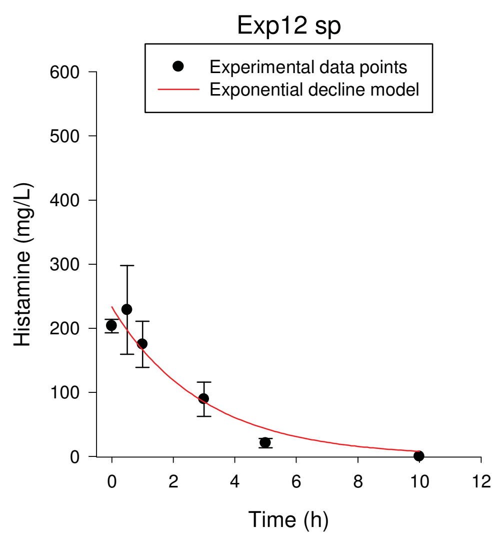
12
Time (h)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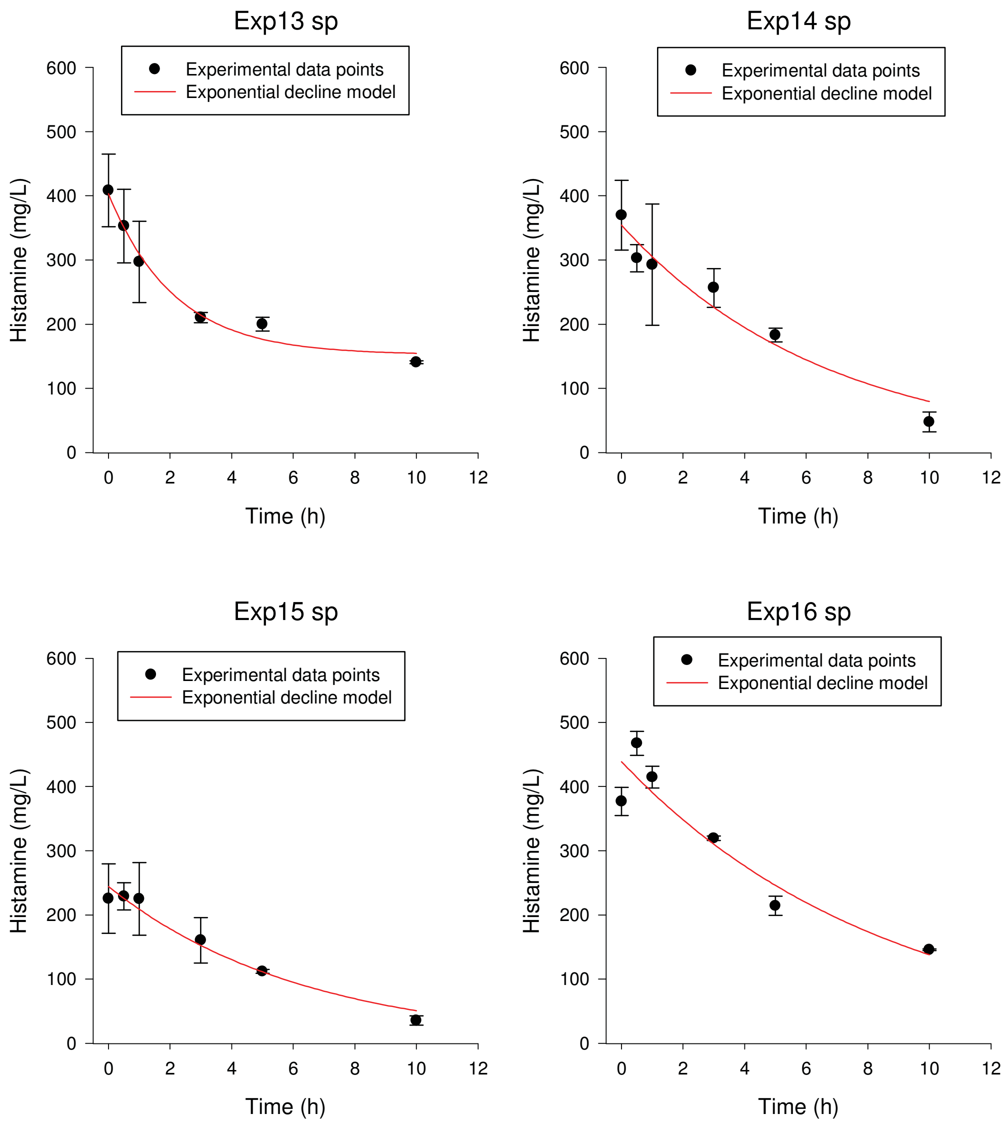
Time (h)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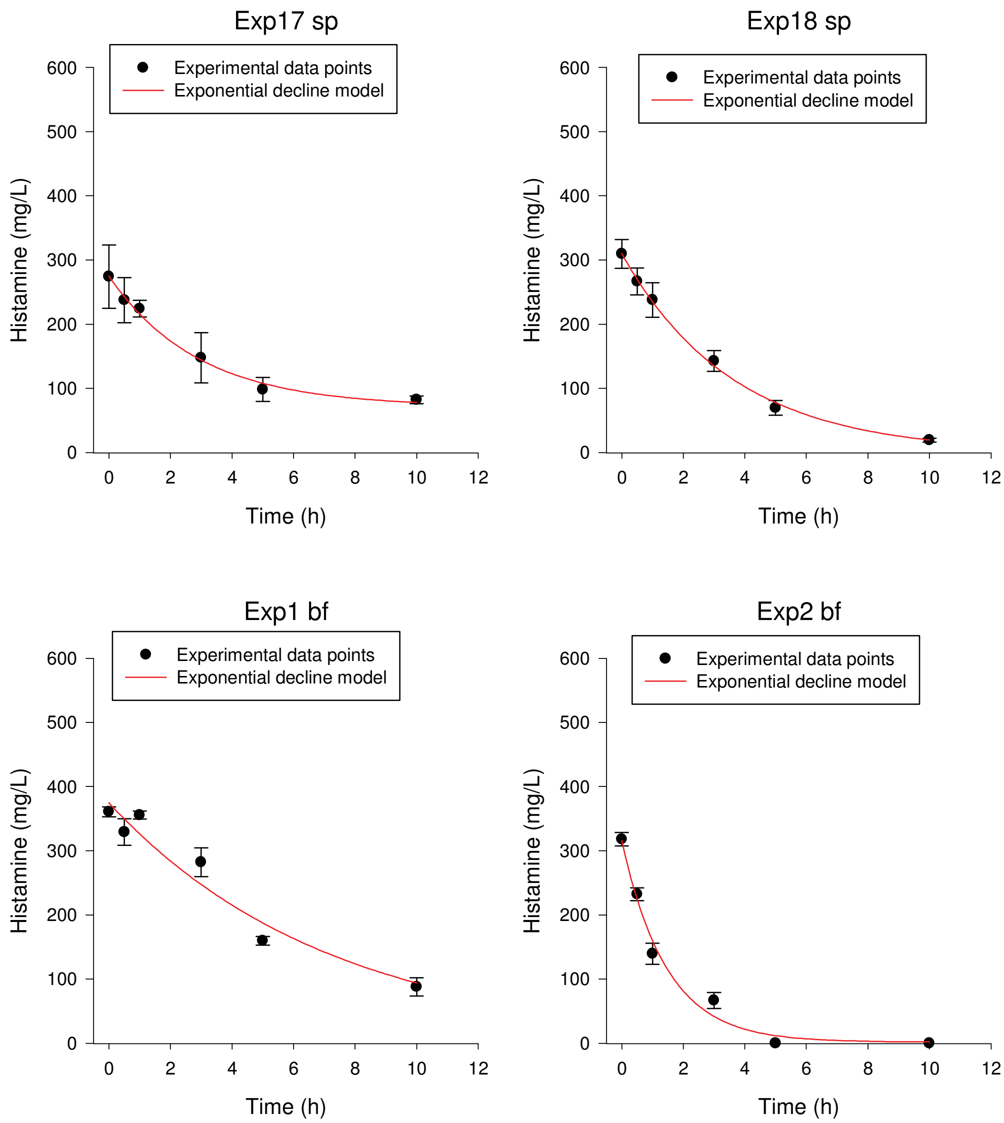
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp15 sp
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp16 sp
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)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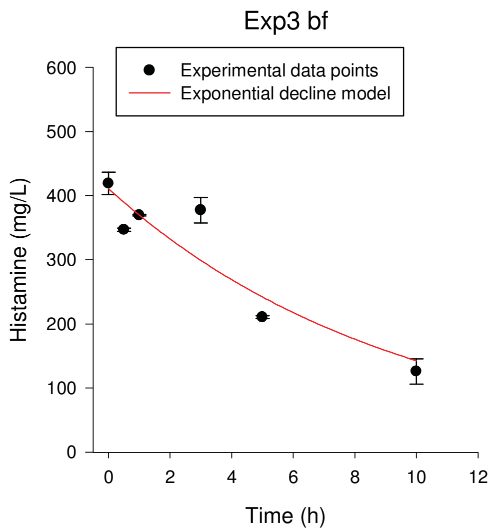
