ABSTRACT

BJORNSDOTTIR, KRISTIN. Detection and Control of Histamine-Producing Bacteria in Fish. (Under the direction of Dr. David P. Green and Dr. Lee-Ann Jaykus).

Histamine (or scombroid) fish poisoning is the most frequently reported human illness associated with consumption of fish despite considerable control efforts. Lack of reliable detection methods and limited measures for control of histamine-producing bacteria (HPB) are partially responsible for the high incidence of this disease. To address these concerns, this dissertation focused on development of a rapid and reliable detection method for quantification of histamine-producing bacteria (HPB) in fish. Objectives were: (i) to compare available methods for use in detection of HPB, (ii) to develop DNA-based probe(s) to detect the histidine decarboxylase (*hdc*) gene in HPB, (iii) to apply the DNA probe(s) with colony-lift hybridization for quantification of HPB, and (iv) to evaluate the effect of pH for control of histamine formation using the colony lift hybridization method and DNA probe(s).

In the first study, 152 histamine- and non-HPB were collected and examined for their histamine-forming capabilities. Strains were divided into high-, low- and non-histamine producing strains based on production of >1000 ppm, 125-500 ppm and <125 ppm of histamine in histidine broth. Histamine production was compared to results produced using Niven's medium, impedance by a potentiometric method and a PCR-based method. Of 152 strains examined, 73 were classified as high-histamine producers, 6 low histamine producers and 73 were non-detectable for histamine. Results demonstrated the potentiometric and PCR methods detected high-HPB but were not able to detect the low histamine-producers. The culture-based Niven's medium was able to detect high and low histamine-producers, but resulted in a large number (38%) of false positive responses.

Next DNA-based probe(s) were evaluated for detection of the histidine decarboxylase (*hdc*) gene from HPB. Two types of dioxigenin (DIG) labeled probes were examined using DNA dot-blot hybridizations: (i) six degenerate probes obtained by comparison of 17 *hdc* genes cloned and sequenced from HPB and (ii) 249 bp and 709 bp PCR DIG-labeled probes generated from four high histamine-producing bacteria. Detection of HPB using the degenerate probes resulted in weak responses from high-HPB. Detection of HPB using the 249 bp and 709 bp probes applied individually did not result in positive response for known histamine-producing strains. However, when 709 bp probes were examined as a mixture of equal proportions from the 4 high histamine-producing bacteria, positive responses were obtained for all high-histamine producing bacteria examined.

The third study applied the *hdc*-probes to colony lift hybridization for rapid detection and quantification of HPB in fish. First, labeled DNA probes were evaluated for detection specificity when applied to 152 histamine- and non-histamine producing bacteria using dot-blot hybridization. The assay was converted to colony lift hybridization format and the efficiency was compared to Niven's agar medium and validated using artificially inoculated fish samples. The *hdc*-probe mix detected all 73 high-histamine producing bacteria. Six low and 73 non-histamine producing bacteria were not detected. The colony lift hybridization method with DNA probes was found to be accurate for quantification of HPB in inoculated fish samples.

The final study evaluated pH for potential control of HPB and histamine formation by phosphate treatment of fish muscle. First, phosphate treament effects on fish muscle pH were examined. Then histamine-producing bacteria inoculated mahi-mahi and tuna samples were vacuumed-packed with 7% trisodiumphospate (TSP) and stored under slightly abusive

temperature (10˚C) for 4 days. Samples were analyzed for surface pH, drip loss, growth of HPB and histamine-production. Phosphate treatment significantly increased fish muscle pH and reduced histamine formation in the inoculated fish samples. However, growth of HPB was not significantly reduced by phosphate treatments.

These studies have resulted in a new colony lift hybridization method for quantification of HPB in fish. The new method can be used to evaluate control measures for prevention of histamine fish poisoning and help in study of the microbial ecology in fish and fishery products leading to histamine fish poisoning.

Detection and Control of Histamine-Producing Bacteria in Fish

by
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DEDICATION

I dedicate this dissertation to my parents, Vilhelmína Ólafsdóttir and Björn Ævarr Steinarsson, for making me the person that I am today and for being a rock to lean on.

BIOGRAPHY

Kristín Björnsdóttir was born in Germany, on February 7, 1978. Her interest in natural sciences started early in her education and shaped her career as a food scientist. She obtained a Bachelor's in Food Science from the University of Iceland in 2003 receiving the highest average grade upon graduation to date. She came to NC State University to pursue a Master's degree in Food Science with a minor in Food Safety under the direction of Dr. Fred Breidt. During her masters program at the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Laboratory, she examined the effect of organic acids on *Escherichia coli* O157:H7 independent of pH under acidified food conditions. After graduating in 2005, she continued her study at NC State University, to pursue her PhD degree in Food Science under co-direction of Dr. David P. Green and Dr. Lee-Ann Jaykus. She joined the NCSU Seafood Laboratory located at the Center for Marine Sciences and Technologies (CMAST) where she studied detection methods and control measures for histamine-producing bacteria in fish. Kristin enjoys outdoor actives including road biking, hiking, Ultimate Frisbee and scuba diving and plays violin in her free time.

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Finally, I am extremely thankful to my family for always being there and giving me the support that I needed to complete this degree. I am especially thankful to my fiancé, Chris Butler, for the support and encouragement throughout my study. I could not have done it without you. Note: By the time this dissertation is completed Chris and I will have tied the knot and cast our future together hopefully somewhere close to the sea.

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Prevention of human disease due to seafood-borne chemical intoxication remains a challenge despite recent efforts for its control. Scombrotoxic food poisoning is one of the most frequently reported human illness associated with seafood *(55)*. The causative agents are biogenic amines, e.g. histamine, putrescine and cadaverine. The primary challenges are the diversity in microflora capable of producing biogenic amines, use of temperature as the primary control measure to prevent microbial growth and the mild nature of the illness. This review summarizes methods used for detection of histamine-producing bacteria and control measures in addition to temperature for potential use in prevention strategies.

Biogenic amines are low molecular weight organic compounds that affect several human physiological functions such as regulation of body temperature, stomach volume and pH, and brain activity *(122, 199)*. In high concentrations, biogenic amines such as ethanolamine, putrescine, cadaverine, spermidine, phenylethlamine, tyramine, and histamine cause allergic-like reactions *(57)*. Most biogenic amines in food are formed by the action of enzymes that are capable of decarboxylating specific amino acids *(199)*. From a food safety perspective, the most important biogenic amines are putrescine, formed from ornithine; cadaverine, formed from lysine; and histamine, formed from histidine. Human illness resulting from consumption of food high in biogenic amines has generally been called histamine poisoning. The disease is often referred to as scombrotoxic fish poisoning or

scombrotoxicosis because of its common association with fish in the suborder *Scombridae* (e.g. tuna and mackerel). These pelagic species of fish are known to contain large amounts of free amino acids (i.e. histidine) in their muscle tissue.

Prevention of scombrotoxin formation in fish is primarily achieved by time and temperature controls to prevent growth of naturally occurring spoilage bacteria, which are capable of producing histamine and other biogenic amines due to the presence of decarboxylase enzymes. Other measures of control that may be used to reduce or inhibit scombrotoxic formation and therefore prevent foodborne illness include sanitation, antimicrobials, histamine degrading enzymes, modified atmospheres, high hydrostatic pressure (HPP) and adequate storage/holding conditions. It is widely recognized that use of two or more control measures in combination are more effective than one control measure for reducing food safety risks and preventing human illness. The hurdle technology approach is a food preservation technique frequently used to inhibit or reduce the risks from potential pathogenic microorganisms, or in the case of scombrotoxic fish poisoning, the risks from potential toxigenic histamine-producing bacteria.

1.2 EPIDEMIOLOGY AND OUTBREAKS

The U.S. Centers for Disease Control and Prevention (CDC) estimate that foodborne diseases cause 76 million illnesses annually in the US, resulting in 325,000 hospitalizations and 5,000 deaths *(143)*. Histamine or scombrotoxic fish poisoning accounts for 5% of all food-borne outbreaks and 37% of all seafood-related, food-borne illnesses reported to the CDC *(159)*;

scombrotoxic fish poisoning being one of the three most frequently reported illnesses associated with the ingestion of fish *(150)*. Ironically, scombrotoxic fish poisoning often goes unreported due to the mild nature of the illness.

From 1973 through 1987, 202 of 697 (29%) foodborne outbreaks reported to the CDC and associated with chemical agents were due to scombrotoxic fish poisoning *(89)*. From 1993 to 1997, 69 outbreaks caused 297 cases of scombrotoxic fish poisoning. Thirty eight percent (341/899) of the seafood associated outbreaks from 1990 to 2003 were caused by scombrotoxin where mahi-mahi and tuna were the most common associated fish species *(55)*. One of the largest scombrotoxic fish poisoning outbreaks in the US occurred in 1973, with 254 cases in eight states. All victims became ill about 45 minutes after consumption of commercially canned tuna. The U.S. Food and Drug Administration (FDA) recalled the suspected lots and determined histamine levels of 680 to 2,800 mg/kg *(147)*. More recently, in 2003 an outbreak of scombrotoxin fish poisoning occurred in California where 42 individuals became ill after consuming escolar fish (*Lepidocybium flavobrunneum*), a type of mackerel *(71)*. Victims became ill within a few minutes after consumption of the fish and the illness lasted up to 2 hours.

Scombrotoxic fish poisoning occurs not only in the United States, but throughout the world. The countries with the highest reported incidence of scombrotoxic fish poisoning, outside of the United States are Japan and the United Kingdom *(122)*. Thirty five outbreaks associated with processed fish and shellfish were reported between 1980 and 1989 in England and Wales. Scombrotoxic fish poisoning was the most common cause of illness,

and gave rise to 20 outbreaks affecting 59 people *(189)*. Between 1992 and 2004, 56 outbreaks of scombrotoxic fish poisoning were reported in England and Wales affecting 296 people *(155)*. Scombrotoxic fish poisoning was recognized as a major cause of illness in Japan during the early 1950s, and remains a major foodborne disease in that country. Forty-two outbreaks involving 4,122 cases were reported by the Ministry of Health and Welfare, Japan, from 1970-1980 *(122)*. The largest recorded outbreak came from Japan in 1973 and involved 1,656 cases associated with the consumption of dried horse mackerel.

1.3 HISTAMINE FOOD POISONING

The most common symptoms of scombrotoxic fish poisoning include rash, flushing and sweating, and burning of the mouth, sometimes described as oral tingling. This manifestation occurs seconds after consumption, declining rapidly in the first 10 minutes, and then slowly afterwards *(43, 154)*. Nausea, vomiting, diarrhea, and stomach pain have also been associated with scombrotoxic fish poisoning. Other possible symptoms include dizziness and swelling of the tongue and face *(154)*. Lethal doses of histamine in guinea pigs produce gasping respiratory movements followed by convulsions, with autopsies revealing perforation of the gastric wall *(26)*. Symptoms usually begin 10 minutes to 2 hours after consumption of toxic substances *(154)*. The severity of symptoms can differ from person to person or with other unknown variables. For example, in one study, fish implicated in an outbreak that led to hospitalization of several people was later fed to volunteers who reported

only mild responses *(43)*. When illness results, histamine levels in implicated fish have commonly been at least 200 ppm and often greater than 500 ppm *(71)*.

Scombrotoxic fish poisoning can be differentiated from an allergic reaction using three main criteria. The first criterion is that the inflicted individual had no previous allergy to the food in question. A second parameter is a high attack rate in outbreaks. Another guideline used for discriminating allergic reactions from scombrotoxic fish poisoning is the presence of a high concentration of histamine in the implicated food. Elevated levels of immunoglobulin E (IgE) in afflicted individuals can also be used to discriminate allergic reactions from scombrotoxic fish poisoning *(199)*.

1.4 AT RISK FOOD PRODUCTS

Many foods can support growth of bacteria resulting in histamine formation. Fermented products such as salami, cheese, and canned sauerkraut have been shown to have histamine concentrations high enough to cause illness, because many of the lactic acid bacteria species needed to produce these products are capable of producing histamine *(198)*. However, fish have been implicated in numerous outbreaks of scombroid fish poisoning. Fish belonging to the families *Scomberesocidae* and *Scombridae* are good candidates for histamine formation because of high concentration of free histidine in muscle tissue *(147, 199)*. However, non-scombroid fish have also been implicated (Table 1. 1). Tuna (*Thunnus*) and mackerel (*Scomber*) have most frequently been involved, but this is partially due to the greater consumption of the these fish world-wide *(197)*. Histidine content can vary within a

single species of fish, and may even differ with stage of reproduction at harvest, leading to a greater or lesser risk of conditions favorable to the production of histamine *(85)*.

Table 1. 1. Common and scientific names of fish most commonly involved in scombroid fish poisoning

	Scientific names	
Common Names	**Family**	**Genus and species**
	Scombridae	
Bonito		*Cybiosarda elegans, Gymnosarda unicolor, Orcynopsis unicolor, Sarda spp.*
Mackerel		*Gasterochisma melampus, Grammatorcynus spp., Rastrelliger kanagurta, Scomber scombrus*
Mackerel, Chub & Spanish		*Scomber spp., Scomberomorus cavalla*
Tuna (small)		*Allothunnus fallai, Auxis spp., Euthynnus spp., Katsuwonus pelamis, Thunnus tonggol*
Tuna (large)		*Thunnus alalunga, Thunnus albacares, Thunnus atlanticus, Thunnus maccoyii, Thunnus obesus, Thunnus thynnus*
Wahoo		*Acanthocybium solandri*
	Scomberesocidae	
Saury		*Cololabis saira, Scomberesox saurus*
	Clupeidae	
Alewife or River Herring		*Alosa pseudoharengus*
Herring		*Etrumeus teres, Harengula thrissina, Ilisha spp., Opisthopterus tardoore, Pellona ditchela, Alosa spp.*
Pilchard or Sardine		*Sardina pilchardus, Sardinops spp.*
Sardine		*Harengula spp., Sardinella spp.*
Shad and roe		*Alosa spp.*
Shad, Gizzard		*Dorosoma spp., Nematalosa vlaminghi*
Sprat or Bristling		*Sprattus spp.*
	Carangidae	
Amberjack or Yellowtail		*Seriola spp.*
Jack		*Caranx spp., Oligoplites saurus, Selene spp., Seriola rivoliana, Urapsis secunda*
Blue Runner		*Caranx crysos*
Jack Crevalle		*Alectis indica*
Rainbow Runner		*Elagatis bipinnulata*
Trevally		*Caranx sexfasciatus*
Jack Mackerel		*Trachurus spp.*
	Engraulidae	
Anchovy		*Anchoa spp., Anchoviella spp., Cetengraulis mysticetus, Engraulis spp., Stolephorus spp.*

Table 1 continued

	Pomatomidae	
Bluefish		*Pomatomus saltatrix*
	Gempylidae	
Escolar or Oilfish		*Lepidocybium flavobrunneum, Ruvettus pretiosus*
Gemfish		*Lepidocybium flavobrunneum*
	Nematistiidae	
Jack or Roosterfish		*Nematistius pectoralis*
	Lutjanidae	
Jobfish		*Aphareus spp., Aprion virescens, Pristipomoides spp.*
Snapper		*Pristipomoides spp.*
	Coryphaenidae	
Mahi-Mahi		*Coryphaena spp.*
	Xiphiidae	
Marlin		*Makaira spp., Tetrapturus spp.*

Source: *(70)*

1.5 HISTAMINE FORMING BACTERIA

There are numerous species of bacteria that produce histamine. The main species responsible for histamine formation in fish belong to the family of *Enterobacteriaceae*, and the genus of *Clostridium*. *Morganella morganii*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterobacter aerogenes, Raoultella planticola, Raoultella ornithinolytica* and *Clostridium perfringens* have all been identified as prolific histamine-formers, producing >1,000 ppm in culture broth. Strains of *Hafnia alvei*, *Citrobacter freundii* and *Escherichia coli* are low producers of histamine, producing histamine at levels <500 ppm *(92, 106, 108)*. Of these, *M. morganii*, *K. pneumonia* and *H. alvei* have most often been implicated in scombrotoxic fish poisoning outbreaks *(108, 197)*. Kanki et al. *(98)* reported that *R. planticola* isolated from fish, produced between 2,810 and 5,250 ppm histamine when overnight culture was incubated in

trypticase soy broth fortified with 1% histamine (pH 5.8) at 30˚C for 18 hours. Lopez et al. *(133)* reported that six strains of *M. morganii* produced on average 2,765 ppm of histamine when incubated in 1% histidine broth (pH 5.3) at 37˚C. *Hafnia alvei* and *C. freundii* produced on average 224 and 90 ppm under the same conditions.

Proteus spp. and *Klebsiella* spp. were isolated and classified as strong histamine producers in samples of skipjack tuna and jack mackerel, with the most abundant species being *Morganella morganii (Proteus morganii) (163). Morganella morganii* and *Proteus mirabilis*, both mesophilic Gram-negative rods, have been isolated from mahi-mahi and both species formed greater than 1,000 ppm when grown in tryptic soy broth supplemented with 2% histidine monohydrochloride for 24 hours at 37˚C *(75). Morganella morganii* and *Proteus mirabilis* were also isolated from sardine, along with *Proteus vulgaris, Providencia stuartii*, and unidentified species of *Proteus (1).* From market samples of mackerel collected from Barcelona, Spain, *Citrobacter freundii, Enterobacter agglomerans, Morganella morganii, Proteus mirabilis, Serratia fonticola*, and *Serratia marcescens* were isolated *(133)*. In addition to these, *Enterobacter cloacae, Enterobacter intermedium, Hafnia alvei, Klebsiella oxytoca, Plesiomonas shigelloides, Proteus vulgaris, Pseudomona fluorescens, Serratia liquefaciens,* and *Serratia plymuthica* have all been isolated from samples of tuna collected from markets in the same area *(133)*. Likewise, bonito samples contained *Enterobacter agglomerans, Enterobacter cloacae*, *Klebsiella pheumoniae*, and *Pseudomona fluorescens*. *Hafnia alvei* and *Proteus* spp. isolated from skipjack tuna and jack mackerel were classified as weak histamine producers *(163)*. *Vibrio alginolyticus*, a mesophilic, Gram negative rod, was isolated from fresh mahi-mahi and determined to be a weak histamine

former, producing less than 100 ppm in tryptic soy broth supplemented with 2% histidine monohydrochloride held for 24 hrs at 37˚C *(75)*. *Stenotrophomonas maltophia* was recently isolated from albacore tuna (*Thunnus alalconga*) *(23)* and *Photobacterium* spp., a psychrophilic organism, has been proposed as a significant histamine-producing organism *(199)*. *Alteromonas putrefaciens*, also a psychrotrophic Gram-negative rod, was isolated from mahi-mahi and determined to be a weak histamine producer, forming less than 100 ppm histamine in histidine supplemented growth medium *(75)*.

The ability to form histamine in fermented foods appears to be limited to a few strains of lactobacilli, including *Lactobacillus* 30a, *L. buchneri*, and *L. delbrueckii (197)*. *Lactobacillus* 30a produced 1402 ppm of histamine in decarboxylase synthetic broth at 28˚C for seven days *(76)*. Chander et al. *(34)* reported that *L. bulgaricus* produced 71 ppm of histamine after 24 hours at 30˚C incubation in milk.