Time (h)
Exp18 sp
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp1 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp2 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)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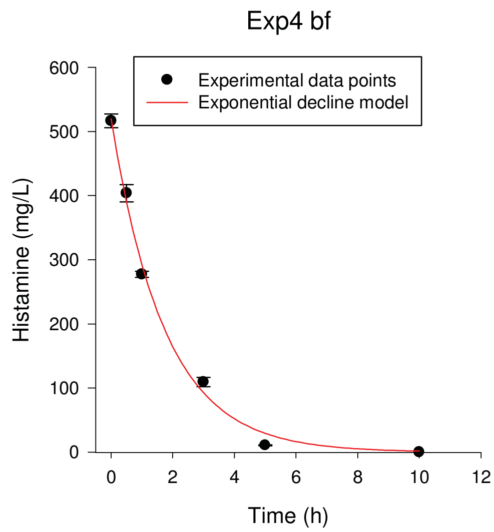
Time (h)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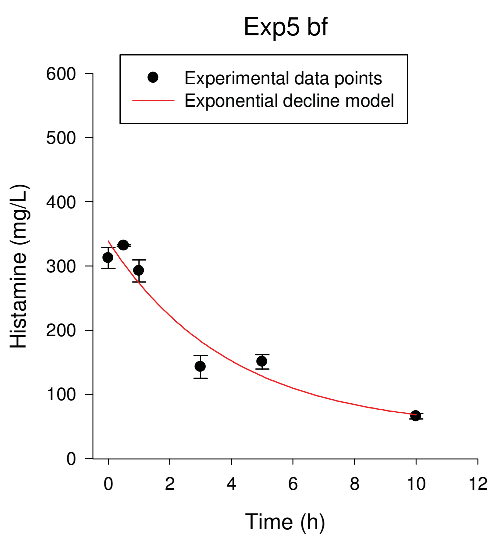