The presence of high numbers of histamine-producing bacteria does not necessarily correlate with high histamine levels in fish samples. In one study, a very low percentage (7%) of isolates from one tuna specimen were shown to be histamine producers, but the level of histamine from that tuna sample was higher than that in a second tuna specimen with a much higher percentage (59%) of histamine-producing isolates *(163)*. The most likely explanation for this observation is that the bacteria responsible for the histamine production were out competed or where inactivated over the course of product storage. Other possible explanations include the presence histamine reducing substances or bacteria, or that the bacterial isolates vary in their ability to produce biogenic amines *(163)*.

1.6 HISTIDINE DECARBOXYLASE

Two different classes of histidine decarboxylase enzymes (HDC) exist: those found in eukaryotic cells and Gram negative bacteria, which require pyridoxal-5'-phosphate as a cofactor, and those found in Gram-positive bacteria, which use a covalently bound pyruvoyl moiety as a prosthetic group *(29, 170, 174)*. Seven histidine decarboxylases isolated from Gram-positive bacteria have been studied. These were derived from the following organisms: *Lactobacillus* 30a *(180)*, *Lactabacillus buchneri (168)*, *Clostridium perfringens (168)*, *Micrococcus* sp. *(167)*, *Tetragenococcus muriaticus (114)* and *Oenococcus oeni (44)*. The most thoroughly studied histidine decarboxylase enzyme is from *Lactobacillus* 30a (EC 4.1.1.22). Histidine decarboxylase from *Lactobacillus* 30a is hexameric enzyme of Mr 208,000 composed of two noncovalently bound subunits, designed α and β, in a $(\alpha\beta)_6$ structure. It is synthesized as a single subunit proenzyme (π) which is activated by cleavage of the π-chain at the amide bond between serine 81 and 82 to yield α and β subunits *(170)*. The mechanism by which histidine decarboxylase functions involves a Schiff base formation with the pyruvyl residue in the catalytic site (Figure 1. 1). Catalysis is initiated by carbinolamine formation followed by dehydration (Figure 1. 1, reaction 1). It is possible, however, that once the catalytic cycle has begun, the enzyme-substrate Schiff base may form by transamination between the enzyme-product Schiff base and histidine (Figure 1. 1, reaction 5), thereby eliminating the hydration and dehydration steps. The enzyme also catalyzes exchange of the hydrogen atom of histamine, which occupies the same position as

the carboxyl group of L-histidine (Figure 1. 1, reaction 3), but at a very slow rate, indicative of either a high energy of activation for proton abstraction from the enzyme-histamine Schiff base or slow exchange of the abstracted proton with the solvent *(170)*. The optimum pH range for enzyme activity of pyruvoyl-dependent histidine decarboxylase is 4.4-5.8 (Table 1. 2).

Of the Gram-negative pyridoxal-5'-phosphate dependent (PLP) enzymes, the histamine decarboxylase from *Morganella morganii* AM-15 *(44)*, *Raoultella planticola*, and *Enterobacter aerogenes* have been studied *(83, 96)*. Histidine decarboxylase from *Morganella morganii* is the most extensively studied of the Gram-negative PLP-dependent enzymes *(195)*. Unlike the histidine decarboxylase from *Lactobacillus* 30a, it is a tetramer of identical subunits each having MW 43,000 kDa, with a total MW of 170,000 kDa. The histidine decarboxylase from *M. morganii* has a pH optimum of 6.5 and the enzyme is not stable above 40˚C *(195)*. *R. planticola* histidine decarboxylase forms dimeric and tetrameric forms, while native HDC of *E. aerogenes* contains two subunits. HDCs from all three organisms are specific for L-histidine; arginine, lysine, and ornithine are inactive as substrates *(83)*.

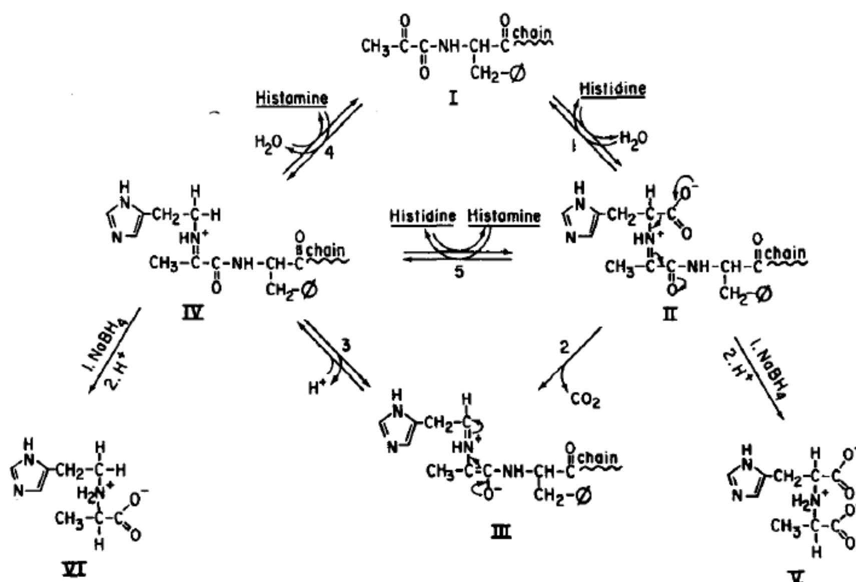


Figure 1. 1. Mechanism of action of histidine decarboxylase from Lactobacillus 30a. The letter Φ denotes the phenyl group. Formation of Schiff base intermediates II and IV has been demonstrated by trapping with sodium borohydride *(169)*. This mechanism is probably applicable to all pyruvate-dependent carboxylases *(170)*. Source: *(169)*

Table 1. 2 Properties of histidine decarboxylases from different sources.

Decarboxylase	Microorganism	pH_{opt}	Reference
	Morganella morganii	6.5	*(96, 195)*
	Raoultella planticola	6.5	*(83, 96)*
Pyrodoxal-P dependent	*Enterobacter aerogenes*	6.5	*(83)*
	Photobacterium damselae	6.0	*(96)*
	Photobacterium phosphoreum	7.0	*(96)*
	Lactobacillus 30a	4.8	*(180)*
Pyruvoyl-dependent	*Lactobacillus buchneri*	5.5	*(168)*
	Clostridium perfringens	4.5	*(168)*
	Micrococcus sp.	4.4-5.8	*(167)*

1.7 OPTIMUM CONDITIONS FOR HISTIDINE DECARBOXYLASE ACTIVITY

Optimum conditions for histidine decarboxylase enzyme (HDC) activity are not completely clear, largely because of the many factors that need to be interpreted, including bacterial cell propagation, initial cell concentrations, and initial composition of the microflora. Substrate-specific decarboxylase enzymes from microorganisms in the food are responsible for the production of amines in foods, but the rate of production is not necessarily linked to bacterial growth *(199)*. For instance, *Morganella morganii* produced substantial amounts of histamine at temperatures between 10 and 25˚C. After incubation at 25˚C for 24 hours, this same organism was found to produce even more histamine during subsequent low temperature storage (0-5ºC), exceeding the level produced during storage at 25ºC. At these low temperatures, *Morganella morganii* did not actively grow but the enzyme produced at high temperature remains active *(111)*. Similarly, histamine was formed by resting cells, or cells that were alive but not reproducing, of *Klebsiella pneumoniae* at 2ºC *(17)*. Haaland et al. *(85)* found that, for bacteria isolated from mackerel, histamine was produced at 2ºC albeit at a lower rate than at 20ºC. Fernandez-Salguero et al. *(73)* also reported the production of toxic levels of histamine at 0ºC in mackerel fillets, minced meat, and liver by day 18 of storage. Kanki et al. found that the histidine decarboxylase enzyme from *Photobacterium phosphoreum*, *P. damselae*, *Raoultella planticola* and *Morganella morganii* was most active at temperatures between 30-40˚C *(96)*.

Not only is there variation in the optimal temperature for histamine production, but there is also variation in the oxygen and pH requirements for production of histamine.

Ferencik *(72)* found that histamine-producing *Hafnia* strains produce the compound under anaerobic conditions, but anaerobic production is associated with a longer lag period compared to aerobic environments. As perhaps expected, there was a shorter lag period when histamine was produced by bacteria inoculated into fish homogenate compared to actual flesh samples *(72)*. On the other hand, Dapkevicius et al. *(49)* reported that a low oxygen environment was best for promoting decarboxylation of histidine. In addition, a low pH was preferred for optimal decarboxylase activity in that same study *(49)*. For example, histidine decarboxylase from *Klebsiella pneumoniae* was found to have an optimum activity at a pH of 4.0 with a reduction to 70% activity at a pH of 6.0 *(17)*. On the other hand, Kanki et al. *(96)* found that optimum activity of *P. phosphoreum*, *P. damselae* and *R. planticola* and *M. morgani* was at pH 7.0, 6.0, 6.5 and 6.5, respectively. These investigators reported that because of the high stability of the HDC from *P. damselae* under acidic environments and survival of the bacteria during frozen storage, it may be a major contributor to histamine-formation in frozen-thawed fish.

1.8 DETECTION METHODS

1.8.1 Detection of Histamine-Producing Bacteria

Detection of histamine-producing bacteria is frequently performed using Niven's agar, a differential media containing tryptone, yeast extract, L-histidine dihydrochloride, sodium chloride, calcium carbonate, agar and the pH indicator bromocresol purple. Purple colonies indicate possible histamine production due to shifting of medium pH *(23, 74, 158)*. There have been modifications in the concentration of these various components over the

years, but mechanisms of detection are the same, i.e., the decarboxylation of histidine to histamine makes acidic conditions more basic, resulting in a color change. After isolation and incubation, further methods for detection of histamine, discussed later, can be used to confirm histamine production *(74)*. Although Niven's agar is a generally accepted method for screening, it has been shown to give not only false positive but also false negative results. For instance, Kim et al. *(102)* cultured an isolate that was negative on Niven's agar but turned out to be the most active histamine producer isolated from the study. These same investigators also found that many positive isolates on Niven's agar produced no detectable histamine. Fletcher et al. *(74)* also reported many false positive isolates, slightly more than 15%, whereas Lopez-Sabater et al. *(133)* recorded over 60% false positives on Niven's agar.

Histamine-forming bacteria can be detected and the activity of their decarboxylase enzymes quantified by measuring changes in the electrical potential of the histidine decarboxylating media in which they are grown. The confirmation of a significant histamine producer can be determined within 24 hours using this method. This method was found to be rather specific and comparatively fast, but it has not been extensively tested, due to the cost of equipment needed to perform the procedure *(112)*.

More recently, molecular methods are becoming widely accepted as an alternative to culture methods for detection and identification of histamine forming bacteria. One method involves amplifying the histidine decarboxylase gene of histamine-producing bacteria. Takahashi et al. *(194)* developed a molecular method for detection of Gram-negative histamine producers by using two oligonucleotide-mixed primers to specifically amplify a

709-bp fragment in the pyridoxal phosphate-dependent (PLP) histidine decarboxylase gene. Of the 37 histamine-producing bacteria tested in this study, all histidine-decarboxylating Gram-negative bacteria produced a polymerase chain reaction (PCR) product, except for one strain of *Citrobacter braakii*. None of the 470 non-histamine-producing strains tested produced an amplification product. Similarly, Kanki et al. *(98)* developed primers on the basis of the sequence of *hdc* genes of *Raoutella planticola,* producing a 724 bp fragment of the PLP-dependent histidine decarboxylase. All 53 histamine producing strains of *R. planticola* and 13 strains *R. ornithinolytic* tested produced PCR product corresponding to amplification of the PLP-dependent histidine decarboxylase gene. However, two strains of non histamine-producing *R. planticola* also showed a positive product.

In addition, oligonucletide primers have been designed to amplify other biogenic amine-forming enzymes. In a study by Rivas et al. *(176)* oligonucleotide primers were designed for the specific detection of genes encoding for histidine, ornithine, or lysine decarboxylase of Gram-negative bacteria. The designed primers allowed for the amplification of a 531, 1,440, and 1,098 bp fragments of the histidine, ornithine and lysine decarboxylase genes, respectively. In another study, a multiplex assay was developed for the simultaneous detection of these enzymes *(177)*.

The pyruvoyl-dependent histidine decarboxylase from Gram-positive bacteria has also been detected by PCR amplification. Le Jeune at al. *(120)* developed four primers (JV16HC, JV17HC, CL1, CL2) based on the histidine decarboxylase gene of *Lactobacillus* 30A, *Lactobacillus buchneri* and *Clostridium perfringens*. They found that using primer set

JV16HC/JV17HC which amplifies a 370 bp fragment of the histidine decarboxylase gene, all strains identified as histamine producers by PCR gave positive signals in histidine decarboxylase activity test. Moreover, strains which did not exhibit histidine decarboxylase activity failed to give a signal in PCR. A 500 pb fragment amplified using primer set CL1/JV17HC was used as a probe in hybridization. All histamine producing lactic acid bacteria were detected by DNA hybridization. However one false-positive was detected in the hybridization assay and *Cl. perfringens* did not give a hybridization signal. The same primers were used by Alves et al. *(5)* to amplify the pyruvoyl-dependent histidine-decarboxylase gene from Gram-positive bacteria present in canned fish. They found non-expected fragments (100 bp, 150 bp and 250 bp) when DNA was amplified with primer set JV16HC/JV17HC. One of the disadvantages of molecular-based method is that both powerful and weak histamine producers are detected equally well, meaning that fish with weak histamine-producing bacteria may be positively identified based on PCR, even though histamine production is minimal. Thus, molecular methods for histamine-production need to take to account these differences.

1.8.2 Detection of Histamine and other Biogenic Amines

Detection of histamine in scombrotoxic fish in the range of 10 to 200 ppm is required to meet quality control measures designed to minimize the chance of histamine poisoning in scombrotoxic fish. Good quality fish contains less than 10 ppm histamine, a level of 30 ppm indicates significant deterioration, and 50 ppm is considered to be evidence of definite

decomposition The defect action level (DAL), the level at which regulatory actions are taken, for histamine is 50 ppm *(178)*. Histamine is usually extracted from the fish tissue using alcohol, water or trichloroacetic acid (TCA).

The original Association of Official Analytical Chemists (AOAC) approved method for detection of histamine involved inoculation of a section of guinea pig intestine. The histamine was first extracted from unknown samples by incubating the homogenized sample in a weak hydrochloric acid solution, followed by filtering and neutralizing steps. The measurement was the muscle’s contraction upon exposure to the unknown solution and this was compared to a standard curve produced using different concentrations of purified histamine *(10)*.

The second AOAC method approved for detecting and quantifying histamine in seafood begins with a complex chemical extraction using methanol, benzaldehyde, a sodium hydroxide solution, and a benzene-n-butanol mixture, after which the extracted sample is passed through a crude cotton acid succinate column. The eluted fraction is collected and quantified by reading at 475 nm using a spectrophotometer *(11)*. Lerke and Bell *(126)* developed the basic design of the most recent official method of histamine determination in seafood, which uses an ion exchange column step for sample extraction, after which is applied a flourescence based detection method *(12)*. Extraction of biogenic amines using 75% methanol, as in the second AOAC method, was later added to the most current AOAC method *(178)*.

Other methods for detection of biogenic amines are available, although they have not been validated for official use. Chromatographic methods are the most commonly used for analyzing biogenic amine concentrations. For instance, similar to the AOAC method, liquid chromatography followed by fluorometric detection is a reliable method with low detection limits (10 ppm) *(22)*. A common method of biogenic amine analysis has been the use of high pressure liquid chromatography (HPLC), which can accurately quantify histamine at concentrations as low as 0.5-2.5 ppm, although for some products, such as canned anchovies in oil, the detection limit is higher (10-55 ppm) *(90, 149, 211)*. Ben-Gigirey et al. *(23)* found that extraction of the biogenic amines using 0.4 M perchloric acid provided better peak separation than the traditional use of 10% trichloroacetic acid when applying HPLC to the detection and quantification of histamine. Thin layer chromatography (TLC) has also been used to separate histamine from fish tissue using ninhydrin or Pauly's reagent to visualize spots. The somewhat poorer detection limit of this method (20 ppm) restricts its use to screening purposes, but due to its speed and low cost it remains a valuable detection approach *(183)*. Putrescine and cadaverine can be quantified using gas chromatography (GC) technology *(178)*.

Histamine can also be quantified based on electrical potential, which is conceptually the same as that used to identify decarboxylase-producing bacteria. The major differences between these methods is that for direct detection of biogenic amines, an enzyme must be added, either monoamine oxidase (MAO) or diamine oxidase (DAO), and a prior perchloric acid extraction is also needed. This method appears to be rather sensitive, being able to

quantify an increase of only 2 μmol/L of biogenic amines in a sample. A problem with this method is that it is not very specific and will respond to all biogenic amines *(58)*. Histamine concentration has also been evaluated using DAO applied to an aqueous extraction from tuna. This results in the formation of hydrogen peroxide, followed by quantification of the hydrogen peroxide using a colormetric method *(127)*. Until recently this method has not been very accurate because other biogenic amines can also be oxidized. However, Lopez-Sabater et al. *(133)* applied it with relatively good results using a perchloric acid extraction method, and a more specific DAO enzyme, reporting a curve that can be correlated with a standard curve in the range of 3-30 ppm. Capillary zone electrophoresis has been used after a methanol extraction to separate histamine from other sample components. This was followed by spectrophotometric detection at 210 nm yielding a quantification range between 0.5 ppm and 100 ppm *(151)*. After a methanol extraction similar to that applied in the AOAC method, the strong copper chelation activity of histamine can also be used to determine histamine concentration. By adding copper and a dye to a purified sample extract, a color change results. This method has a detection limit in the low ppm range, making it useful as an industry confirmation method because it provides sensitivity without the need for additional equipment *(20)*. Odor detection has even been applied to the detection of certain biogenic amines. Putrescine was found to have an odor threshold of about 10^{-5} M, while spermine and spermidine were found to both have thresholds of less than 10^{-4} M in soybean flour *(202)*. However since sensory thresholds vary from person to person, this is not a very accurate or reliable method for screening of biogenic amines.

Histamine test kits are now available that are based on enzyme-linked immunosorbent assay (ELISA) technology. Most of these tests are good for little more than screening, but some, such as Neogen Corporation's (Lansing, MI) Veratox® histamine kit have been shown to accurately measure a wide range (2-50 ppm) of histamine concentrations *(179)*.

1.9 FEDERAL REGULATIONS OF HISTAMINE IN SEAFOOD

In September 1982, the US Food and Drug Administration (FDA) established a defect action level (DAL) of 200 ppm for histamine in raw and cooked fish, meaning that when a product was found to have 200 ppm or more histamine, corrective action (discard) had to be taken. In 1995, the FDA reduced the histamine DAL to 50 ppm *(68)*. The reduced DAL occurred due to the variability in histamine concentrations from section to section within a fish. For example, if there is a 50 ppm concentration of histamine in one sample, the FDA reasoned that there could be a sufficiently high enough concentration to cause illness (500 ppm) in another section of the same fish or lot. Also in 1995, the FDA promulgated new food safety regulations based on the Hazard Analysis Critical Control Points (HACCP) approach that required industry to implement written HACCP plans by December 1997 to reduce and/or eliminate food safety risks in fish and fishery products *(70)*.