Time (h)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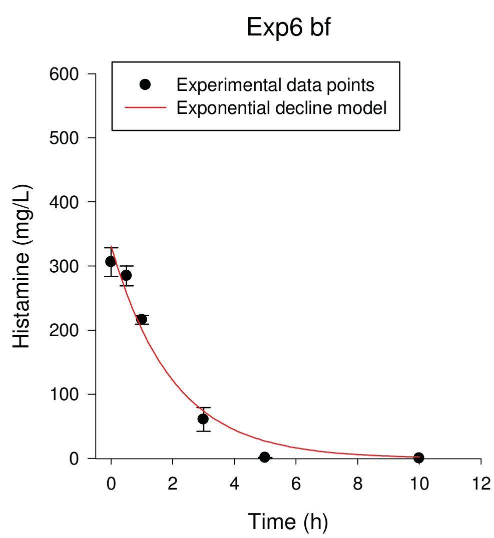
Time (h)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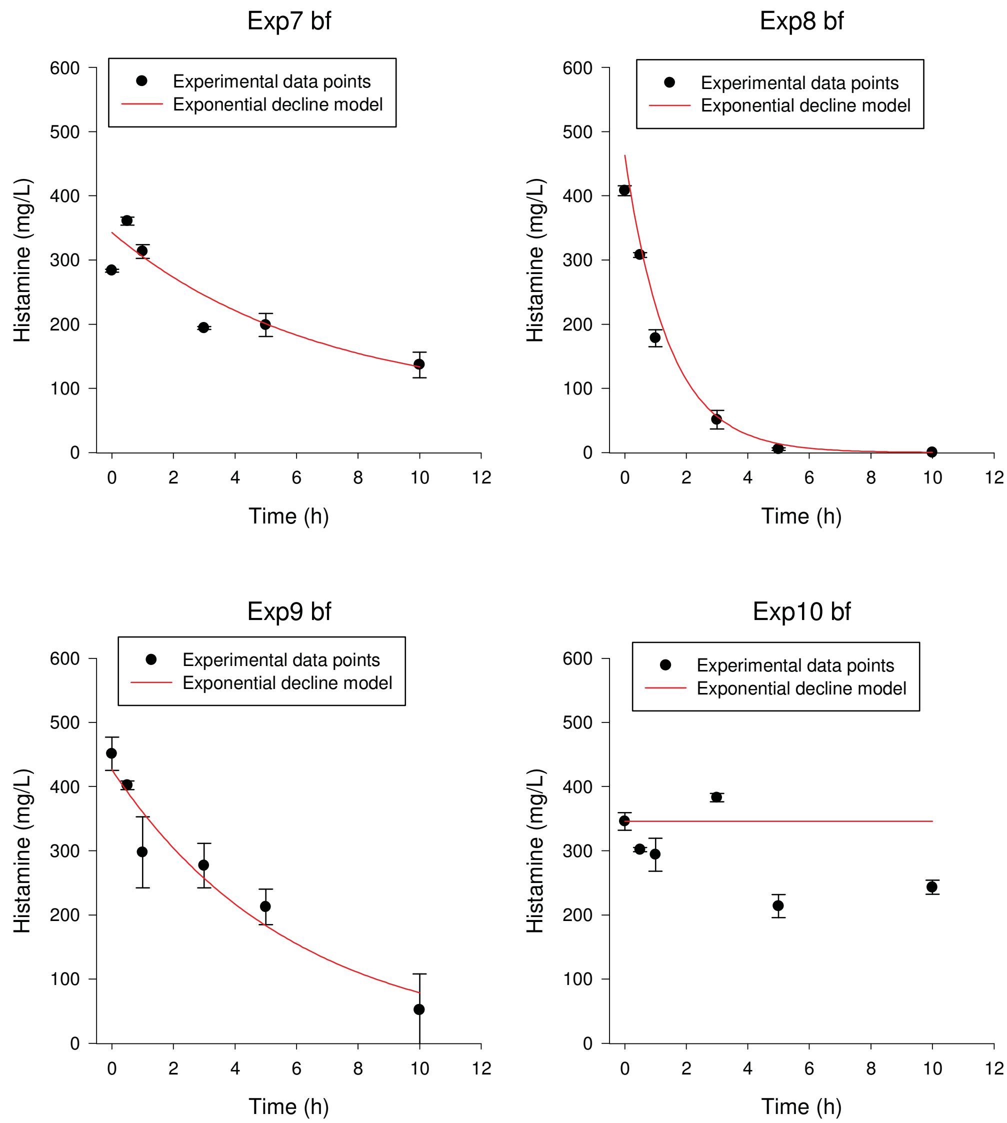
Time (h)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Exp8 bf
Exp9 bf
Exp10 bf
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Histamine (mg/L)
Time (h)
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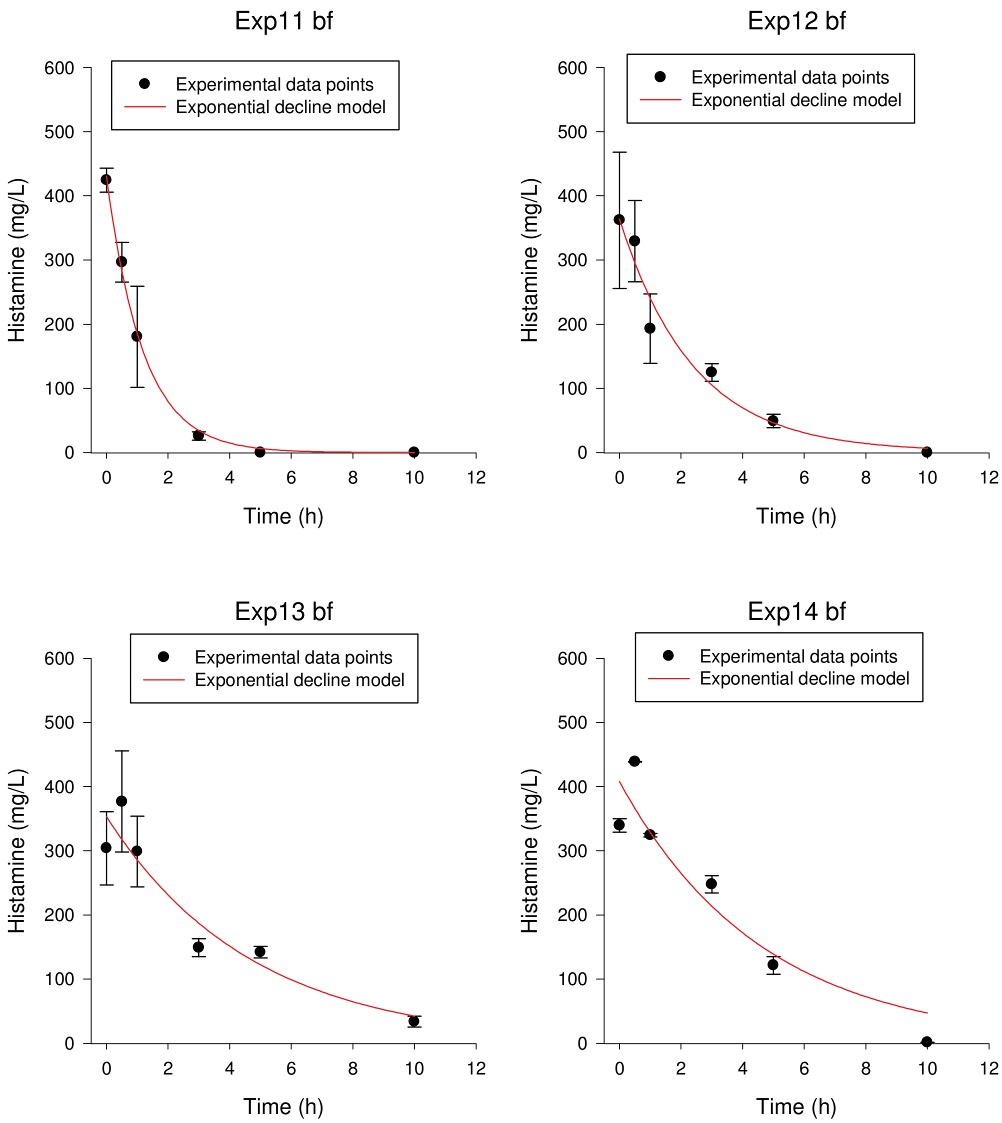