HACCP is based on seven principles which are designed to set limits at points in a process to control the risk of a health hazard, and then make sure the limits are met by monitoring and record keeping *(94)*. For fish susceptible to histamine formation, the FDA has suggested three critical points where controls should be exercised: (1) receiving by the

primary processor (includes first receiver); (2) receiving by the secondary processor; and (3) during processing (including storage). The limits suggested for the primary processor depend mainly on the water temperature at harvest, the size, and the species of fish. For harvesters that do not collect records at capture, the FDA suggests that a sample of the fish be tested for histamine levels to ensure compliance with DAL specifications. On receipt by the secondary processor, either transportation records showing that the fish were held at or below 4.4˚C, or else the presence of sufficient cooling media surrounding the product at the time of delivery, are suggested. Critical limits for processing depend on whether the fish have been previously frozen. If the fish were not previously frozen, they should not be exposed to temperatures above 4.4˚C for more than 8 cumulative hours. If at any time fish are exposed to temperatures above 21˚C, the cumulative time above 4.4˚C cannot exceed 4 hours. If the fish were previously frozen, they should not be exposed to temperatures above 4.4˚C for more than 24 cumulative hours. If at any time the temperature is above 21˚C, the cumulative time above 4.4˚C cannot exceed 12 hours *(199)*. The FDA is expected to update guidance for implementing HACCP control for fish in 2009.

Lehane and Olley published in 2000 a review on scombrotoxic fish poisoning in a risk-assessment framework, see *(122)*. In this review, the hazard of scombrotoxic fish poisoning is identified, dose-response assessment and exposure assessment are discussed quantitatively. They reported that the key for keeping bacterial numbers and histamine levels low is the rapid cooling of fish after harvest and the maintenance of adequate refrigeration during handling and storing.

1.10 STRATEGIES OF CONTROL

1.10.1 Hurdle Technology

Hurdle technology is well established for the realization of safe, stable, nutritious, tasty, and economical foods. At a very basic level, hurdle technology consists of the deliberate combination of multiple preservation techniques (existing and novel) to establish a series of inhibitory conditions (hurdles) that limit the growth and/or survival of microorganisms in foods. The type of technologies used depends upon both the food and the organisms of concern *(192)*. Since the microorganism present in a food should not overcome the hurdles present during the storage of a product, the use of multiple hurdles frequently results in improved product shelf life and reduced risk of pathogen contamination and subsequent foodborne disease. By placing a number of sublethal stresses (hurdles) on a microbial cell, the organism expends energy to overcome the hostile environment, potentially leading to metabolic exhaustion and death. Thus, hurdle technology aims at improving the overall quality and safety of foods by application of an appropriate mix of controls. An important consideration in hurdle technology is that different hurdles used together result in synergistic rather than additive effects *(60)*. Combination techniques allow the use of hurdles or barriers of lower intensity, minimizing their effect on product quality *(192)*. The physiological responses of microorganisms during food preservation (i.e. their homeostasis, metabolic exhaustion, and stress reactions) are the basis of the application of advanced hurdle technology *(124)*.

At present, more than 60 potential hurdles for improving food product stability, safety and/or quality have been described (Table 1. 3) *(124)*. The most commonly used hurdles (also called barriers) are temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), competitive microorganisms (lactic acid bacteria) and preservatives (nitrite, sorbate and sulfite). Emerging hurdles include ultrahigh pressure, irradiation, modified-atmosphere packaging, bacteriocins and edible coatings *(125)*. When the traditional preservation factors are combined with some of the newer technologies, greater efficiencies in food preservation may be possible.

Table 1. 3 Some potential hurdles for use in the preservation of foods

Temperature (high or low)	MA (modified atmosphere)
pH (low or high)	Pressure (high or low)
aw (low or high)	Ultrasound (high)
Eh (low or high)	Radiation (UV, Microwaves, ionizing radiation)
Oxygen tension (low or high)	Competitive microflora (lactic acid bacteria etc.)
Microstructure (e.g. water-in-oil emulsion)	
Preservatives (organic acids, sodium lactate, sodium acetate, sodium ascorbate, trisodium phosphate, potassium sorbate, parabens, fatty acids and their esters, glycerol, propylenen glycol, ethanol, spices, nitrite, sulfite, smoke, antioxidants, chelating agents, Maillard reaction product, pimaricin, and other antibiotics, lysozymes, nisin and other bacteriocines, etc.)	

Source: *(123)*

1.10.2 Temperature

In the seafood industry, histamine formation is controlled primarily by the use of low (refrigeration and freezing) storage temperatures. Optimal temperature for histamine production is variable *(69)* due to differences in the types and levels of microbial flora in fish. For example, Behling and Taylor *(21)* reported that the optimum temperature for

histamine production by *Eschericha coli* and *Citrobacter freundii* was 37˚C; 30˚C for *Morganella morganii* (*Proteus morganii*) strain 110SC2, *Klebsiella pneumoniae*, and *Hafnia alvei*; and 15˚C for *Morganella morganii* (*Proteus morganii*) strain JM. The lower temperature limits for production of toxicologically significant levels of histamine were 7˚C for *K. pneumoniae*; 15˚C for both *M. morganii* strains; and 30˚C for *H. alvei*, *C. freundii*, and *E. coli*.

Increased histamine levels have been reported in fish stored at high temperatures. For instance, Gingerich et al. *(80)* determined changes in histamine concentrations in bluefish fillets stored at 5, 10, and 15˚C for 7 days. The greatest accumulation of histamine was observed in the fish stored at 15˚C, reaching 938 ppm in 3 days. The histamine concentration of samples stored at 10 and 15˚C did not increase considerably over time. Du et al. *(59)* found the highest level of histamine (764 ppm) in mahi-mahi stored for 5 days at 12.8˚C. Histamine levels in mahi-mahi stored at 1.5 and 7.2˚C were 5 and <15 ppm, respectively. Kim et al. *(101)* reported that the optimum temperature for histamine formation in albacore tuna was 25˚C, where histamine levels in the fish were higher at 25˚C than at 30˚C or 37˚C. Kim et al. *(105)* isolated histamine-producing bacteria from albacore stored at 0, 25, 30, and 37˚C. The highest histamine former was identified as *M. morganii* and was isolated from albacore muscle completely spoiled after storage at 25˚C for 7 days. The optimum temperature for growth of histamine-producing bacteria was 25˚C. Neither microbal growth nor histamine formation was detectable at 4˚C.

Storage at 0˚C or below can prevent histamine formation and reduce histamine in fish muscle. Kim et al. *(101)* found no histamine in whole or dressed albacore stored in ice for up to 18 days. When albacore was frozen prior to storage, a reduced amount of histamine resulted. Middlebrooks *(148)* found low levels of histamine in mackerel during storage at 0˚C for 16 days, with mean levels remaining less than 0.6 mg/100g. Guizani et al. *(84)* found that histamine levels dropped in yellowfin tuna stored at 0˚C, from a initial level of 2.78 mg/100 g on day one to 0.61 mg/100 g at day 17, which is below FDA standards. The reduction of histamine most likely occurs because of the action of histamine decomposing bacteria. Ryser et al. *(182)* isolated 60 psychotropic bacteria from raw tuna which were identified as *Pseudomonas fluorescens*, *Pseudomonas putida* and non-fluorescent *Pseudomonas* spp. Of the isolates, 28% of *P. putida*, 21% of *P. fluorescens* and 62% of non-fluorescent *Pseudomonas* spp. produced low levels of histamine, with the maximum amount of 320 ppm, which is below the minimum level of 500 ppm believed necessary to induce symptoms of histamine toxicity. Klausen and Huss *(111)* found no growth or histamine formation in mackerel inoculated with *M. morganii* during storage at 0˚C. It has been reported that after storage of albacore tuna for 3 months at either –18˚C or –25˚C, histamine levels decreased dramatically, but after 9 months of storage in –18˚C, the levels rose to their original concentration *(23)*. A possible explanation for this phenomenon is that components, chemical or enzymatic, break down the biogenic amines faster than they can be produced at these temperatures, however such components may be exhausted or inactivated faster than the histidine decarboxylase enzymes.

Significant histamine formation has been reported to occur at low temperatures. Emborg et al. *(63)* found that the initial microbiota of vacuum packed yellowfin tuna from the Indian ocean was dominated by *P. phosphoreum* (60%) and *B. thermosphacta* (40%). After 17 days of storage at 1.7±0.7˚C, the microbiota of the MAP tuna was dominated by *Carnobacterium* spp. (30%) and psychrotolerant *M. morganii*-like bacteria (30%). Screening studies at 10˚C showed that the psycrotolerant *M. morganii*-like isolates from the spoilage microbiota were most likely responsible for the formation of histamine in MAP tuna at 1.7±0.7˚C. The substantial histamine formation at such a low temperature was surprising, particularly as numerous studies found *M. morganii* unable to produce toxic concentrations of histamine below 7-10˚C. The same authors later identified this new psychrotolerant strain as *Morganella psychrotolerance* sp. nov *(61, 62)*. This new species grew faster than *M. morganii* at temperatures below 15˚C and grew at temperatures between 2-37˚C with optimum growth at 26-27˚C.

Temperature is the most effective method for controlling the growth and formation of histamine by relevant bacterial strains *(69)*. However, studies have shown that histamine can form at temperatures as low as 2˚C. In addition, slight temperature abuse can stimulate histamine production. Thus, consistent with the hurdle concept, other methods in addition to time/temperature may be needed to control histamine formation in fish and fishery products.

1.10.3 Sanitation

Histamine-forming bacteria can originate from the marine environment or from post-harvest contamination. Post-harvest contamination with histamine-forming bacteria can occur at several levels: aboard the fishing vessel, at the processing plant, in the distribution system and at the user level *(122)*. Lopez-Sabater et al. *(134)* reported that the level of *Enterobacteriaceae* in tuna increased over the course of handling during a canning process until it represented 2.18% of the total bacterial load. Allen et al. *(4)* found histamine-producing bacteria on fish contact surfaces and on a knife used to cut fish on-board a fishing vessel. Certainly, post-harvest contamination of histamine-forming bacteria must be minimized to prevent the formation of histamine in fish. Fishermen and processors must thoroughly wash hands before handling fish as well as after handling contaminated material and using toilet facilities. Food contact surfaces and equipment should be properly sanitized to prevent contamination by biogenic amine-producing bacteria. Since the bacteria that produce histamine can be found in high numbers in the digestive tracts of fish, care must be taken when gutting whole fish. Specifically, the digestive tract should not be cut or spilled into the gut cavity or other parts of the fish, and care should be taken to avoid cutting or puncturing the edible portions of the fish *(69)*.

1.10.4 Inhibition of Histidine Decarboxylase

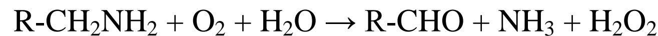
Inhibiting the activity of the histidine decarboxylase enzyme (HDC) can prevent or reduce the formation of histamine. Histamine itself is a good competitive inhibitor of the pyruvoyl enzymes *(180)*, but not of the pyridoxal-P enzymes. Other competitive inhibitors of pyruvoyl HDC include imidazolepropionic acid, urocanic acid, imidazole acetic acid, imidazole carboxylic acid, imidazole, and N-methylimidazole. Cyanide and p-chloromercuribenzoate provided a noncompetitive inhibition of the decarboxylase *(180)*. Methyl-substituted histidines such as α-methylhistidine and particularly α-fluoromethylhistidine are potent inhibitors of the pyridoxal-P dependent HDC, but not the pyruvoyl enzymes *(83)*.

Several investigators have examined the inhibitory effect of food components on HDC. Soy flavones, compounds from soy sauce, show intense inhibitory activity on HDC from *Clostridium perfringens* even in the presence of 2.7 M NaCl *(109)*. Wendakoon et al. *(208)* reported that ethanol extract of allspice, nutmeg, sage, and cinnamon and clove are very effective in inhibiting histidine decarboxylase activity in a crude extract from *Enterobacter aerogenes*. In this case, more than 90% of the HDC activity was diminished in the presence of ethanol extract of cinnamon, allspice and sage. The presence of NaCl accelerated the inhibitory action. Of the compounds present in the spices, cinnamic aldehyde and eugenol were the most effective inhibitors. Histidine decarboxylase from *Lactobacillus higardii* 5w shows opposite regulation when exposed to two organic acids commonly present in wines: L-malic and citric acid. Histidine decarboxylase production is inhibited up to 71%

by the addition of 2.0 g/L-malic acid but the specific activity of the enzyme is increased with increased citric acid concentration up to 300 mg/L *(66)*. These studies suggest that specific inhibitory substances may be used to prevent histamine formation in fish. Optimal inhibitory compounds should have a generally recognized as safe status (GRAS) and hence be safe for human consumption.

1.10.5 Histamine Degradation (Biocontrol)

The use of diamine oxidase to degrade histamine is another means by which to reduce histamine contamination of fish. Indeed, investigators have used histamine degrading starter cultures in fermentations. In these studies, inactivation of biogenic amines was achieved by the addition of microorganisms possessing amine oxidase activity. Amine oxidases are a large group of enzymes catalyzing oxidative deamination of amines:



$$R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + NH_3 + H_2O_2$$

where R are aromatic or aliphatic radicals. Primary amines are thus oxidized to the corresponding aldehydes, ammonia and hydrogen peroxide, where aldehydes can further be used as nutrient source. Dapkevicius et al. *(49)* screened starter cultures for their ability to degrade biogenic amines in fish silage and assessed the ability of the diamine oxidase (DAO) to degrade histamine under the conditions prevailing in fish silage. Four of the 48 lactic acid bacteria (LAB) isolates examined could degrade histamine in model systems. Histamine

degradation by two of these isolates was also observed in the ensiled fish slurry. Leuschner et al. *(128)* examined microorganisms suitable for food fermentation with regards to their potential to degrade histamine and tyramine. Out of the 64 LAB tested, 27 degraded histamine and one degraded tyramine *(141)*.

Determination of the optimal conditions for DAO treatment is difficult, since each organism has its own optimum condition for producing DAO and the DAOs produced by different organisms each have their own requirements for optimum activity. In one study, purified DAO from an unidentified bacterial isolate performed optimally at 37˚C with a range of activity from 20˚C to 63˚C *(49)*. The activity of DAO decreased when the pH was reduced to 4.5. A relatively thermostable DAO produced by *Vigna radiara* seedlings had reactivity between 25˚C and 85˚C and could withstand heat treatment of 85˚C for 30 minutes with no significant loss of activity *(40)*. Draisci et al. *(58)* found that optimal deterioration of different biogenic amines is pH-dependent when using a single DAO. Leushner et al. *(128)* reported a negative effect on amine degradation by glucose, NaCl, low temperatures and low pH values. Bacterial cultures isolated from the same type of product for which they will be subsequently used as biocontrol agents may have the greatest chance of success in controlling pathogens *(30)*. Clearly, the use of histamine-degrading microorganisms or the addition of diamine oxidase to fish has the potential of controlling histamine formation.

1.10.6 Antimicrobials and Preservatives

Antimicrobials are a category of preservatives used to prevent or delay the microbiological spoilage of foods. These include various organic acids, parabens, sulfites, ozone, chitosan and nitrite as well as some naturally occurring antimicrobials and antibiotics *(78)*. One of the disadvantages of using preservatives is that they can have a negative perception by consumers. However, preservatives may be used to prevent or delay the production of biogenic amines in fish either by inhibiting the growth of microorganisms or the activity of enzymes that produce biogenic amines.

Organic acids are used extensively in the meat industry to inhibit microbial growth. For example lactic acid, acetic acid, and citric acid can be used in the final wash that is applied to livestock carcasses after trimming and inspection but before chilling. The most important factor affecting the activity of organic acids is the pH of the food in which they are used. This is because the undissociated form of the acid is the one with antimicrobial activity, and the extent of dissociation is impacted by the pK_a of the acid and the pH of the food product. Organic acids are not used to the same degree by the seafood industry, most likely because of differences in muscle structure and the potential for color loss. Bal'a and Marshall *(16)* examined the effect of dip solutions consisting of 2% acetic, citric, hydrochloric, lactic, malic, or tartaric acid at 4˚C to treat catfish fillets inoculated with *L. monocytogenes*. Bleaching occurred on the acid treated fillets, causing them to become lighter and yellower in color than untreated controls. A 2.2 and 1.4 log unit reduction in *L. monocytogenes* occurred for samples treated with acetic acid and hypochloric acid,

respectively. Shalini et al. *(184)* examined the effects of vacuum-packaging with and without a potassium sorbate dip (1, 1.5 and 2%, 30 min) on the shelf life of *Lethrinus lentjan* fillets. The shelf life of control packs was about 7-8 days, whereas the potassium sorbate-treated fillets had an extended shelf life of 20 days. Tassou et al. *(196)* reported that the initial load of *Salmonella* was reduced by 1 log cfu/g in carp and red mullet treated with 5% potassium sorbate.

Polyphosphates may be added to foods to inhibit the growth of microorganisms, presumably because these compounds chelate cations essential for growth. The polyphosphates inhibit the growth of Gram-positive bacteria and molds and are classified as orthophosphates, pyrophosphates, tripolyphosphates and polyphosphates *(78)*. Polyphosphates have been shown in the literature to have different antimicrobial activities. Statham et al. *(191)* reported that polyphosphates (10%) alone or in conjunction with potassium sorbate had no significant effect on the bacterial flora of morwong fillets caught in Australia and stored at 4˚C. On the other hand, Badr et al. *(15)* found that treatment with 3% sodium monophosphate for 15 min extended the shelf life of common carp fillets during storage at 5˚C by 9 days.

Ozone is a gas with strong antimicrobial activities and is generally recognized as safe (GRAS) by FDA for bottled water but is regarded as food additive in food applications *(100)*. It decomposes in solution producing hydrogenperoxyl (•H_2O), hydroxyl (•OH), and superoxide (•O_2^-) radicals. The reactivity of ozone is attributed to the oxidizing power of these free radicals. Ozone has been shown to act on the bacterial cell surface, causing

cellular protein to flocculate. It can also act as a general protoplasmic oxidant, destroying dehydrogenating enzyme systems, oxidizing sulfhydryl groups on enzymes, and causing damage to the genetic material of microorganisms. For a review, see Kim et al. *(100)*. Ozone inactivates Gram-negative and Gram-positive bacteria and both vegetative cells and spores. It has been tested as a means of reducing the bacterial flora of fish and extending product shelf life. Gelman et al. *(77)* examined the effect of ozone pretreatment (6 ppm) of live tilapia on the shelf life and storage characteristics of the muscle meat during storage at 0 and 5˚C. The shelf life of ozone-treated fish was prolonged by 12 days when stored at 0˚C and 3 days at 5˚C, as determined by sensory analysis. During storage at 0˚C, total bacterial counts on the surface of the pretreated fish were 2 to 3 $\log_{10}$ CFU/m^3 lower compared with untreated controls, whereas at 5˚C no difference was observed in bacterial count. Crapo et al. *(32)* examined the effect of ozonated water (0.6-1.5 ppm) for sanitizing food contact surfaces and for treatment of raw seafood. Ozone was not effective at reducing the bacterial load present on processed salmon roe and fillets and actually accelerated the development of rancidity in frozen salmon roe and fillets, resulting in reduced shelf life. These investigators recommended using ozone as a sanitizer for only cleaned seafood contact surfaces *(32)*.