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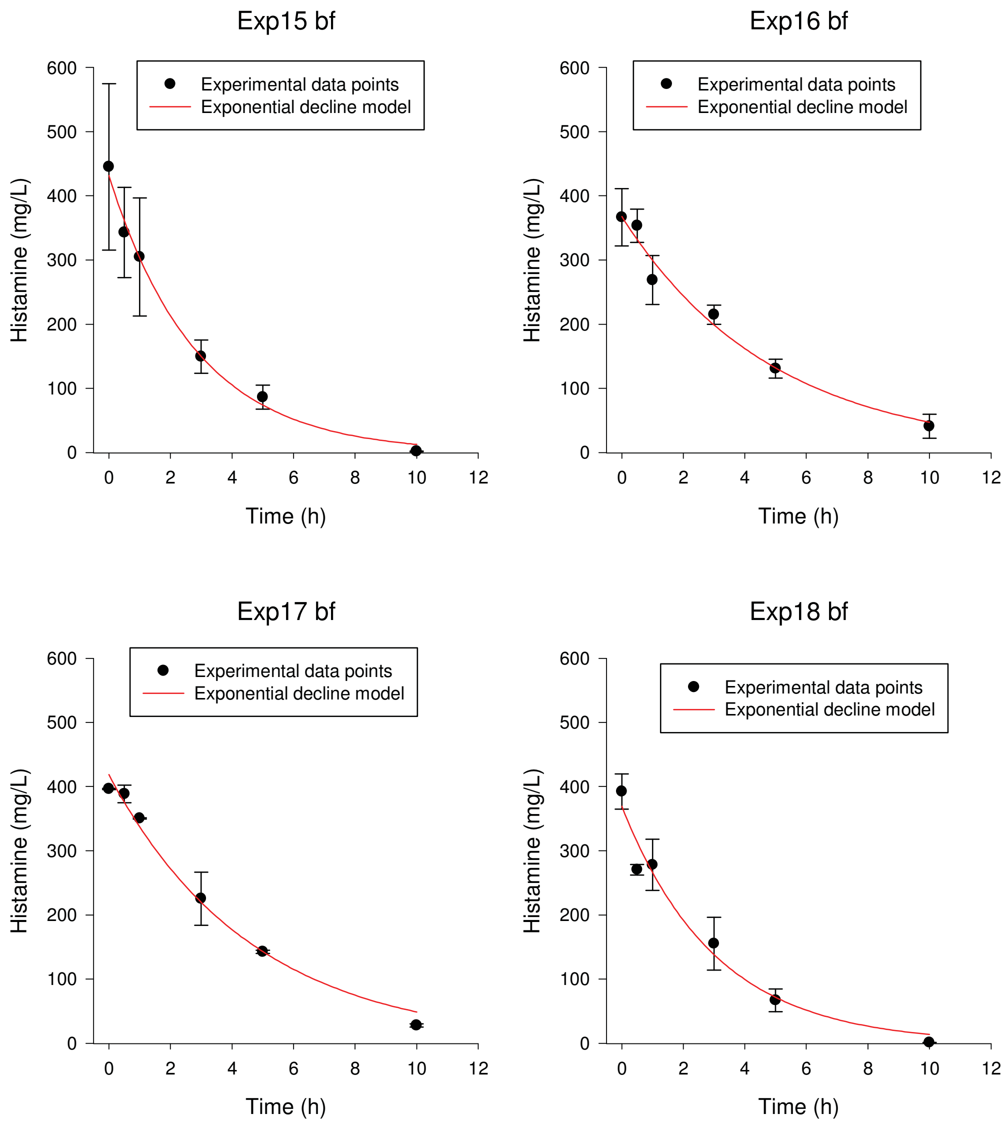
Exponential decline model
Histamine (mg/L)
Time (h)
Exp12 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp13 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp14 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)