Chitosan, a deacetylated derivative of chitin, is a natural antimicrobial found in the exoskeleton of crustaceans and can be obtained from the shell waste of crab, shrimp, and crawfish processing industries. The antibacterial mechanism of chitosan is theorized to be associated with the positive charge on the NH_3^+ glucosamine monomer, which at pH <6.3 interacts with negatively charged microbial cell membranes leading to leakage of

intracellular constitutes *(130).* Chitosan has broad activity against Gram-positive and Gram-negative bacteria, as well as yeast and molds. Xiaohui et al. *(204)* examined the antimicrobial activity of five chitosan-zinc complexes against 11 species of bacteria and fungi. Minimum inhibitory concentration (MIC) of chitosan-zinc complexes in nutrient broth was used to compare the antimicrobial activity. The complexes showed a wide spectrum of antimicrobial activities, which were 2-8 and 4-16 X higher than those of chitosan and zinc sulfate alone, respectively. Among the bacteria tested, *E. coli* and *Corynebacterium* were most significantly inhibited by chitoan-Zn(c), both of which had a MIC value of 0.00031%.

The combined effect of two or more preservatives has been shown in various studies to be synergistic. Wendakoon and Sakahuchi *(207)* examined the effect of potassium sorbate (5% w/v) and sodium gluconate (1% w/v) dips on gilt head sea bream stored under modified atmosphere (40:30:30 CO_2:O_2:N_2) at 1˚C. The Gram-positive bacteria count, which dominated during storage of untreated samples, was about 5 $\log_{10}$ units higher than the count in treated samples. Similar reductions (2-3 log CFU/g) were obtained with the Gram-negative bacteria on treated samples. A clear synergistic effect between the two preservatives was observed. Nykanen et al. *(162)* reported that combinations of lactic acid (LA), nisin (Nis) and sodium chloride (NaCl) retarded bacterial growth more effectively than each ingredient alone when applied to rainbow trout stored at 3˚C. The most effective treatment combinations were LA/NaCl and Nis/LA/NaCl, both yielding microbial counts of 4.5 log CFU/g by the 10th day of refrigerated storage as compared to counts of 9.0 log CFU/g in the control. However, lactic acid enhanced fishy and metallic flavors, astringency,

and sourness of the fish samples. Statham et al. *(191)* reported that a polyphosphate dip with or without potassium sorbate had no significant effect on the bacterial flora of morwong fillets stored at 4˚C. The treatment of the fillets with potassium sorbate, CO_2 and polyphosphate effectively extended the shelf life. Cinnamma and Perigreen *(39)* examined the use of chemical preservatives (propionate, sodium benzoate, a 1:4 mixture of ascorbic and citric acid, and spices like clove, cardamom, ginger and tumeric) to extend the frozen shelf life of mackerel. All the spice-treated samples had the characteristic flavor of the spice which may be a problem unless the fish is to be highly spiced. Glazing with clove, cardamom or ginger extract extended the storage life of *R. kanagurta* at -18˚C from 20 to at least 30 weeks.

The effect of antimicrobials on histamine formation has been studied to some degree. Wendakoon et al. *(207)* examined the inhibitory effect of clove (0.5%) and sodium chloride (1-5%) on the growth and biogenic amine (histamine and cadaverine) production of *Enterobacter aerogenes* in mackerel muscle broth at 30˚C. After application of a combination of NaCl (2% or more) and clove, no amines were detected during the experimental period and the bacterial load fell to a level of 10^2 CFU/ml. However, sodium chloride levels up to 1% favored the growth of the bacteria. Krizek et al. *(115)* studied a preservative agent (Purac) composed substantially of lactic acid (20, 30 and 50 g/Kg), on histamine production in carp (*Cyprinus carpio*) muscle stored at 3, 15 and 25˚C. Lactic acid at 20 mg/Kg extended storage times by about 5 days at 3˚C, about 1 day at 15˚C, and less than one day at 25ºC. The investigators found that temperature was more distinct than the

effect of lactic acid on histamine-formation. Mejlholm and Dalgaard *(144)* examined the antimicrobial effect of nine essential oils (EO; basil, cinnamon, clove, lemongrass, marjoram, oregano, sage, thyme) on *P. phosphoreum* and determined the effect of oregano oil on the shelf-life of modified atmosphere-packed (MAP) cod fillets. *P. phosphoreum* is a histamine-forming bacteria that has been identified as a specific spoilage organism in cod *(47)*. EO's inhibited the growth of *P. phosphoreum* both in liquid media and in naturally contaminated cod-muscle blocks. Oils of oregano and cinnamon had the strongest antimicrobial activity, followed by lemongrass, thyme, clove, bay, marjoram, sage and basil oils. Oregano oil (0.05%, v/w) extended the shelf-life of naturally contaminated modified atmosphere package (MAP) cod fillets from 11-12 days to 21-26 days at 2˚C. However, these EO´s had no effect on the growth of *P. phosphoreum* in MAP salmon, which is a much fattier fish. Most likely the active components of oregano oil, primarily thymo and carvacrol, dissolved in the lipid phase of salmon fillets and therefore had no effect on *P. phosphoreum* growth in the aqueous phase. Baranowski et al. *(19)* examined the inhibitory effect of hypochlorite and chlorine dioxide in seawater on the deterioration of mahi-mahi by incubating fish in seawater containing 100 ppm available chlorine for 18 h at 32˚C. Neither histamine formation nor quality loss was inhibited when sodium hypochlorite or chlorine dioxide was added to the seawater. In addition, the skin of the chlorine-treated fish was bleached and appeared almost silver-white.

The main impediments to finding a preservative system to prevent histamine formation in fish are consumer acceptance and efficacy at inhibiting the growth of a diverse population of bacteria showing varying degrees of enzymatic activity.

1.10.7 Modified Atmosphere

Modified atmosphere packaging (MAP) has become an increasingly popular method to extend the shelf life of fresh refrigerated foods. MAP is defined as the packaging of a perishable product in an atmosphere which has been modified so that its composition is other than that of air *(88)*. Vacuum packaging (VP) can be defined as the packaging of product in a high barrier package from which air is removed to prevent the growth of aerobic spoilage organisms, shrinkage, oxidation and color deterioration *(88)*. Although MAP is frequently viewed as an alternative to vacuum packaging, vacuum packaging is actually a form of MAP because the removal of air is in itself a modification of the atmosphere. Vacuum packaging is often also regarded as MAP in the sense that elevated levels (10-20%) of carbon dioxide are produced within vacuum packages by microorganisms on the food as they consume residual oxygen or by respiration *(88, 190)*. FDA classified MAP and VP as “reduced oxygen packaging” or “ROP” *(70)*.

Gases commonly used in modified atmosphere packing are N_2, O_2, and CO_2 *(14, 172, 190)*. Each of these gases plays a specific role in extending the shelf life of fresh seafood products. Nitrogen is used as an inert gas to prevent package collapse in products that absorb CO_2 because of its low solubility in water and fat, but it has no antimicrobial properties. CO_2

is used to replace O_2 in packages to delay oxidative rancity and inhibit the growth of aerobic microorganisms, as an alternative to vacuum packaging *(14)*. Oxygen is generally avoided in gas packaging mixtures used for high-fat fish. However, it may be used in low concentration in both high and low-fat fish products to prevent anaerobic conditions and thereby limit the growth of potentially harmful anaerobes, specifically *Clostridium botulinum (14)*. For some products, oxygen could or should be used. For example, high levels of oxygen are used in red meat and red fish meat (e.g. tunas) to maintain the red color of the meat and reduce and retard browning caused by the formation of metmyoglobin *(185)*. Carbon monoxide (CO) can also be used to retain the red color of the meat of dark fish prior to vacuum packaging or in modified atmosphere packaging, although it is not currently approved for use in fish by the European Union *(41)*.

Carbon dioxide is the most important gas used in MAP because of its bacteriostatic and fungistatic properties. It inhibits the growth of many spoilage bacteria and the degree of inhibition increases with increased CO_2 concentration *(13, 50, 79)*. Gill et al. *(79)* reported that the major effect of CO_2 is to impose a greatly extended lag phase on the *Enterobacteriaceae* component of the initial flora. Carbon dioxide is highly soluble in water and fat, where it forms carbonic acid which may lower the pH of the product and change the flavor of fish *(14)*. For example, in one study, the initial headspace CO_2 concentration of MAP tilapia was 75%, but this decreased to a range of 40 - 47% after one day as the CO_2 dissolved into the fillets *(172)*. This phenomenon can have undesirable effects on the product since the water capacity of the flesh may then decrease and excessive amounts of muscle

exudate or drip may occur during storage. CO_2 may also dissolve in the fish tissue causing deformation or collapse of the package, and may affect the color of the product by interacting with pigments in the flesh *(172)*. The solubility of CO_2 increases with decreased temperature. Therefore the activity of the gas is dependent on product storage temperature, with increased inhibition of bacterial growth as temperature is decreased *(185)*. Sivertsvik et al. *(186)* examined the solubility and diffusion of carbon dioxide into salmon, cod, anglerfish, wolf-fish and tuna fillets and found that the average effective diffusion coefficient for CO_2 into raw fish fillets was 1.69×10^{-5} cm^2/s in the temperature range of -2 to 4˚C. The ratio between the volume of gas and the volume of food product (G/P ratio) is important in MAP because a certain amount of CO_2 has to dissolve in the food product to inhibit bacterial growth. The G/P ratio should be 2:1 or 3:1. Such high G/P ratios are also necessary to prevent package collapse because of the CO_2 solubility in wet foods *(185)*.

Most often, the shelf life extension that is observed for MAP fish falls in the range 30-60% *(185)*. For example, the shelf life of tilapia packed in 75% CO_2:25% N_2 atmosphere and stored at 4˚C was 25 days, but only 9 to 13 days when stored in 100% air *(172)*. The shelf life of tuna in 40% CO_2:60% O_2 atmosphere, as evaluated by both microbial condition and product color, doubled when the product was stored at 2˚C compared to tuna stored in 20% CO_2: 80% air and 40% CO_2: 60% air at 2˚C *(131)*. Color was the limiting factor influencing the shelf life of refrigerated tuna stored in CO_2-enriched atmospheres. The shelf life of cod was 20 days in 48% CO_2-stored at 0˚C, 6-7 days longer than vacuum packed cod *(48)*. Compared to meat products, limited or no extension of shelf life is obtained by packing

fish in a CO_2 atmosphere. For example, the shelf life of beef was 12 weeks when packed in vacuum but 21 weeks when packed in modified atmosphere stored at 1˚C *(79)*. As different gas mixtures and packaging systems have to be tailored for each product type application *(14)*, it is often difficult to compare MAP studies to one another.

The major cause of seafood spoilage is bacterial activity *(185, 190)*. The bacterial group causing the important chemical changes associated with fish spoilage often consists of a single species or specific spoilage organism (SSO). SSO's are dependent upon fish species, origin of the fish and storage conditions. Under aerobic iced storage, the SSO is mainly composed of *Pseudomonas spp*. and *Shewanella putrefaciens (82, 193)*. Modified atmosphere stored marine fish originating from temperate waters are spoiled by the CO_2 resistant *Photobacterium phosphoreum*, whereas Gram-positive bacteria are likely to be the cause of spoilage in CO_2 packed fish harvested from fresh or tropical waters *(82)*. *P. phosporeum* dominated the spoilage microflora of fresh MAP (60% CO_2/40% N_2) salmon stored at 2˚C *(64)*. Freezing inactivated *P. phosphoreum* and caused LAB to become the SSO. Similarly, *P. phosphoreum* was identified as the SSO of MAP packed cod with different CO_2 concentrations stored at 0˚C *(47, 48)*. Psychrotolerant *M. morganii*-like bacteria dominated the spoilage microbiota of fresh MAP tuna (60% CO_2/40% N_2) held for 24 days at 1.7˚C *(63)*.

The main safety concern of vacuum and MAP fish is the growth of pathogens and their potential for significant toxin production, particularly when storage temperature is not strictly controlled. The pathogens of most concern are *Clostridium botulinum* type E and

Listeria monocytogenes (136, 137, 171, 190). C. botulinum type E is generally found in marine and estuarine environments. As a result, fresh fish is likely to be contaminated with *C. botulinum* type E. It is a strict anaerobe and can grow and produce toxin at temperatures as low as 3.3˚C, which means that the potential for temperature abuse is a concern *(172)*. Toxin production may develop in MAP and vacuum packed fish before sensory rejection. Although Post et al. *(166)* reported that VP or MAP flounder stored at 12˚C and 8˚C deteriorated rapidly and was rejected by sensory evaluation prior to toxin accumulation, *C. botulinum* toxin was present either prior to or simultaneously with sensory rejection in cod and whiting fillets for all vacuum or modified atmosphere treatments and temperature regimes examined. Other studies have come to a different conclusion. Reddy et al. *(172)* examined the margin of safety between shelf life (onset of sensory spoilage) and potential time to toxin production by *Clostridium botulinum* type E in retail type packages from fresh marine cod fillets packaged in high barrier film in 100% air and in modified atmosphere (MA) containing 75% CO_2:25% N_2 and vacuum and stored at 4, 8 and 16˚C. Margin of safety (days) was determined by subtracting sensory spoilage (days) from onset of toxin development (days). Sensory spoilage preceded the onset of toxin production in the cod fillets packaged in any of the atmospheres and at all storage temperatures tested, with the highest margin of safety at 4˚C. Lyver et al. *(136)* inoculated *Clostridium botulinum* type E spores into raw, cooked and sterilized surimi nuggets, packed in air and under MAP condition, and stored at 4, 12, and 25˚C for 28 days. *C. botulinum* decreased in raw and cooked nuggets during the 28 day storage period. However, *C. botulinum* increased in the

sterilized nuggets. In the raw nuggets, the microflora consisted mainly of lactic acid bacteria and *Bacillus* species, whose growth resulted in a rapid pH decrease and inhibition of *C. botulinum*. The pH in the cooked nuggets did not decrease, but the inhibition of growth and toxin production could have been due to the presence of heat resistant *Bacillus* species found in relatively high numbers. The growth of *C. botulinum* in sterilized surimi nuggets could be attributed to the absence of *Bacillus* spp., either because it may be a natural biopreservative or because of the absence of competition.

Similar studies have been conducted with *Listeria monocytogenes*. Lyver et al. *(137)* examined the safety of value-added raw and cooked seafood nuggets inoculated with 10^3 cfu/g of *Listeria monocytogenes* packed in air or 100% CO_2, with and without Ageless SS, an oxygen absorbent, and stored at 4 or 12˚C. The results showed that most packaging atmospheres failed to inhibit the growth of *L. monocytogenes*. The growth of spoilage microorganisms, i.e., LAB and *Bacillus* spp., which resulted in a decrease in pH, particularly in raw nuggets, appeared to have little or no effect on the growth of *L. monocytogenes*. Product still had acceptable odor and appearance scores at 4 and 12˚C, yet levels of *L. monocytogenes* increased throughout storage. Davies *(50)* came to a different conclusion when he examined the growth/survival of psychrotrophic pathogens including *Listeria monocytogenes* on MAP meat (beef, cooked ham, dry cured ham) and fish (rainbow trout, cod, breaded cod). For all fish samples examined, in no instance was the growth/survival of any of the pathogens greater than that in the aerobically stored control, and frequently growth was reduced by MAP. These investigators concluded that for beef and fish, the risks

associated with the growth of food-borne pathogens was no greater for MAP product and frequently less than control (aerobic/vacuum packed) product. Taken together, these studies suggest a potential for growth and toxin production of pathogens in MAP and vacuum packed fish, but this can largerly be controlled by strict adherence to low refrigeration temperature.

Few studies have been conducted on the effect of modified atmosphere packing on histamine formation in fish. *P. phosphoreum* and *M. morganii*, both histamine producers, have been isolated from vacuum packed tuna harvested from the Indian Ocean and involved in an outbreak of scombrotoxic fish poisoning in Denmark *(63)*. These bacteria were able to form histamine at low temperatures (1.7˚C). Interestingly, the growth of both the psychrotolerant aerobic *M. morganii*-like bacteria and CO_2 resistant *P. phosphoreum* was strongly inhibited in modified atmosphere (40% CO_2:60% O_2). Similarly, Lopez-Galvez et al. *(131)* reported that bacterial growth was inhibited when tuna steaks were packed in atmospheres enriched in both 20% and 40% CO_2. Watts and Brown *(205)* studied the effect of modified atmosphere storage of mackerel on amine formation. The use of 80% CO_2 modified atmosphere storage did not lead to increased histamine formation in Pacific mackerel. At 20˚C, this atmosphere mildly inhibited, at least initially, the formation of potentially toxic amines. Vacuum packing of tuna, on the other hand, did not inhibit or slow bacterial growth at 2 and 10˚C *(206)*. Tuna samples subjected to vacuum packaging had higher histamine levels than non-vacuum packed product when stored at 2 and 10˚C, although histamine levels were greatly reduced at 2˚C. At the time of spoilage, Lopez-

Galvez et al. *(131)* found similar levels of histamine in tuna packed in modified atmosphere and air (20% CO_2/80% air, 40% CO_2/60% air and 40% CO_2/60% O_2). They concluded that the risk of scombrotoxic fish poisoning associated with the consumption of tuna packaged in modified atmosphere was no higher than that for tuna stored aerobically. Emborg et al. *(64)* found that *P. phosphoreum* was the SSO of chilled fresh MAP salmon. Inactivation of *P. phosphoreum* by freezing resulted in a substantial reduction in biogenic amine formation and a marked extension of shelf life. Taken together, this research suggests that modified atmosphere can inhibit or slow the growth and toxin production of histamine-producing bacteria. However, the degree of inhibition is dependent upon atmosphere, temperature, and microflora.

1.10.8 High-Pressure Processing

High-pressure processing (HPP) is a nonthermal preservation technique that, depending on the pressure, processing time/temperature and product characteristics, results in microbial inactivation without changing the sensory characteristics of foods as drastically as treatments using heat. HPP uses pressure up to 900 MPa to kill many of the microorganisms found in foods and can be a batch or continuous process. In a typical batch process, products are placed in a plastic bag and surrounded by a pressure transmitting medium (often water), and then held in a pressure vessel. Pressure is intensified with a hydraulically driven piston. Process pressures, hold times and temperatures are controlled electronically. The changes induced in microbial cells by pressure treatment have been extensively studied and include

alteration in the cell membrane, changes to cell morphology, effects on proteins (including enzymes), and effects on genetic mechanisms *(165)*(for reviews see *(139, 165)*). Some high pressure processed products are already commercially available in Japan (fruit juices, jam, soybeans, pasta, rice, and seaweeds), the United States (avocado spread and oysters) and Europe (orange juice in France and ham in Spain) *(200)*. HPP has the potential of being used to control histamine formation in fish by either inactivating microorganisms or the enzymes responsible for histamine production.