Exp15 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp16 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp17 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)
Exp18 bf
Experimental data points
Exponential decline model
Histamine (mg/L)
Time (h)

Primary and secondary models' rate of reaction of DAO in buffer and tuna soup

			buffer				Tuna soup			
					Primary model[c]	Secondary model[d]			observed[c]	predicted[d]
exp	**pH**	**salt**	**r[e]**	**r-fit[f]**	**rate (ppm/h)**	**rate (ppm/h)**	**r**	**r-fit**	**rate (ppm/h)**	**rate (ppm/h)**
1[a]	5.5	2	0.14	0.14	65.32	65.32	0.21	0.19	94.71	86.52
2	6.5	2	0.69	0.58	249.21	220.05	0.4	0.34	164.84	144.11
3	5.5	4	0.11	0.14	52.08	65.32	0.117	0.17	55.21	78.17
4	6.5	4	0.58	0.58	220.05	220.05	0.19	0.32	86.52	136.93
5	5.5	2	0.25	0.14	110.6	65.32	0.18	0.19	82.37	86.52
6	6.5	2	0.5	0.58	196.73	220.05	0.43	0.34	174.75	144.11
7	5.5	4	0.15	0.14	69.65	65.32	0.13	0.17	60.95	78.17
8	6.5	4	0.7	0.58	251.71	220.05	0.41	0.32	168.17	136.93
9	6	3	0.17	0.35	78.17	147.66	0.21	0.15	94.71	69.65
10	5	3	0	0	0	0	0.28	0.2	122.11	90.63
11	7	3	0.84	0.83	284.14	281.98	0.46	0.49	184.36	193.69
12	6	1	0.42	0.35	171.48	147.66	0.34	0.36	144.11	151.16
13	6	5	0.21	0.35	94.71	147.66	0.47	0.36	187.5	151.16
14	6	3	0.22	0.35	98.74	147.66	0.15	0.15	69.65	69.65
15	6	3	0.35	0.35	147.66	147.66	0.16	0.15	73.93	69.65
16[b]	5.6	1	0.21	0.18	94.71	82.36	0.12	0.37	56.54	154.63
17	5.9	1.5	0.22	0.31	98.74	133.28	0.34	0.28	144.11	122.11
18	6.1	2	0.33	0.4	140.54	164.84	0.28	0.23	122.11	102.73