HPP treatment has been reported to adversely affect color and texture of fish. Lakshmanan et al. *(118)* reported that sliced and vacuum packed cold smoked salmon that had been HP processed at 200 MPa became pale or light pink, had reduced smoke odors and a texture that differed markedly from the control sample. High pressure treated turbot fillets lost their transparency and appeared as cooked when pressure treated at 100, 140, and 200 MPa for 15 and 30 min at 4˚C *(37)*. When vacuum packaged hake was pressure treated at 200 and 400 MPa (7˚C), two types of product were produced: one having a cooked appearance following pressurizing at 400 MPa, and the other having a fresh appearance after pressurizing at 200 MPa *(91)*. When cod was treated at 200 MPa, it lost some translucency, but at higher pressures the redness was lost and it became white and opaque *(7)*.

Microorganisms vary in their response to high pressure. Bacterial spores are the most resistant group and they cannot be significantly inactivated by pressure alone. Viruses appear to be less resistant than bacterial spores, although that might be dependent upon the type of virus. Yeast, mold, and vegetative bacteria vary in their response to pressure,

depending on factors such as species, strain, processing temperature and substrate *(165)*. In most cases, the effect of HPP on Gram-positive bacteria is less pronounced than on Gram-negative bacteria. Unlike classic thermal inactivation, the inactivation of microorganisms by HPP is not a linear relationship (first order kinetics). Patterson et al. *(165)* reported that the inactivation curve of five different vegetative pathogens did not follow first order kinetics but tended to be exponential, with an initial rapid decrease in numbers during the first 15 min of treatment followed by a tail, indicating that a small fraction of the population was more pressure resistant. Patterson et al. *(165)* examined the effect of high hydrostatic pressure (up to 700 MPa) at 20˚C on the survival of six pathogens (*Yersinia enterocolitica*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Escherichia coli* O157:H7 and *Staphylococcus aureus*) in 10 mM phosphate buffer (pH 7.0), ultra high-temperature-treated (UHT) milk, and poultry meat. Treatments of 350, 375, 450, 700, and 700 MPa for 15 min were needed to achieve equivalent reduction in *S.* Typhimurium, *L. monocytogenes*, *S.* Enteritidis, *E. coli* O157:H7 and *S. aureus*. The organisms were more resistant to pressure when treated in UHT milk than in poultry meat or buffer. Lakshmanan and Dalgaard *(118)* reported that HPP was unable to reduce levels of *L. monocytogenes* without changing the characteristic sensory properties of sliced and vacuum packed cold smoked salmon. *L. monocytogenes* was not inactivated by HPP at 150-250 MPa, 9˚C for 20 min. Novella et al. *(160)* reported that microbial load was 1 and 2 $\log_{10}$ units lower after pressurizing at 200 and 400 MPa, respectively, in vacuum packaged hake muscle stored at 7˚C. HPP inactivated *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio cholerae*, *Vibrio hollisae*, and *Vibrio*

mimicus at treatments of 200 to 300 MPa for 5 to 15 min at 25˚C. To date, limited research has been done to evaluate the effect of HPP on histamine-forming microorganisms.

The impact of HPP on biogenic amine formation in meat and cheese has been investigated. With respect to the formation of biogenic amines, two possible outcomes can be expected: (i) microbial inhibition/destruction produced by high pressure could lead to lower levels of biogenic amines; or (ii) protein denaturation induced by high pressure could cause an increase in proteolysis, thereby yielding higher amounts of free amino acids, which are the precursors of biogenic amines, in which case higher amine production would be expected *(161)*. Ruiz et al. *(181)* examined the biogenic amine content in 20 kinds of Spanish retail meat products treated by HPP and MAP. They found lower amine levels in HPP-treated Chorizo, cooked ham and butifarra meat. Spermine was the amine present in the greatest concentration in the different products, and high concentrations of tyramine were observed in fermented chorizo samples. Novella *(161)* compared the profiles of biogenic amines between cheeses made from pressurized (500 MPa, 15 in, 20˚C) and thermally pasteurized (72˚C, 15s) milk. Total amine concentration increased progressively throughout the ripening. No significant difference ($p > 0.05$) was found in total biogenic amine contents between cheeses made from pressurized versus pasteurized milk. The use of HPP treatment as a potential alternative to pasteurization did not lead to higher production of undesirable biogenic amines. However, the initial quality of the milk could have more to do with biogenic amine accumulation in ripened cheeses than the kind of treatment applied.

HPP of food can either deactivate or enhance enzyme activity. Most studies on the effect of HPP on enzymatic activity in fish have evaluated enzymes responsible for texture. For instance, Ashie and Simpson *(14)* examined the effect of HPP (100-300 MPa) on enzyme extract (cathepsin C, collagenase, chypotrypsin and trypsin-like enzymes) from bluefish and sheepshead and compared this to bovine enzymes. The fish-derived enzymes were generally more susceptible to hydrostatic pressure inactivation than their mammalian counterparts. For example, in the case of cathepsin C, application of 300 MPa pressure for 30 min had little or no effect on the bovine enzyme, but sheepshead and bluefish enzymes lost as much as 80% and 91% of their original activities. Under the same conditions, trypsin- chymotryps-like enzymes from fish retained 30-40% of thie original activity. Cheret et al. *(36)* examined the effect of HPP on the activity of proteoloytic enzymes (cathepsin and calpains) in fish muscle. High pressure processing up to 500 MPa enhanced the activity of cathepsin B, H, and I, whereas the activity of cathepsin D increased up to 300 MPa and decreased above 300 MPa. During the high-pressure treatment, the cathepsins were released from lysosomes by disruption, which explains the increase in their activities. High pressure lead to decreased activity of calpain, which was non-detectable after treatment above 400 MPa. Similarly, the activities of the three enzymes cathepsin B-like, cathepsin B+L-like, and calpain were reduced at all pressure levels up to 300 MPa in crude enzyme extracts prepared from cold-smoked salmon. Calpain was almost completely inactivated at 300 MPa *(118)*.

Two phenomena were observed in reference to the high hydrostatic pressure effect on enzymes: enzyme activation observed at comparatively low pressures (approx. 100 MPa) and

enzyme inactivation occurring at much higher pressures (>300 MPa). Angsupanich and Ledward *(7)* showed that the proteolytic activity of cod muscle at pH 6.6 decreased rapidly at pressures above 200 MPa, while the activity at pH values of 3.3 and 9.0 appeared to increase slightly after pressure treatment at 200 MPa. These activities decreased at higher pressures. The same authors noted that at pressures above 400 MPa, lipid oxidation increased due to the release of metal ions. Limited studies have been reported in the literature on the effect of HPP on enzymes responsible for the formation of histamine in fish. Thus, HPP might be used to control histamine formation in fish, but research needs to be done to determine its effect on microorganisms and histamine-forming enzymes.

1.10.9 Irradiation

Ionization radiation involves the application of electromagnetic waves or electrons to foods. Radiation sources are either gamma rays from cobalt-60, electron beams or X rays, and the amount of radiation absorbed by a food is measured in kGy (1Gy = 1 J/kg) *(139)* Cobalt-60 produces electromagnetic γ-rays which are similar to light but with much higher energy *(116)*. The critical target for ionizing radiation is the chromosome. Hydroxyl radicals formed by the radiolysis of water react with the sugar-phosphate backbone of the DNA molecule causing elimination of a hydrogen atom from the sugar. This causes the scission of the phosphate ester bonds and subsequent appearance of single strand breaks. Double strand breaks occur when two single strand breaks take place in each chain of the double helix at a close distance *(139)*. There is a linear relationship between the increase of ionizing

radiation dose and the reduction in viable microorganisms *(146)*. Generally, Gram-negative bacteria are more sensitive than Gram-positive organisms such as *Lactobacillus* and *Micrococcus (121)*. *Campylobacter*, *Yersinia* and *Vibrio* spp. have a low resistance to ionizing radiation; *E. coli* is also quite susceptible, while *Salmonella* serotypes vary in their radiation sensitivity *(67)*.

Irradiation can be used as a method for reducing histamine-forming-bacteria in fish. Mendes et al. *(145)* irradiated blue jack mackerel (0, 1, 2 and 3 kGy) and stored the fillets on ice for 23 days. The production of histamine was significantly reduced after irradiation of the fish. Histamine in the control lot exceeded 100 ppm after 7 days, while in the irradiated lots, a maximum value of 54 ppm of histamine was found after 23 days. This was most likely caused by reduction of histamine-producing bacteria; indeed, non-irradiated fish samples had 5 to 10 times as many bacteria as their irradiated counterparts on the same day of the study (day 13). Similarly, Mendes et al. *(146)* irradiated Atlantic horse mackerel at 1 and 3 kGy and stored on ice for 23 days. Histamine levels in the irradiated lots were undetetable at the end of the storage period while the histamine level in the control lot remained low (<40 ppm).

1.11 CONCLUSION

Scombroid fish poisoning is a significant health and safety concern worldwide. The high incidence of the illness may in part be attributed to the lack of measures for its control. The primary control measure used for the prevention of scombrotoxin formation in fish is

temperature. FDA implemented time and temperature as a control strategy in the Hazard Analysis Critical Control Points (HACCP) approach for preventing scombrotoxin formation. However in some instances, histamine can form at temperatures as low as 2˚C. In addition, no preparation of the fish once histamine has been formed, including, freezing, canning or smoking, will eliminate the toxin. It is evident that additional measures for the control of scombroid fish poisoning are needed.

In order to effectively evaluate secondary control measures, an accurate, reliable and rapid method for detection and quantification of histamine-producing bacteria is required. However, present culture methods used have been reported to result in a high incidence of false-positive and false-negative results. More recently, rapid molecular methods have been developed based on the detection of the genes coding the decarboxylase enzymes. Although these methods are more rapid, they have not been adapted for the quantification of histamine-producing bacteria.

In this review, potential secondary control measures for scombroid fish poisoning were discussed. Several of these measures have applications in terms of control for histamine formation in fish. For instance, food grade inhibitors for use with histidine decarboxylase that do not alter the sensory characteristics of the product merit further investigation. In addition, the use of histamine degrading enzymes in fish needs further study. The combination of such additional control measures along with FDA's temperature and time strategy in a hurdle approach may be more powerful than temperature alone. To be

able to examine secondary control measures as an additional control strategy, a rapid and reliable method for detection and enumeration of histamine-producing bacteria is needed.

CHAPTER 2

DETECTION OF GRAM-NEGATIVE HISTAMINE-PRODUCING BACTERIA IN FISH: A COMPARATIVE STUDY

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2.1 ABSTRACT

Poisoning due to ingestion of foods with elevated levels of biogenic amines (histamine, putrescine, cadaverine, tyramine) is well documented. Histamine fish poisoning occurs largely due to growth of naturally-occurring bacteria associated with scombroid fish species. There is a need for a rapid and reliable method to screen for the presence of histamine-forming bacteria in fish. This study compared three methods for the detection of histamine-producing bacteria. Specifically, a total of 152 histamine and non-histamine producing bacteria from multiple sources were screened using a modified Niven's agar, a potentiometric method, and a PCR-based assay targeting a 709 bp fragment of the histidine decarboxylase gene. Histamine production of bacteria isolates was confirmed by HPLC; detection by HPLC was classified into the following categories: high histamine-producing strains; low histamine-producing strains; and non-producing strains. Of the 152 strains tested, 128/152 (84%) were positive by Niven's, 73/152 (48%) were positive using the potentiometric technique, and 74/152 (49%) were positive by PCR. Overall, a 38% false positive rate was observed for the modified Niven's medium, although this method was able to detect both low and high histamine producing strains. There was a high degree of concordance (>99%) between the potentiometric and PCR methods, but neither of these were able to detect low histamine-producing bacteria. These observations support the need for a simple and straightforward yet sensitive method to detect histamine-producing bacteria in seafood and environmental samples.

2.2 INTRODUCTION

Poisoning due to the ingestion of foods with elevated levels of biogenic amines (histamine, putrescine, cadaverine, tyramine) is well documented *(8)*. Of these agents, histamine is a common cause of foodborne disease, particularly in association with the consumption of fish. For example, between 1990 and 2003, histamine fish poisoning accounted for 7.5 % of all foodborne disease outbreaks and 38% of all seafood-related illnesses reported to the U.S. Centers for Disease Control and Prevention (CDC) *(54)*.

Excess histamine in foods occurs as a result of amino-acid specific enzymes derived from spoilage bacteria, and has been associated with fermented products such as salami, cheese, sauerkraut and wine. In fish, histamine is sometimes called scombrotoxin because of its common association with scombroid fish (i.e., tunas, mackerels, and bonitos) but has also been associated with non-scombroid fish (mahi-mahi and bluefish) which have high levels of amino acid precursors in the flesh. Histamine fish poisoning occurs largely due to the growth of naturally-occurring Gram-negative bacteria, e.g. *Morganella morganii*, *Raoultella planticola*, *Enterobacter aerogenes*, among others. Most of these organisms are mesophiles. Therefore, tight temperature control is important for the prevention of histamine formation in fish, as reflected in the U.S. Food and Drug Administration‘s (FDA) Hazard Analysis Critical Control Points (HACCP) guidelines *(8).* Unfortunately, rapid chilling alone may not prevent the formation of high levels of biogenic amines by a few pychrotrophic histamine-producing bacteria *(86)*. Further, once formed, histamine is resistant to commonly used food preservation methods including freezing, cooking, retorting, or smoking *(65)*.

There continues to be a need for a rapid and reliable method to screen for the presence of histamine-forming bacteria. Historically, fish products have been screened for the presence of histamine-producing bacteria using Niven's agar, a differential growth media containing the pH indicator bromocresol purple as well as tryptone, L-histidine hydrochloride and a few other components *(35, 158, 212)*. Histamine accumulation occurs during the growth of bacteria and results in a rise in pH which induces a color change, allowing for visualization of positive bacterial colonies. The method has advantages in that it is relatively easy to use and inexpensive. Unfortunately, some studies have reported loss of histamine-production in bacteria strains after cultivation in culture-based media *(119)*. In addition, false-positive reactions are frequent, largely because of the formation of other (non-histamine) alkaline compounds during microbial metabolism *(18, 35, 133)*. Investigators have sought to reduce such false positive reactions by various modifications to the differential media. For instance, Mavromatis and Quantick *(142)* produced a modified Niven's medium which increased the selectivity by manipulating media pH, and incubation time and temperature.

The development of streamlined methods to detect histamine and/or Gram-negative histamine-producing bacteria from fish has been an area of active research. For instance, a potentiometric method in which histamine is detected based on an increase in conductance after growth of histamine-producing microorganisms in histidine decarboxylase broth has been reported *(112)*. More recently, investigators have focused efforts on the development of molecular techniques (reviewed by Landete et al. *(119)*) which usually target the genes

encoding the histidine decarboxylase enzyme (*hdc) (51, 52, 102, 103, 194)*. For instance, Takahashi et al. *(194)* developed a rapid molecular method for detection of Gram-negative histamine-producing bacteria using PCR followed by single-strand conformation polymorphism analysis.

The purpose of this study was to compare three different methods used for the detection of histamine-producing bacteria. Specifically, Gram-negative bacteria with and without histamine production capabilities were screened by modified Niven's agar, by conductance change after incubation in histidine decarboxylase broth, and using a PCR-based identification assay. These results were compared directly with histamine production levels obtained using high performance liquid chromatography (HPLC).

2.3 MATERIALS AND METHODS

2.3.1 Culture library

Histamine and non-histamine producing bacterial strains were obtained from multiples sources, including the American Type Culture Collection (ATCC, Manassas,VA, USA), Dr. Graham Fletcher (The New Zealand Institute for Plant and Food Research Limited, Auckland, NZ), Dr. Rachel Nobles (University of North Carolina Chapel Hill Institute of Marine Sciences, Morehead City, NC, USA), Dr. John Kaneko (PacMar Inc., Honolulu, HI, USA) and Dr. George Flick (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) or isolated from fish [gills, belly cavities and muscle tissues of yellowfin tuna (*Thunnus albacares*), mahi-mahi (*Coryphaena hippurus*) bluefish

(*Pomatomus saltatrix*) and wahoo (*Acanthocybium solandri*)] and environmental samples collected in North Carolina and Hawaii.

Media components were obtained from Becton Dickinson (BD; Franklin Lakes, NJ) unless otherwise specified. For isolation of naturally-occurring histamine-producing bacteria, surface swabs (32 cm^2) or tissue samples (5g) were enriched in 10 ml histidine broth (pH 6.5) containing 1% bacto proteose peptone, 0.3 % yeast extract, 1.5% NaCl (all obtained from EMD, Gibbstown, NJ) and 0.5% histidine (L-histidine hydrochloride monohydrate; Acros, Morris Plains, NJ) at 37˚C for 24 h. A 1 ml sub-sample of the enrichment culture was incubated in 9 ml trypticase soy broth (TSB) containing, 2% histidine, 2% NaCl and 0.0005% pyridoxal-HCl (TSB+; Alexis, Plymouth Meeting, PA; pH 5.8) for 24 h at 37˚C. Histamine formation in the subculture was tentatively identified using the Veratox histamine test kit (Neogen Corp., Lansing, MI). Those enrichment cultures testing positive by Verotox were serially diluted in saline (0.85% NaCl) and 0.1 ml spread on trypticase soy agar plates containing 2% NaCl ($TSAN_2$). Ten representative isolates were identified based on a combination of cell morphology, gram stain, and results from the Enteric and Nonfermenter BBL Crystal™ Identification Test Kit (Becton Dickenson). Gram-negative histamine- and non-histamine producing bacteria were used for further screening.

2.3.2 Histamine determination

HPLC chemicals were obtained from J.T. Barker (Hayward, CA) unless otherwise specified. Histamine content was determined by the HPLC method of Cinquina et al. *(42)*. Bacterial isolates were first inoculated in duplicate into TSB+ (pH 6.5) and incubated at 37˚C for 48 h. A 200 µl aliquot of the culture broth was extracted in 800 µl of 1M perchloric acid (VWR, West Chester, PA). The diluted sample was vortexed for 1 min, sonicated for 15 min and centrifuged for 15 min at 4,160 x *g* at 4˚C. The supernatant was filtered through a 0.45 µm PTFE (polytetrafluoroethylene, VWR) filter and diluted 1:10 in HPLC grade water. This was then injected onto a 4.6 x 250 mm Luna C18(2) column (Phenomenex, Torrance, CA, USA) using an Agilent 1050 HPLC and separated under isocratic conditions using 85% Eluent A (85% phosphate buffer pH 6.9 and 15% methanol) and 15% Eluent B (acetonitrile). Flow rate was set at 0.5 ml/min and detection was achieved using a diode-array detector (DAD) at 214 nm. The detection limit for this method was 125 ppm based on a histamine standard curve using a histamine suspension serially diluted in the range of 2.5-200 ppm.

2.3.3 Culture-based method

Bacterial strains (n=152) from the culture library were streaked on modified Niven's agar *(158)* by Mavromatis and Quantick *(142)* containing 0.5% tryptone, 0.5% yeast extract, 2.7% histidine, 0.5% NaCl, 0.1% $CaCO_3$ (Mallinckrodt, Hazelwood, MO), 3% agar, and 0.006% bromocresol purple (Fisher Scientific, Pittsburgh, PA), and incubated at 30˚C for 48

h. Isolates were considered positive when the color of the media changed from green to purple.

2.3.4 Potentiometric-based method

Strains were evaluated for their ability to increase conductance after growth in histidine decarboxylase broth (HDB), in accordance with the method developed by Klausen and Huss *(112)* with slight modifications. Briefly, single isolates were incubated in 9 ml of TSB for 24 h at 30˚C. Samples were diluted 1:10 in TSB and 1 ml of the culture was added to 9 ml of HDB containing 0.2% peptone, 0.1% Lab-Lemco (Oxoid, Hampshire, UK), 0.81% histidine, 0.5% NaCl and 0.0005% pyridoxal·HCl in potentiometric vials. Vials were placed into the BacTrac instrument (SY-Lab, Neupurkersdorf, Austria) and conductance was measured automatically at 10 minutes intervals for 24 h at 30˚C. Results were expressed graphically as percent increase in media conductance (%M) vs. time. Positive isolates were defined as those that increased the conductance of the HDB above 5% of baseline within 24 h.

2.3.5 Molecular-based method.

Culture strains were inoculated in 5 ml TSB containing 2% NaCl ($TSBN_2$) for 24-48 hours and DNA was isolated using UltraClean™ Microbial DNA Isolation Kit (MoBio, CA, USA) in accordance with manufacturer instructions. Absorbance was recorded at 260 and

280 nm (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) in a 96-well quartz plate and DNA concentration and purity was calculated using standard formulae.

A 709 bp fragment of the histidine decarboxylase (*hdc*) gene was amplified using primers designed by Takahashi et al. *(194)*: *hdc-f* (5'-TCH ATY ARY AAC TGY GGT GAC TGG RG-3') and *hdc-r* (5'-CCC ACA KCA TBA RWG GDG TRT GRC C-3'). Amplification was performed in 50 µl reactions that included: 25 µl PCR master mix (50 units/ml Taq DNA polymerase, 400µM of each of the four deoxynucleoside triphosphate, reaction buffers, 3 mM $MgCl_2$, pH 8.5 (all provided by Promega Corp., Madisom, WI)), 75 pmol of each primer and 20 ng DNA template. Amplifications were carried out for 40 cycles (94˚C for 1 min, 52˚C for 1 min, and 72˚C for 1 min) in a GTC-2 thermal cycler (Precision Scientific, Chicago, IL). PCR products were separated on a 1% agarose gel at 86V in 1x TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) for 1 h. Gels were stained with ethidium bromide (0.3 µg/ml) and visualized using a UV transilluminator (UVP, Upland, CA). Product size was confirmed using 100 bp molecular weight markers (Invitrogen, Carlsbad, CA).

2.4 RESULTS AND DISCUSSION

A total of 152 isolates were screened in this study. On the basis of HPLC analysis (which is considered the "gold standard'), the isolates were sub-classified into three groups: high (>1000 ppm) histamine producers (73/152 or 48%); low (126 - 500 ppm) histamine producers (6/152 or 4%); and non- (<125 ppm) producers (73/152 or 48%). Using the

modified Niven's method, 128/152 (84%) of the cultures screened positive and 24/152 (16%) screened negative. The 128 Niven's positive isolates consisted of 73 high producers (57%), 6 low producers (5%), and 49 non-producers (38%); all 24 Niven's negative isolates were found to be non-producers (Table 1). In short, when compared to the HPLC gold standard, modified Niven's screening produced a 38% (49/128) false positive rate. This is consistent with the work of Lopez-Sabater et al. *(133)* and Fletcher et al. *(74)* who similarly reported that detection of histamine-producing bacteria using Niven's agar resulted in 63% and 15% false positive rates, respectively. False-positives are likely due to the production of one or more basic compound(s) capable of increasing the pH of the medium, resulting in the characteristic color change shown by histamine-producing strains *(2)*.

Using the potentiometric method, 73/152 (48%) strains tested positive; this included all of the high histamine producers. The remaining 79/152 (52%) strains screened negative by the potentiometric method and included all the strains in the library which fell into the low and non-histamine producing groups as determined by HPLC. This is consistent with the work of Klausen and Huss *(112)* who also found positive response using the potentiometric method when screening high-histamine producing strains of *Morganella*, but negative response for non-histamine producing strains of *Pseudomonas* and *Alteromonas*.

Using the PCR assay targeting the *hdc* gene, 74/152 strains (49%) screened positive and 78/152 (51%) screened negative. The isolates screening positive by PCR included all of strains designated as high histamine producers by HPLC, and also a single isolate (*Citrobacter freundii* HW7.4) which tested as a non-histamine producer by both the

potentiometric method and HPLC. The strains which screened negative by PCR included the remaining 72 non-producers and the 6 low producers as evaluated by HPLC. Takahashi et al. *(194)* similarly found that all high histamine producers in their study produced positive PCR reactions when targeting the *hdc* gene using the same primer set. In addition, these investigators identified low histamine producing strains of *Citrobacter braakii* and *Hafnia alvei* that did produce positive and negative PCR products, respectively. Using different primers based on the *hdc* gene of *Raoultella planticola* HDC protein, Kanki et al. *(97)* successfully amplified a 724 bp fragment from all the histamine-producing *R. planticola* and *R. ornithinolytia* strains in their library, as did De las Rivas et al. *(52)* for a 534 bp amplification product. However, neither of these groups examined low histamine-producing bacteria such as *C. freudii* and *H. alvei* for amplification of the *hdc* gene.

Clearly, both the potentiometric and PCR methods compared favorably to the HPLC "gold standard" when screening strains which produce high amounts of histamine; in fact, concordance between either of these methods and HPLC exceeds 99%. Similarly high concordance is observed when comparing the potentiometric and PCR-based methods to one another. It is well documented that the high producers are more likely than the low producers to produce toxic levels of histamine if temperature abuse occurs *(104)*. It can therefore be postulated that the presence of high histamine-producing bacteria suggests a higher likelihood for product adulteration, a good justification for the use of either the potentiometric or the PCR-based methods for routine screening of histamine-producing bacteria in natural environments.

On the other hand, neither the potentiometric nor the PCR method was acceptable for the identification of low histamine-producing bacteria. In the case of the potentiometric method, the small amounts of histamine (<500ppm) produced by these strains may have been insufficient to result in detectable changes in conductance. An alternative explanation is that other bacterial amino acid decarboxylases, such as the arginine decarboxylase, were able to metabolize histidine in addition to their natural substrates; such a phenomenon has been observed for strains of *Salmonella typhimurium* and *Escherichia coli (28, 46)*. Either (or both) of these may explain the potentiometric results. However, we were also unable to detect the *hdc* gene in these same low histamine producers. The significance of this is currently unknown, as the *hdc* gene associated with similar low producing stains belonging to species such as *C. freundii* and *H. alvei* has yet to be identified and may in fact, be plasmid-associated *(56, 119)*. Histamine-producers have been reported to lose their ability to form histamine during prolonged storage or cultivation of isolated strains in synthetic media *(135)*. This loss of histamine production may in fact be related to loss of plasmids containing the histidine decarboxylase gene. Potential explanations for these findings are the absence of an *hdc* gene, presence of an alternative *hdc* gene sequence, and/or loss of a plasmid-borne gene.

Comparatively speaking, the modified Niven's method is the easiest and least costly of the three approaches evaluated in this study. Additional advantages of the modified Niven's method is its adaptability to quantitative assay, making it useful for the enumeration of histamine-producing bacteria from natural (fish and environmental) samples. Furthermore, it is much better at identification of low producing strains although these strains

may not produce histamine of toxicological significance (>500 ppm). Nonetheless, the high incidence of false positives means that detection of histamine producers using modified Niven's medium should be considered presumptive only, requiring further confirmation. This, of course, complicates the assay and calls into question the interpretation of enumerative results. On the other hand, the potentiometric and PCR methods require more expensive equipment, a higher degree of training on the part of the user, but are faster (<24 h). Although they do not reliably detect strains producing low amounts of histamine, they accurately detect high-histamine producing strains that are more likely to produce histamine levels of toxicological significance. These observations support the need for a straightforward method by which to combine cultural methods with potentiometric or molecular methods. A logical approach would be colony lift hybridization which uses the combined power of microbiological culture with nucleic acid hybridization. This method is uniquely suited to situations in which the performance of selective and/or differential media is less than perfect, as it provides more accurate quantitative results because the target organism can be confirmed without the need for sub-culturing. Efforts to develop such a method are currently underway.

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Table 2. 1 Strains, histamine production and detection response by modified Niven's medium, potentiometric- and PCR-based measurements.

Species	Nivens*	BacTrac*	PCR*	Histamine (ppm)**		Source***
				Mean	Range	
Morganella morganii	30/30	30/30	30/30	4466	(2880-6353)	ATCC, NC, HI, NZ, NCBI
Providencia rustigianii	3/3	3/3	3/3	4269	(3778-4383)	NC, HI
Proteus mirabilis	2/2	2/2	2/2	4114	(4024-4204)	NC
Raoutella planticola	1/1	1/1	1/1	6143	(5459-5789)	ATCC
Raoutella ornithinolytica	2/2	2/2	2/2	5624		NC
Enterobacter aerogenes	6/6	6/6	6/6	6660	(2833-7779)	ATCC, NC, HI
Enterobacter gergoviae	2/2	2/2	2/2	5634	(3508-7760)	HI
Photobacterium damselae	27/27	27/27	27/27	3372	(1622-7711)	NC, HI
Klebsiella oxitoca	1/1	0/1	0/1	<125		ATCC
Hafnia alvei						
[ATCC 13337, NZ1]	2/2	0/2	0/2	181	(171- 204)	ATCC, NZ
[HW46.3]	1/1	0/1	0/1	<125		HI
Vibrio alginolyticus	2/2	0/2	0/2	<125		ATCC, NC
Citrobacter freundii	21/21	0/21	1/21			
[BO216, A4077, A4086, FT761]	4/4	0/4	0/4	265	(126-448)	NC
[ATCC 8090, BR121, BR129, BO240, BO251, BO201, BO222, HPP304, A4083, FT730, BR902, HW12.1, HW16.3, HW17.1, HW24.1, HW1.4, HW7.4†]	17/17	0/17	1†/17	<125		ATCC, NC HI
Citrobacter amalonaticus	1/1	0/1	0/1	<125		HI
Serratia marcescens	1/1	0/1	0/1	<125		HI
Vibrio mimicus	1/1	0/1	0/1	<125		NC
Pseudomonas putida	1/1	0/1	0/1	<125		NC
Shewanella putrefaciens	6/6	0/6	0/6	<125		NC
Enterobacter cloacae	11/11	0/11	0/11	<125		NC
Escherichia hermannii	2/2	0/2	0/2	<125		HI
Vibrio parahemolyticus	1/1	0/1	0/1	<125		NC
Vibrio fluvialis	2/2	0/2	0/2	<125		HI
Pseudomonas aeruginosa	2/6	0/6	0/6	<125		
[ATCC27853, HW19.3	2/2	0/2	0/2	<125		HI, ATCC
HW22.3, HW28.6, NZ8, NZ10]	0/4	0/4	0/4	<125		HI, NZ
Esherichia coli	0/13	0/13	0/13	<125		ATCC, NC
Listeria innocua	0/3	0/3	0/3	<125		NC
Shigella flexneri	0/1	0/1	0/1	<125		ATCC

** Results represent no. of strains positive / no. strains tested*
*** Histamine-production of isolates were determined by HPLC: mean values are average in histamine-production of species from two independent replica of each strain; range of histamine produced by each species*

****ATCC, American Type Culture Collection, USA; ICPB, International Collection of Phytopathogenic Bacteria, USA; NC, North Carolina, USA; HI, Hawaii, USA; NZ, New Zealand* † PCR positive isolate

CHAPTER 3

COLONY LIFT HYBRIDIZATION METHOD FOR ENUMERATION OF HISTAMINE-PRODUCING BACTERIA IN FISH

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3.1 ABSTRACT

Detection of histamine-producing bacteria in fish is commonly performed using a microbiological method utilizing modified Niven's agar known to result in a high number of false positives and false negatives. A new method utilizing Dioxigenin (DIG) labeled DNA probes was developed to detect and enumerate histamine-producing bacteria in fish by the use of colony lift hybridization. The DNA probes were evaluated using 152 histamine and non-histamine producing bacteria. The probes reacted specifically (100%) to high histamine-producing bacteria producing >1000 ppm in histidine broth and gave no false positive response. To further assess the specificity and sensitivity of the DNA probes, seafood homogenate of tuna and mahi-mahi inoculated with known concentrations of four histamine-producing bacteria were plated, hybridized and compared to enumeration by the Niven's method. Counts obtained with DNA probes were not significantly different from control samples while the Niven's agar counts were significantly lower.

3.2 INTRODUCTION

Histamine (or scombroid) fish poisoning remains a significant cause of food borne disease in the United States *(55)*. For example, between 1990 and 2003 this disease accounted for 7.5% of all food borne disease outbreaks and 38% of seafood-related food borne illnesses reported to the Centers for Disease Control and Prevention (CDC) *(55)*. Scombroid poisoning is caused by the consumption of fish containing hazardous levels of histamine and other biogenic amines (putrescine and cadaverine). Allergic-like symptoms such as headache, dizziness, swelling of the tongue, nausea, vomiting, diarrhea, and stomach pain usually occur rapidly after ingestion of contaminated product *(93, 138)*. Fortunately, the symptoms are usually short-lived and recovery is complete.

Histamine is produced by certain spoilage microorganisms through action of the enzyme histidine-decarboxylase (HDC) which converts the amino acid histidine to histamine. Scombroid fish have high levels of free histidine in their flesh, making these species of particular concern because toxic levels of histamine can accumulate rapidly. There are two different classes of HDC enzymes. The first group is found in eukaryotic cells and Gram-negative bacteria while the second is found in Gram-positive bacteria; both have slightly different mechanisms of action *(29, 170)*. Histamine is produced by a wide range of bacteria but the major histamine-producing bacteria in fish are Gram-negative mesophilic enteric and marine bacteria *(104)*. Examples include strains of *Morganella morganii*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterobacter aerogenes, Raoultella planticola, Raoultella ornithinolytica* and *Photobacterium damselae*, some of which are particularly high histamine

producers, yielding over 1,000 ppm histamine in culture under optimal culture conditions. Strains of other species, including *Hafnia alvei*, *Citrobacter freundi, Vibrio alginolyticus* and *Escherichia coli*, are weak histamine-producers, producing concentrations <500 ppm *(92, 106, 108, 194)*.

Refrigeration aimed at preventing microbial growth and hence prevention of histamine formation is the primary control measure in seafood. For example, the U.S. Food and Drug Administration's (FDA) seafood HACCP rule includes time and temperature control guidelines, as well as a defect action level (DAL) of 50 ppm histamine *(70)*. Even so, it is often difficult to chill large fish (such as tuna) rapidly enough to prevent histamine formation or to maintain adequate cold storage on boats during long-term fishing expeditions. This is exacerbated by the fact that a minority of histamine-producing bacteria are capable of growth and histamine production at temperatures as low as 2.5˚C *(62, 63, 85)*.

Therefore, there remains a need for a reliable, rapid, and accurate detection and quantification metrhod of histamine and/or histamine-producing bacteria *(70)*. The quantification of histamine using high pressure liquid chromatography might be considered a "gold standard," but the method is complicated, time-consuming, and expensive. The simplest method relies on the detection of histamine-producing bacteria using Niven's media *(158)*. This differential growth medium contains the pH indicator bromocresol purple as well as tryptone, L-histidine hydrochloride and few other components. Histamine accumulation, which occurs during the growth of the target organism(s), results in an elevated pH. This causes a color change which allows discrimination of presumptively

positive histamine-forming bacteria. Although this media is good for initial screening, it usually produces a large number of false positive isolates which are later confirmed as histamine non-producers *(17, 35, 133)*. In addition, the low pH of the media (pH 5.3) inhibits growth of some histamine-producing bacteria *(2, 212)*. Several researchers have modified Niven's agar in an effort to improve its detection but with unsatisfactory results *(35, 142)*.

More recently, molecular-based methods have been developed for the detection of Gram-negative histamine-producing bacteria in fish. For example, Takahashi et al. *(194)* and Kanki et al. *(98)* developed a nucleic acid amplification method targeting 709 bp and 724 bp fragments of the histidine decarboxylase gene (*hdc*). Another PCR-based method was developed for the detection of the prolific histamine producer *M. morganii*, based on the amplification of the variable region of the 16S rDNA region *(102, 103)*. Similar methods have been developed for amplification of the *hdc* from Gram-positive histamine-producing bacteria *(5, 120)*. Methods such as these can be used for confirmation of bacterial cultures which screen positive on Niven's media, but they do not ultimately reduce the number of false positive reactions obtained using differential growth agar.

Colony lift hybridization is a method which uses the combined power of microbiological culture with nucleic acid hybridization. This method is uniquely suited to situations in which the performance of selective and/or differential media is less than perfect, as it provides more accurate quantitative results because the target organism can be confirmed without the need for sub-culturing. In addition, once a colony is identified by this

method, it can be isolated and used in further testing or as part of a strain bank. Colony lift hybridization methods have been applied for the detection and quantification of various food borne pathogens including *Vibrio parahaemolyticus*, *V. vulnificus*, *Campylobacter* spp., and *Listeria monocytogenes (3, 33, 53, 173)*. In a recent report for *V. parahaemolyticus,* investigators claimed that the colony lift approach was faster and more efficient than the most-probable-number (MPN) methods frequently used for detection and quantification of this pathogen *(3, 81, 157)*.

This work is based on the hypothesis that colony lift hybridization methods may be applicable to the quantification of Gram-negative histamine-producing bacteria by virtue of the specificity of a probe targeting the *hdc* gene of these organisms. Therefore, the purpose of this study was to develop a colony lift hybridization method for the detection and quantification of high histamine-producing bacteria in fish. In initial phases, labeled DNA probes were evaluated using dot blot hybridization for their detection specificity as applied to 152 Gram-negative bacteria with varying degrees of histamine production. The assay was then converted to a colony lift hybridization format, the efficiency of which was validated using artificially inoculated fish samples.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial cultures

A culture library of 152 Gram-negative bacteria, some of which were histamine-producers and some of which were not, were obtained from multiples sources, including the

American Type Culture Collection (ATCC, Manassas,VA, USA), Dr. Graham Fletcher (New Zealand Institute for Plant & Food Research, Christchurch, New Zealand), Dr. Rachel Nobles (University of North Carolina Chapel Hill Institute of Marine Sciences, Morehead City, NC, USA), Dr. John Kaneko (PacMar Inc., Honolulu, HI, USA) and Dr. George Flick (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA). Other strains were isolated directly from fish [gills, belly cavities and muscle tissues of yellowfin tuna (*Thunnus albacares*), mahi-mahi (*Coryphaena hippurus*) bluefish (*Pomatomus saltatrix*) and wahoo (*Acanthocybium solandri*)], and environmental samples collected in North Carolina and Hawaii as previously described *(27)*.