[a] 1-15 Central Composite Design (CCD) experiments

[b] 16-18: validation by interpolation

[c] primary model data (exponential decline model)

[d] secondary model data (RSM model)

[e] rate of reaction of primary model

[f] rate of reaction of secondary model

Appendix II:

Publications

Several papers were published during the course of this research. This appendix comprises of the first page of four publications that have been used to compile Chapters 3, 4 and a part of Chapters 2, 7.

Control of Biogenic Amines in Food—Existing and Emerging Approaches

Aishath Naila, Steve Flint, Graham Fletcher, Phil Bremer, and Gerrit Meerdink

Abstract: Biogenic amines have been reported in a variety of foods, such as fish, meat, cheese, vegetables, and wines. They are described as low molecular weight organic bases with aliphatic, aromatic, and heterocyclic structures. The most common biogenic amines found in foods are histamine, tyramine, cadaverine, 2-phenylethylamine, spermine, spermidine, putrescine, tryptamine, and agmatine. In addition octopamine and dopamine have been found in meat and meat products and fish. The formation of biogenic amines in food by the microbial decarboxylation of amino acids can result in consumers suffering allergic reactions, characterized by difficulty in breathing, itching, rash, vomiting, fever, and hypertension. Traditionally, biogenic amine formation in food has been prevented, primarily by limiting microbial growth through chilling and freezing. However, for many fishing based subsistence populations, such measures are not practical. Therefore, secondary control measures to prevent biogenic amine formation in foods or to reduce their levels once formed need to be considered as alternatives. Such approaches to limit microbial growth may include hydrostatic pressures, irradiation, controlled atmosphere packaging, or the use of food additives. Histamine may potentially be degraded by the use of bacterial amine oxidase or amine-negative bacteria. Only some will be cost-effective and practical for use in subsistence populations.

Keywords: biogenic amines, food additives, high hydrostatic pressure (HHP), irradiation, packaging, scombroid poisoning, starter cultures, temperature

Introduction

Biogenic amines and polyamines have been reported in variety of foods, such as fish, meat, cheese, vegetables, and wines, and are described as organic bases with aliphatic, aromatic, and heterocyclic structures (Lorenzo and others 2007). Biogenic amine formation through the microbial decarboxylation of amino acids is dependent on the specific bacterial strain(s) present, the level of decarboxylase activity, and the availability of the amino acid substrate (Suzzi and Gardini 2003; Rivas and others 2008). Histaminolytic (histamine oxidizing) bacteria may allow an equilibrium to develop between histamine production and destruction in foods containing high amounts of histamine (Lenistea 1971). The most common biogenic amines found in foods are histamine, tyramine, cadaverine, 2-phenylethylamine, spermine, spermidine, putrescine, tryptamine, and agmatine. In addition octopamine and dopamine have been found in meat and meat products and fish (Hernandez-Jover and others 1996). Polyamines, such as putrescine, cadaverine, agmatine, spermine, and spermidine, are naturally present in food and are involved in growth and cell proliferation (Hernandez-Jover and others 1997; Kalac 2009; Kim and others 2009). These amines in the presence of nitrites can be potential carcinogens when converted to nitrosamines (Kim and others 2009). Nitrosamines from polyamines may not necessarily pose a health risk as toxicity is reached only after consumption of large amounts, more than expected in a daily meal (Kalac 2009). The aromatic biogenic amines, tyramine, and 2-phenylethylamine have been reported to be initiators of dietary-induced migraine and hypertensive crisis (Stratton and others 1991). Tyramine, 2- phenylethylamine, and putrescine are versoactive amines and increase blood pressure that can lead to heart failure or brain hemorrhage (Til and others 1997; Kalac 2009; Mohan and others 2009).