3.3.2 Culture conditions

Media components were obtained from Becton Dickinson (BD; Franklin Lakes, NJ) unless otherwise specified. For pure culture work, strains were inoculated in 5 ml trypticase soy broth (TSB) containing 2% NaCl (EMD, Gibbstown, NJ; $TSBN_2$) for 24-48 hours. For histamine analysis of culture collections, bacterial isolates were inoculated in duplicate into TSB containing, 2% histidine (L-histidine hydrochloride monohydrate; Acros, Morris Plains, NJ), 2% NaCl and 0.0005% pyridoxal-HCl (TSB+; Alexis, Plymouth Meeting, PA; pH 6.5; TSB+) and incubated at 37˚C for 48 h. Inoculum to be used in the fish seeding studies was prepared by combining 0.25 ml of overnight cultures of *Morganella morganii*, *Raoultella planticola* and *Enterobacter aerogenes* and *Photobacterium damselae* (total volume of 1 ml) and washing by centrifugation at 13,700 *x g* for 1 min and resuspending the pellet in 1 ml of

saline (0.85% NaCl). For quantification of histamine-producing bacteria in inoculated fish homogenates, samples were serially diluted in saline and spread plated on Niven's agar *(158)* with modifications described by Mavromatis and Quantick *(142)*; incubation was done at 30˚C for 24 hours. The same samples and corresponding dilutions were also spread-plated on $TSAN_2$ with incubation at 30˚C for 24 h followed by colony lift hybridization as described below.

3.3.3 Confirmation of histamine production

Each of the 152 bacterial strains was evaluated for the ability to produce histamine using the HPLC method of Cinquina et al. *(42)*. All HPLC chemicals were obtained from J.T. Barker (Hayward, CA) unless otherwise specified. Briefly, a 200 µl aliquot of the 48 hour culture broth was extracted in 800 µl of 1M perchloric acid (1:5; VWR, West Chester, PA). The diluted sample was vortexed for 1 min, sonicated for 15 min and centrifuged for 15 min at 4,160 *x g* at 4˚C. The supernatant was filtered through a 0.45 µm PTFE filter (polytetrafluoroethylene, VWR, West Chester, PA) and diluted 1:10 in HPLC grade water. This was then injected (50 µl) onto a 4.6 x 250 mm Luna C18(2) column (Phenomenex, Torrance, CA, USA) using an Agilent 1050 HPLC and separated under isocratic conditions using 85% Eluent A (85% phosphate buffer pH 6.9 and 15% methanol) and 15% Eluent B (acetonitrile). Flow rate was set at 0.5 ml/min and detection was achieved using a diode-array detector (DAD) at 214 nm. The detection limit for this method was 125 ppm based on

a histamine standard curve using a histamine suspension serially diluted in the range of 2.5-200 ppm.

3.3.4 DNA extraction

DNA from pure cultures was isolated using the UltraClean™ Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA) in accordance with manufacturer instructions. Briefly, bacterial cells were chemically/mechanically lysed in bead solution containing detergents. The released DNA was then bound to a silica spin filter, washed and recovered in Tris-buffer. Absorbance was recorded at 260 and 280 nm (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) in a 96-well quartz plate and DNA concentration and purity was calculated using standard formulae.

3.3.5 Probe labeling

DNA probes based on the *hdc* gene were produced using the dioxigenin (DIG)-labeled PCR DIG Probe Synthesis Kit (Roche, Indianapolis, IN). Two different probes were PCR-labeled using a DNA template from four high-histamine producing strains: *M. morganii* (ATCC 35200), *P. damselae* (mahi-mahi isolate), *E. aerogenes* (ATCC 13048) and *R. planticola* (ATCC 43176). First, a 709 bp probe corresponding to position 249-958 of the *hdc* gene of *M. morganii* (GenBank accession number J02577) was labeled using primers previously described by Takahashi al. *(194)* [*hdc-f* (5'-TCH ATY ARY AAC TGY GGT GAC TGG RG-3') and *hdc-r* (5'-CCC ACA KCA TBA RWG GDG TRT GRC C-3')]. Also,

a 249 bp probe corresponding to position 709-958 of the *hdc* gene of *M. morganii*, (GenBank accession number J02577) was DIG-labeled using the primers *hdc*-r by Takahashi et al. (44) and a primer designed for this study designated *hdc*2-f (5'-AYG CBG AYG CSG CDC TRA GYG GHA TGA-3') by sequence alignment (FastPCR, University of Helsinki, Helsinki, Finland) of the partial *hdc* gene from *M. morganii, P. damselae*, *E. aerogenes*, and *Raoultella spp.* [GenBank accession numbers FJ469557-73]. The *hdc* probes generated from the four histamine-producing bacteria were used in equal mixture (1:1:1:1) in the hybridization solutions. Amplifications were carried out for 40 cycles (94˚C for 1 min, 52˚C for 1 min, and 72˚C for 1 min) in a GTC-2 thermal cycler (Precision Scientific, Chicago, IL). PCR products were separated on a 1% agarose gel at 86V in 1x TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) for 1 h. Gels were stained with ethidium bromide (0.3 µg/ml) and visualized using a UV transilluminator (UVP, Upland, CA). Product size was confirmed using 100 bp molecular weight markers (Invitrogen, Carlsbad, CA).

3.3.6 DNA dot-blot

To determine the specificity of the DNA probes, dot-blot hybridization was done using DNA derived from pure cultures of all 152 histamine- and non-histamine producing isolates used in this study. Dot blots were prepared using a 96-well Bio-Dot Microfiltration apparatus (Biorad, Hercules, CA). A positively charged nylon membrane (Roche, Indianapolis, IN) was equilibrated in sterile distilled water for 10 min before being applied to the dot-blot apparatus. The membrane in the dot-blot apparatus was pre-washed with 500 µl

of sterile distilled water by vacuum filtration prior to DNA addition. Aliquots (2 µl) of purified DNA at a standardized concentration of 10 ng/µl were denatured by the addition of 1M NaOH and 200 mM EDTA (pH 8.2) solution to a final concentration of 0.4 M NaOH/10 mM and heated at 100˚C for 10 min. Denatured DNA solutions were briefly centrifuged and placed on ice until application. The 200 µl denatured DNA sample was applied to the positively charged nylon membrane in the dot-blot apparatus by vacuum filtration and then washed with 500 µl 0.4 M NaOH by vacuum filtration. The DNA dot-blot membrane was then removed from the dot-blot apparatus, rinsed in 2x SSC, air dried and cross-linked in UV-crosslinker (UVP, Upland, CA).

3.3.7 Hybridization

Dot-blot and colony lift membranes were prehybridized in DIG Easy Hyb solution (Roche, Indianapolis, IN) at 40˚C for 1 hour. The prehybridization solution was replaced with hybridization solution (DIG Easy Hyb) containing 5.0 µl/ml DIG-labeled PCR probe mix (1.25 µl/ml each probe) and the membranes incubated overnight at 40˚C. The membranes were washed twice in 2X SSC/0.1% SDS at room temperature for 5 min and twice in 0.5X SSC/0.1% SDS at 55˚C for 15 min. After a brief final wash in a proprietary washing buffer (Roche, Indianapolis, IN), the membranes were blocked in using a solution of 100 mM maleic acid/150 mM NaCl (pH 7.5) containing 1X blocking reagent (Roche) for 30 min at room temperature and then exposed to the same buffer containing 1:5000 of anti-digoxigenin–alkaline phosphatase Fab fragments (Roche) for 30 min. After washing twice in

washing buffer for 15 min, membranes were developed by exposure to 20µl/ml of NBT/BCIP (Roche) suspended in detection buffer (0.1 M Tris-HCl/0.1M NaCl, pH 9.5).

3.3.8 Evaluation of colony-lift hybridization method

Mahi-mahi (*Coryphaena hippurus*) and yellowfin tuna (*Thunnus albacores*), obtained from local North Carolina fish dealers, were homogenized (20 g) in 180 ml of saline (0.85% NaCl). Background flora in the uninoculated fish homogenate was determined by spread plating on $TSAN_2$ plates. The histamine-producing bacterial inoculum was diluted 1:10, 1:100, 1:1000 in saline and inoculated (200 µl) to the fish homogenate so as to obtain approximate concentrations of 2.0-3.0, 3.0-4.0, and 4.0-5.0 $\log_{10}$ CFU/ml histamine-producing bacteria. Counts immediately after inoculation were determined by spread plating on $TSAN_2$ plates.

Histamine-producing bacteria were quantified in the inoculated fish homogenate using modified Niven's agar method and the colony lift hybridization method as applied to $TSAN_2$ plates. Briefly, colony lift was done using positively charged nylon membranes (Roche, Indianapolis, IN) which were applied to agar plates for 1 min, then exposed to denaturation solution (0.5M NaOH/1.5 NaCl) for 5 min, neutralization solution (1.5M NaCl/1.0M Tris-HCl, pH 7.4) for 5 min, 2X SSC for 5 min, dried and crosslinked exposin to UV (UVP, Upland, CA). The membrane was incubated at 37˚C for 1 hour in 0.5 ml proteinase K (Roche, Indianapolis, IN) diluted to 2 mg/ml in 2X SSC prior to hybridization. For the colony lifts, a suspension of *M. morganii*, *R. planticola*, *E. aerogenes* and *P.*

damselae was used as a positive control while *Escherichia coli* and *Shewanella putrefacience* were used as negative controls.

3.3.9 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and discrimination of means by Tukey honest significant difference (HDS) by STATISTICA (StatSoft, Inc., Tulsa, OK). Dot-blot experiments were performed in duplicate and colony lift evaluation was done in triplicate.

3.4 RESULTS

3.4.1 Performance of DIG-labeled DNA probe mixes

Initial experiments focused on determining optimal DNA concentrations for use in dot blot hybridization. DNA extracts obtained from four strains, i.e., *M. morganii* (ID 1), *R. planticola* (ID 36), *E. aerogenes* (ID 39), and *P. damselae* (ID 47), were applied to the dot-blot at total amounts ranging from 5 – 100 ng DNA. After dot-blot, these were hybridized with each of the two probe mixes (709 and 249 bp probes) (Figure 3. 1). For both probe mixes, weak signals were obtained with 5 ng DNA from all strains, but strong signals were obtained using 25-100 ng DNA. The amount of DNA used in subsequent dot blot experiments was determined to be 25 ng.

All 152 isolates were evaluated for histamine production by HPLC. They were categorized into three groups: high- (>1000 ppm), low- (125-500 ppm) and non- (<125 ppm) histamine producers. Specifically, of the 152 individual isolates, 73 were high histamine-

producers, 6 were low producers and 73 did not produce histamine under the culture conditions employed. The dot blots corresponding to each of these strains were then hybridized using the 709 bp and the 249 bp *hdc*-probe mixes, and these results compared directly to HPLC (Table 3. 1 and Figure 3. 2). Positive dot blot hybridization signals were obtained for all 73 high histamine-producing (>1,000 ppm) isolates from *Morganella morganii*, *Providencia rustigianii*, *Proteus mirabilis*, *Raoultella planticola*, *Enterobacter aerogenes*, *Enterobacter georgoviae*, and *Photobacterium damselae* using the 709 bp (Figure 3. 2A-B) and 249 bp (data not shown) *hdc*-probe mixes. All 6 low (125-500 ppm) and all 73 non- (<125 ppm) histamine-producing bacteria could not be detected using either the 709 bp or 249 bp *hdc*-probe mixes. The 249 bp *hdc*-probe mix showed a higher degree of unspecific binding than did the 709 bp *hdc*-probe mix, therefore the 709 bp *hdc*-probe mix was used subsequent colony lift hybridization experiments.

3.4.2 Enumeration of histamine-producing bacteria in inoculated samples

To examine the practical application of the *hdc*-probe mix for quantification of histamine-producing bacteria, seafood homogenates (tuna and mahi-mahi) were inoculated with known numbers (in the range of 10^2 to 10^5 CFU/ml) of four high-histamine producers (*M. morganii*, *R. planticola*, *E. aerogenes*, and *P. damselae*). These samples were analyzed using both modified Niven's media and colony lift hybridization using the 709 bp *hdc*-probe mix. Colony-lift signals from inoculated mahi-mahi and tuna homogenate and positive controls (suspension of *M. morganii*, *R. planticola*, *E. aerogenes* and *P. damselae*) were

similar (Figure 3. 3A,C). No detection occurred on colony lifts from the negative control (*E. coli* or *S. putrefacience*) and uninoculated fish homogenates at any inoculation level (Figure 3. 3B,D). The background flora determined by aerobic plate count (APC) on $TSAN_2$ from uninoculated mahi-mahi and tuna samples was between 4.1-4.3 and 4.2-4.5 log CFU/ml, respectively (Figure 3. 4).

At all inoculation levels, there was no statistically significant difference between colony lift hybridization counts at any one level of inoculation when comparing fish type (mahi-mahi vs. tuna), indicating no matrix effect for the assay (Figure 3. 4A-B). Furthermore, there was no statistically significant difference comparing inoculum levels with colony lift hybridization counts ($p < 0.05$). There were, however, statistically significant differences when comparing quantification of histamine-producing bacteria by modified Niven's media to that obtained by either inoculum level or colony lift hybridization (Figure 3. 4A-B). This observation was consistent for both fish species and at each of the three seeding levels. The 709 bp *hdc*-probe did not appear to react with any of the indigenous microbial population in the seafood homogenate as evaluated when colony lift hybridization was applied to uninoculated control samples. Counts as low as 2.5×10^2 CFU/g of high histamine-producing bacteria could be detected using colony lift hybridization and 709 bp *hdc*-probe mix.

3.5 DISCUSSION

The epidemiological importance of histamine fish poisoning supports the need for continued control and monitoring of the growth of histamine-producing bacteria in fish. However, no reliable methods currently exist for rapid quantification of histamine-producing bacteria. The results in this paper demonstrate that a DIG labeled *hdc*-probe mix can be used to specifically detect high histamine-producing bacteria in fish. This method has significant advantages over conventional identification methods in terms of labor and time.

Detection of histamine-producing bacteria by traditional culture-based methods has been reported to be unspecific and time consuming. For instance, detection by the frequently used Niven's differential media has shown between 15-63% false positives (*(74, 133)*, Chapter 2). Compared to the Niven's method, DNA dot-blot hybridization with *hdc*-probe mix greatly reduced the incidence of false-positives although low histamine-producing bacteria (<300 ppm) were not detected. *M. morganii*, *Providencia rustiganii*, *Proteus mirabilis, Raoultella planticola*, *R. ornithinolytica*, *Enterobacter aerogenes*, *E. gergoviae* and *Photobacterium damselae* all produced >1,000 ppm (3372-6143 ppm) of histamine in culture broth and were all detected by the probe mix. However, three *H. alvei* strains and twenty-one *C. freundii*, low and non histamine-producers, were not detected by the *hdc*-probe mix. The three *Hafnia alvei* strains examined in this study produced 204, 171 and <125 ppm histamine in 1% histidine broth at 37˚C for 48 hours. Similarly, Ozogul et al. *(164)* reported that *H. alvei* produced 4.2 and 115.0 ppm histamine in 1% histidine decarboxylase broth at 30˚C for 48 h. Eighteen *H. alvei* and forty-one *C. freundii* strains

produced on average 286 and 413 ppm histamine when incubated in broth containing 1% histidine at 37˚C for 18 h *(132)*. Thus, *H. alvei* and *C. freundii* are poor histamine producers compared to the high histamine-producers detected by the DIG-labeled *hdc*-probe mix. Therefore, only bacteria that produce quantities exceeding 500 ppm (sufficient to cause scombrotoxic fish poisoning) were detected.

Variable results in the detection of low-histamine producing bacteria by molecular methods have previously been reported. Takahashi et al. *(194)* were unable to detect one *Citrobacter braakii* strain by amplification of the *hdc* gene despite the strains ability to produce 311 ppm histamine. However, they were able to amplify the *hdc* gene from one low histamine producing *H. alvei* strain. In this study, the twenty-one *C. freundii* and three *H. alvei* strains were not detected by the *hdc*-probe mix in DNA dot-blots, although four *C. freundii* and two *H. alvei* strains were able to produce between 126-296 ppm histamine.

Similarly to the molecular-based methods, detection of low-histamine-producing bacteria by culture-based methods has been difficult. As a result of their low histamine production, longer incubation times are generally required which increases the occurrence of false positive detections. For instance, Mavromatis et al. *(142)* recommended incubation of Niven's agar plates at 30˚C for 48 hours even though *M. morganii* produce a pronounced color change within 24 hours at 37˚C. Incubation at 30˚C allowed time for low-histamine producing bacteria such as *H. alvei* to grow and produce histamine. Similarly, Chen et al. *(35)* reported delayed color development of *H. alvei* on Niven's agar where color change was

evident in 48 h compared to 24 h for the high histamine producing strains of *M. morganii*, *Klebsiella pneumonia* and *Klebsiella oxytoca* strains.

It is evident that detection of low histamine producing bacteria by culture and molecular based methods is unreliable. The variation in the detection of low histamine producers by these methods may have several roots. Firstly, the low histamine produced by these strains may be as a result of unspecific activity of other amino acid decarboxylases present. In fact, arginine decarboxylases from *Salmonella typhimurium* and *Escherichia coli*, have been shown to metabolize histidine in addition to their natural substrates *(28, 46)*. Therefore, low histamine-production may occur despite the absence of the *hdc* gene. Secondly, the *hdc* gene may be plasmid associated in low histamine producing bacteria. For example, in *Vibrio anguillarium* the histidine decarboxylase gene is present on a 65 kb pJM1 plasmid that encodes an important iron uptake system required for its virulence *(56, 119)*. In addition, the histidine decarboxylase gene from low histamine-producing bacteria has yet to be sequenced.

Available molecular-based detection methods for histamine-producing bacteria are mainly based on the detection by amplification of the histidine decarboxylase and other amino acid decarboxylase by PCR *(102, 103, 175, 176, 194)*. Currently, these methods have not been adapted for the quantification of histamine-producing bacteria in fish samples. This study is the first attempt in the development of a molecular based method for the quantification of histamine-producing bacteria in fish. Quantification of high histamine-producing bacteria in inoculated samples by colony lift hybridization was in good agreement

with inoculum levels. However, Niven's agar counts were significantly lower than the inoculum counts. Similarly, Niven et al. *(158)* reported that the bacterial count on Niven's agar were approximately one order lower compared to plate count agar. They suggested that the low pH of the Niven's agar medium (pH 5.3) may exclude growth of some histamine-producing bacteria. This observation is consistent with our results. The colony lifts were performed on $TSAN_2$ plates with a pH of about 7.0 and therefore less risk for growth inhibition is present as evident by the higher counts. In addition, in order to be able to quantify high-histamine producing bacteria by Niven's method, a shorter incubation time of 24 hours compared to 48 hours recommended by Mavromatis et al. *(142)* was used. The shorter incubation time was chosen since Niven's agar plates turned completely purple during a 48 hour incubation period inoculated wih high-histamine producing bacteria making it impossible to distinguish positive colonies from negative once. The low pH of the Niven's media and the reduced incubation time may explain the lower counts obtained by this method. Consequently, the lower number of histamine-producing bacteria counts obtained by the Niven's method could underestimate the number of histamine-producing bacteria in fish samples and ultimately underestimate the risk of histamine fish poisoning.