Histamine poisoning (scombroid poisoning) is a worldwide problem (Russell and Maretic 1986) that occurs after the consumption of food containing biogenic amines, particularly histamine at concentrations higher than 500 ppm (Gonzaga and others 2009). Histamine poisoning manifests itself as an allergen-type reaction characterized by difficulty in breathing, itching, rash, vomiting, fever, and hypertension. People having deficient natural mechanisms for detoxifying biogenic amines through genetic reasons or through inhibition due to the intake of antidepression medicines, such as monoamine oxidase inhibitors (MAOIs) are more susceptible to histamine poisoning (Hernandez-Jover and others 1997; Yongmei and others 2009). Histamine alone may not cause toxicity at a low level, but the presence of other biogenic amines such as putrescine and cadaverine, at concentrations 5 times higher than histamine, enhance the toxicity of histamine (Stratton and others 1991; Hernandez-Jover and others 1997; Emborg and Dalgaard 2006) through the inhibition of histamine oxidizing enzymes. Oral toxicity levels for putrescine, spermine, and spermidine are 2000, 600, and 600 ppm, respectively. The acute toxicity level for tyramine and cadaverine is greater than 2000 ppm. The no observed adverse effect level (NOAEL) is 2000 ppm for tyramine, putrescine, and cadaverine; 1000 ppm for spermidine; and

MS 20100316 Submitted 3/23/2010, Accepted 6/29/2010. Authors Naila and Flint are with Inst. of Food Nutrition and Human Health, Massey Univ., Private Bag 11-222 Palmerston North, NZ. Author Fletcher is with Food Safety & Preservation, New Zealand Inst. for Plant & Food Research Limited, Private Bag 92169, Auckland, NZ. Author Bremer is with Dept. of Food Science, Univ. of Otago, PO Box 56, Dunedin, NZ. Author Meerdink is with Dept. of Food Manufacture and Process Technology, Univ. of Lincoln—Holbeach Campus, Park Rd., Holbeach PE12 7PT, U.K. Direct inquiries to author Naila (E-mail: A.Naila@massey.ac.nz/a_naila@hotmail.com).

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informa
healthcare

Chemistry and microbiology of traditional Rihaakuru (fish paste) from the Maldives

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Abstract
Rihaakuru is a traditional Maldivian side dish consumed mainly with rice. It is a thick brown fish paste, made from tuna after prolonged heating. Samples tested were found to have a low water activity (0.55–0.8), slightly acidic pH (5.62–6.18) and moderate salt content (1.4–1.6%). The product was found to be rich in polyunsaturated fatty acids such as omega-3, had high protein content (56–59%) and an energy level of 13.8 kJ/g. The product had a low microbial count (1.54–2.31 $\log_{10}$ cfu/g). The bacteria isolated belonged to the *Bacillaceae* (Genus *Clostridium, and Bacillus*), *Streptococcaceae* (Genus *Streptococcus*), *Micrococcaceae* (Genus *Staphylococcus*), and *Corynebacterium*. The product appears to be a nutritious and shelf-stable product.

Keywords: *Maldives, tuna, Rihaakuru (fish paste), amino acids, omega fatty acids, proteins*

Introduction

Rihaakuru, a traditional side dish in the Maldives, is a thick brown tuna-based paste consumed on a daily basis. While it has traditionally been produced by individual households, commercial manufacture is starting to become more common. The Rihaakuru is prepared by boiling tuna in salted water, removing the cooked fish and concentrating the remaining material by heating until it becomes a thick paste. The raw materials—gutted tuna, sometimes headless, or just chunks—are cooked for about 45 min, in water [1:1 fish:water or water is added until all fish are fully submerged] containing approximately 1% salt. The scum (filleyo) that forms is continuously removed by scooping off the surface during the cooking process. The cooked fish is removed and processed into Maldive dried fish, and then the remaining soup is filtered using cheese cloth to remove scales, bones and other fish debris that may have remained in the soup. This soup is further cooked on a low heat/flame/fire (approximately 70–90°C) with continuous stirring with a wooden/stainless steel spatula until most of the water has evaporated, leaving a thick, brown paste. This takes approximately 4–8 h to concentrate to the desired level (determined by dipping a utensil and judging its thickness from the drip/flow) depending on the volume of Rihaakuru to be made. This method is a summary from five recipes obtained from different parts of the Maldives and from personal communication with different homemade and commercial manufacturers. The fish are cooked in this manner in large aluminum open pans as a single batch of fish or batches of fish each boiled for 45 min in the same soup. Once the paste temperature falls to ambient temperature (in Maldives, 25–30°C), it is generally bottled into clean glass or plastic bottles with lids, and sold to the general public through retail outlets. The paste is kept in ambient temperature; no special storage condition is required. Commercial manufactures label the shelf-life as 6 months, while homemade manufacturers state a year as the shelf life; however,

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Biogenic amines and potential histamine – Forming bacteria in Rihaakuru (a cooked fish paste)

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ABSTRACT

The biogenic amine concentration in Rihaakuru (a fish paste) ($n = 28$), obtained from different parts of the Maldives (North, South, and Central), was determined by HPLC. Ten biogenic amines were detected; agmatine, not detected (ND) – 161 ppm; cadaverine, ND – 387 ppm; histamine, ND – 5487 ppm; putrescine, ND – 290 ppm; phenylethylamine, ND – 23 ppm; serotonin, ND – 91 ppm; spermine, ND – 329 ppm; spermidine, ND – 79 ppm; tryptamine, ND – <5 ppm; and tyramine, ND – 50 ppm. Nine biogenic amines were found in 3 samples, 8 in 10 samples, 7 in 6 samples, 6 in 3 samples, 4 in 5 samples, and 1 was found in 1 sample. Histamine was detected at levels that are regarded as a risk to human health. Fourteen isolates were selected from two randomly selected samples out of the 28 samples of Rihaakuru and screened for histamine production. Twelve of the 14 isolates produced histamine, with the highest histamine producers being *Bacillus massiliensis* Nai5 (6.65 ppm) and *Bacillus polyfermenticus* (5.58 ppm); while *Bacillus malacitensis* produced the least (<0.5 ppm).

1. Introduction

The fish paste Rihaakuru, which is an important condiment in the Maldives, could contain high concentrations of biogenic amines, due to raw tuna, from which the product is made from, being subjected to temperature abuse (Naila, Flint, Fletcher, Bremer, & Meerdink, 2010). Biogenic amines could cause scombroid poisoning, with symptoms including rash, facial flushing, itching, lip swelling, headache, vomiting and fever (Motil & Scrimshaw, 1979; Russell & Maretic, 1986). Biogenic amines, such as histamine have been reported to occur in fish and fish products, such as fermented fish paste (Fardiaz & Markakis, 1979). While there are a limited number of reports (Fardiaz & Markakis, 1979; Tsai et al., 2006) of fermented fish paste products causing histamine poisoning, this may possibly reflect underreporting rather than absence of cases (Tsai et al., 2006).

The tuna used in the production of Rihaakuru include skipjack (*Katsuwonus pelamis*), yellowfin (*Thunnus albacores*), big eye (*Thunnus obesus*), frigate (*Auxis thazard*) and little tunny (*Euthynnus affinis*) or a mixture of these tunas (Naila, Flint, Fletcher, Bremer, & Meerdink, 2011). These may be held without chilling for up to 18 h before processing, which could promote the growth and activity of a range of histamine producing bacteria (Arnold, Price, & Brown, 1980; Behling & Taylor, 1982; Taylor, Guthertz, Tillman, & Lieber, 1978). The formation and diffusion of histamine in fish depend upon the growth of histamine forming bacteria that varies depending on which of these bacteria are present (Tao, Sato, Yamaguchi, & Nakano, 2009). When fish are held without chilling for 16 h, high histamine levels have been found (Personal Communication, Mohamed Sobah). This supports the work of Taylor and Speckhard (1983), where histamine producing bacteria were reported to grow and multiply enough to produce a toxic concentration of histamine within 24 h.

Live fish contains bacteria on the skin and gills but once the fish is dead the loins become the main focus for bacterial proliferation. Tuna loins contain high concentrations of free histidine which is decarboxylated into histamine by histamine producing bacteria (Yoshinaga & Frank, 1982). Histamine producing bacteria found in Skipjack tuna (*Katsuwonus pelamis*) include: *Clostridium perfringens*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Vibrio alginolyticus* (Arnold et al., 1980). Fish has been reported to contain 10 different biogenic amines: agmatine, cadaverine, histamine, putrsecine, phenylethylamine, spermine, spermidine, serotonin, tyramine and tryptamine (Yen & Hsieh, 1991).

To make Rihaakuru, tuna is boiled in water (1:1, fish:water) containing approximately 1% salt, for about 45 min, the cooked fish is removed (for further processing into Maldive dried fish) and the remaining liquid is filtered through layers of cheese cloth or a

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Degradation of histamine in tuna soup by diamine oxidase (DAO)

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Abstract

Histamine is a biogenic amine, which can cause food poisoning when present at high concentrations (>500 ppm). In situations where the formation of histamine in food cannot be prevented through traditional methods such as refrigeration, diamine oxidase (DAO) enzyme may be a suitable method to reduce histamine concentration to safe levels. The aim of this work was to apply the enzyme to cooked tuna soup, which is one of the manufacturing steps of Rihaakuru, which often contains high levels of histamine. The DAO activity in tuna soup containing 500 ppm of histamine, at various pH values (5-7) and salt concentrations (1-5%) were examined. A central composite design (CCD) was used which contained a total of fifteen experiments. Histamine was completely degraded ($L = 0$) at pH 7 and 6.5, and at salt level of 2 and 3%. The rate of histamine reduction was optimum ($r > 5$) at pH 7 and salt level 3%. To obtain complete histamine degradation and optimum rate of degradation simultaneously, salt 3% and pH 7 was suggested.
Keywords: diamine oxidase, enzyme, histamine, tuna soup, rihaakuru, biogenic amines, maldives, fish paste, HPLC, histamine degradation.

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