Both enumeration methods have their advantages. The Niven's method is a low cost technique requiring basic microbial equipment and skills. However, because of the different histamine-production capabilities of different histamine-producing strains, identifying purple colonies in mixed cultures is difficult. Mavromatis et al. *(142)* suggested the number of colonies on plates should range from 1 to 80 for quantitative analysis of histamine producing

bacteria on Niven's agar. However, if high-histamine producing bacteria are present the Niven's agar plate will turn purple within the incubation period making it impossible to distinguish positive from negative colonies and give quantifiable results. In addition, Niven's agar has shown to give high false positives at longer incubation times *(133)*. Colony lift hybridization on the other hand requires expensive equipment and more advanced laboratory skills. However, as this study has shown it was more accurate in enmueration of high histamine-producing bacteria

At present, no reliable methods exist for rapid quantification and detection of histamine-producing bacteria in fish. Quantification of high histamine-producing bacteria can be achieved by colony lift hybridization using a *hdc*-probe mix. This new method is expected to improve the isolation and quantification of histamine-producing bacteria and will be useful in future studies for the development of adequate control measures and alternative strategies for reducing the risks associated with histamine-formation in scombroid fish.

3.6 ACKNOWLEDGEMENTS

This study was funded in part by grants from the National Fisheries Institute's Fisheries Scholarship Foundation, North Carolina Sea Grant, and North Carolina Agricultural Research Service. We thank Dr. Graham Fletcher at The New Zealand Institute for Plant and Food Research Limited, Dr. Rachel Nobles at the University of North Carolina Chapel Hill Institute of Marine Sciences, Dr. John Kaneko at PacMar Inc. and Dr. George Flick at Virginia Polytechnic Institute and State University for culture and sample donations and Drs.

Jay Levine, Fred Breidt, and Damian Shea at North Carolina State University for the loan of lab equipment used in this study.

Table 3. 1 Strain, histamine production and response to detection by DNA dot-blot hybridizations

ID	Species (no. tested)	Dot-Blot Hybridization with*: DIG-labeled PCR probe mix (709 bp)	Dot-Blot Hybridization with*: DIG-labeled PCR probe mix (249 bp)	Histamine* (ppm)	Source**
1-30	*Morganella morganii* (30)	30/30	30/30	4466	ATCC, NC, HI, NZ, ICPB
31-33	*Providencia rustigianii* (3)	3/3	3/3	4466	NC, HI
34-35	*Proteus mirabilis* (2)	2/2	2/2	4114	NC
36	*Raoultella planticola* (1)	1/1	1/1	6143	ATCC
37-38	*Raoultella ornithinolytica* (2)	2/2	2/2	5624	NC
39-44	*Enterobacter aerogenes* (6)	6/6	6/6	6660	ATCC, NC, HI
45-46	*Enterobacter gergoviae* (2)	2/2	2/2	5600	HI
47-73	*Photobacterium damselae* (27)	27/27	27/27	3372	NC, HI
74	*Klebsiella oxytoca* (1)	0/1	0/1	<125	ATCC
75-77	*Hafnia alvei* (3)	0/3	0/3	<125-128	ATCC, HI, NZ
78-79	*Vibrio alginolyticus* (2)	0/2	0/2	<125	ATCC, NC
80-101	*Citrobacter freundii* (21)	0/21	0/21	<125-265	ATCC, NC HI
102	*Citrobacter amalonaticus* (1)	0/1	0/1	<125	HI
103	*Serratia marcescens* (1)	0/1	0/1	<125	HI
104	*Vibrio mimicus* (1)	0/1	0/1	<125	NC
105	*Pseudomonas putida* (1)	0/1	0/1	<125	NC
106-111	*Shewanella putrefaciens* (6)	0/6	0/6	<125	NC
112-121	*Enterobacter cloacae* (11)	0/11	0/11	<125	NC
122-123	*Escherichia hermannii* (2)	0/2	0/2	<125	HI
124	*Vibrio parahemolyticus* (1)	0/1	0/1	<125	NC
125-126	*Vibrio fluvialis* (2)	0/2	0/2	<125	HI
127-132	*Pseudomonas aeruginosa* (6)	0/6	0/6	<125	HI, NZ
133-138	*Escherichia coli* (13)	0/13	0/13	<125	ATCC, NC
149-151	*Listeria innocua* (3)	0/3	0/3	<125	NC
152	*Shigella flexneri* (1)	0/1	0/1	<125	ATCC

* Results represent no. of strains positive / no. tested
** ATCC, American Type Culture Collection, USA; ICPB, International Collection of Phytopathogenic Bacteria, USA; NC, North Carolina, USA; HI, Hawaii, USA; NZ, New Zealand

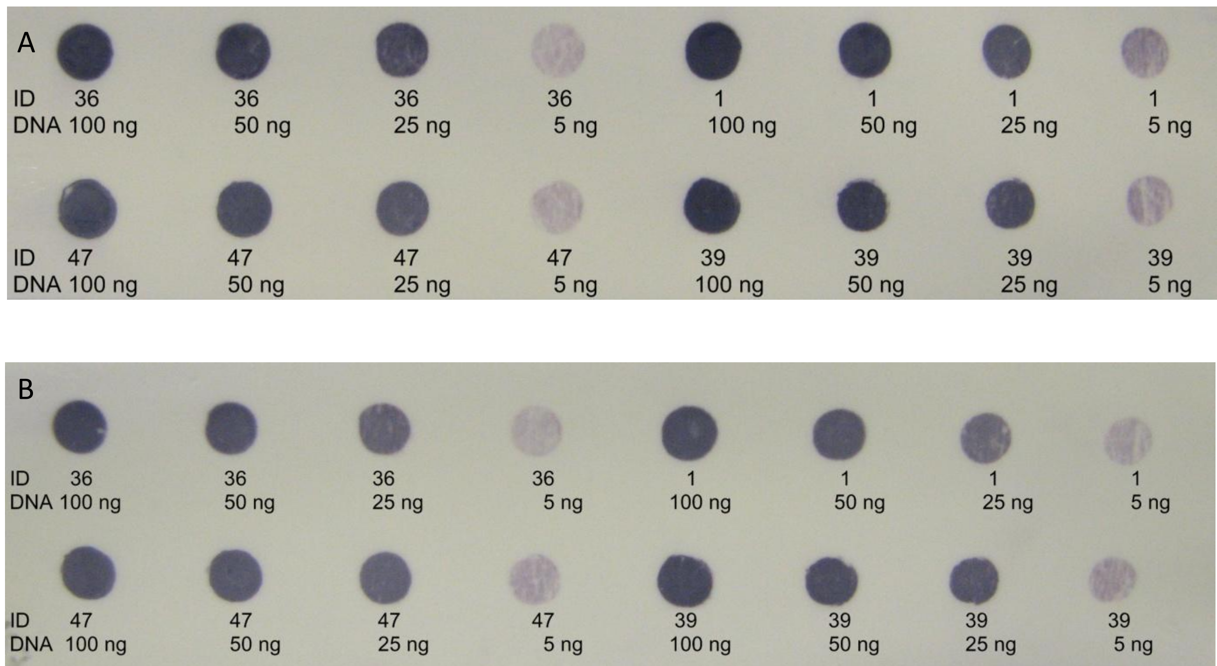
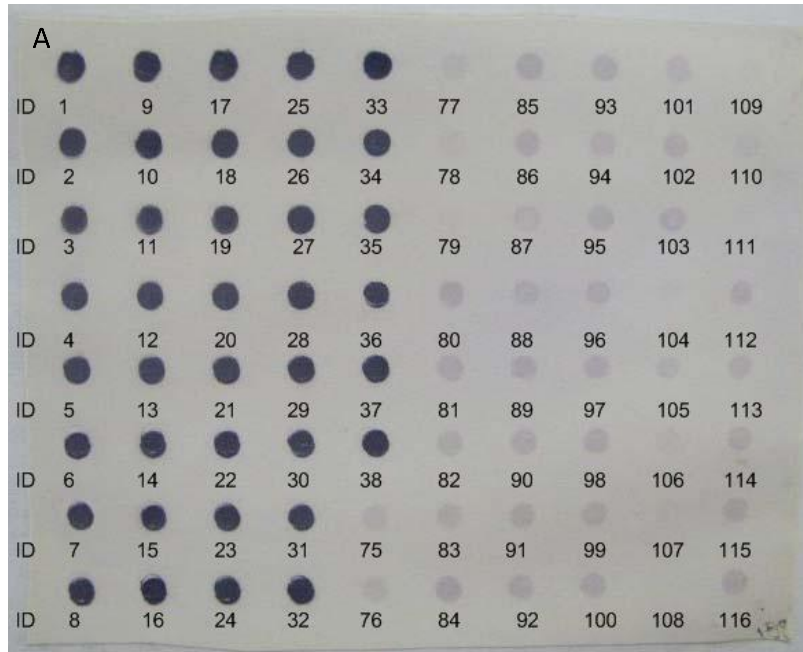


Figure 3. 1 Determination of optimal DNA concentration from high-histamine producers [*M. morganii* (ID 1), *R. planticola* (ID 36), E. *aerogenes* (ID 39), and *P. damselae* (ID 47)] for use in dot-blot hybridization using (A) the 709 bp *hdc*-probe mix; and (B) the 249 bp *hdc*-probe mix.



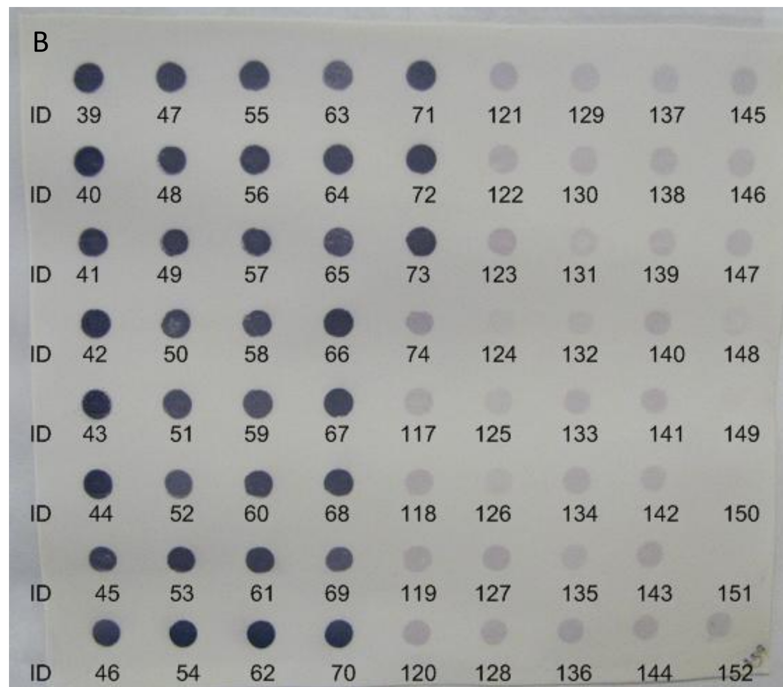


Figure 3. 2 Dot-blot hybridization of the 709 bp DIG-labeled probe mix with 20 ng of purified DNA from high and low histamine-producing bacteria in Table1 (A-B).

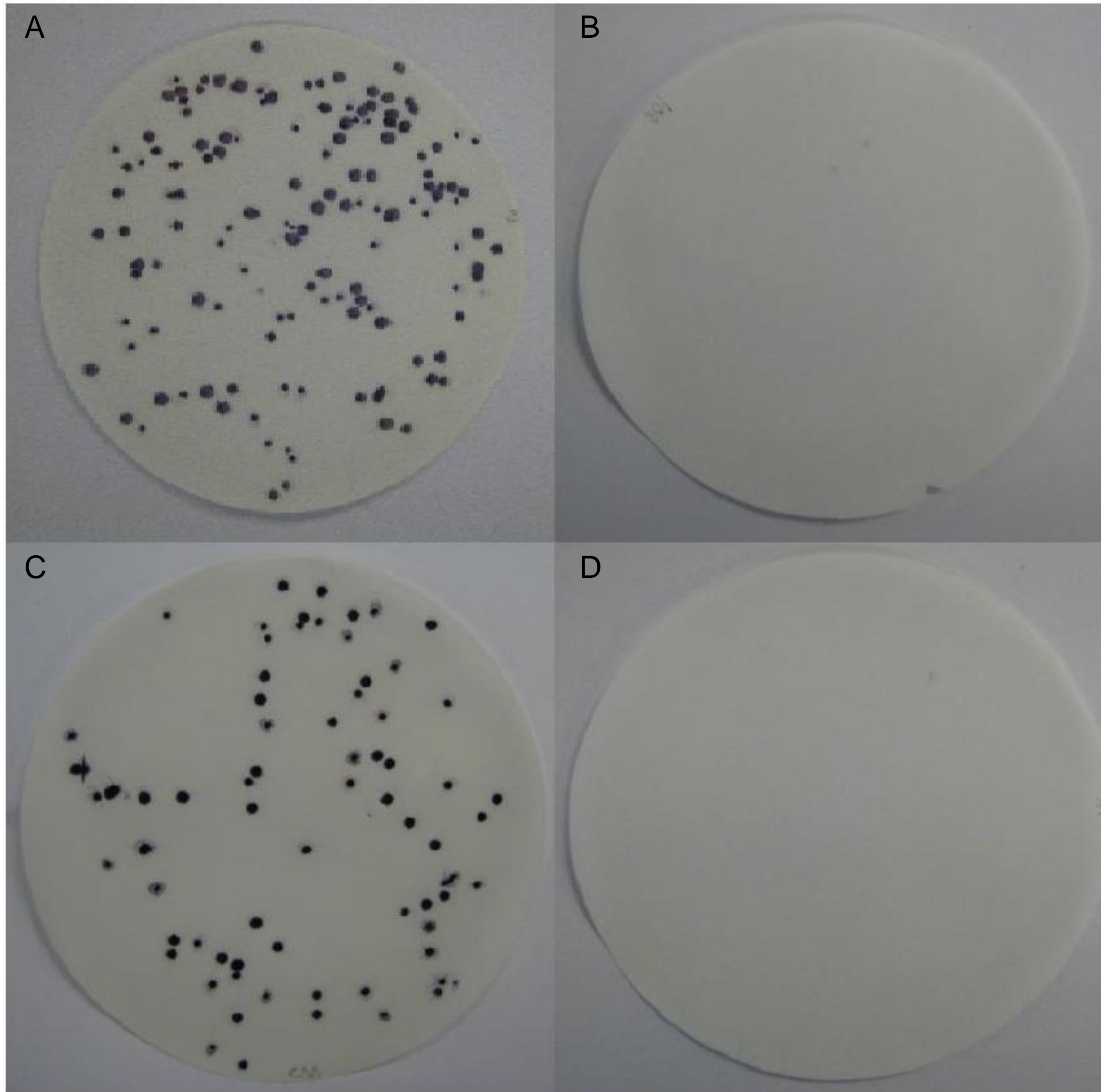


Figure 3. 3 Sample colony lift blots from inoculated fish sample (A), uninoculated sample (B), positive control (suspension of *M. morganii*, *R. planticola*, *E. aerogenes* and *P. damselae)* (C), and negative control (*Shewanella putrefaciens)* (D)

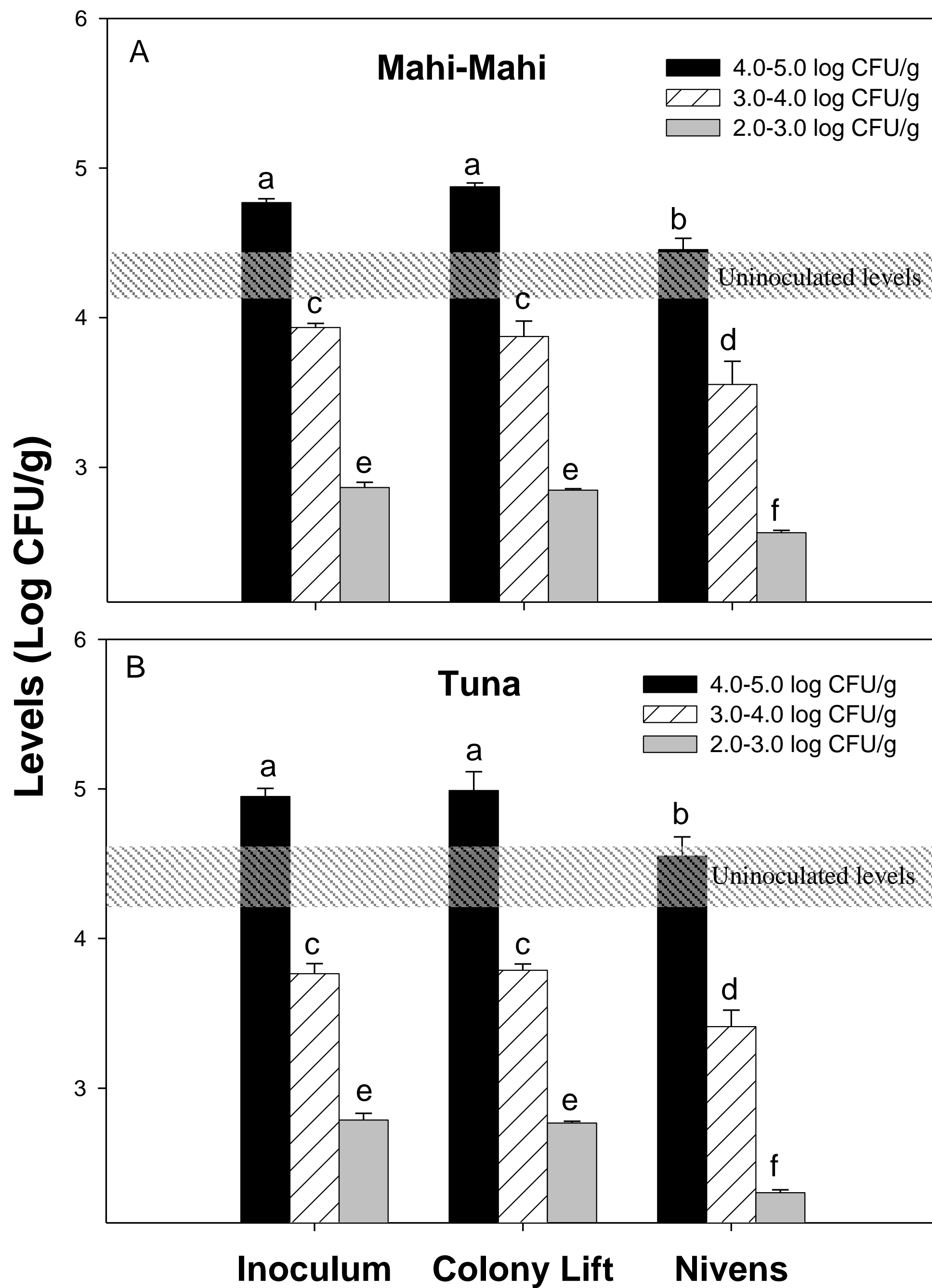


Figure 3. 4 Comparison of detection of inoculated mahi-mahi (A) and tuna (B) samples by colony lift hybridization and Niven's agar methods. Samples were inoculated with known number of histamine-producing at three different levels (2.0-3.0, 3.0-4.0, and 4.0-5.0 log CFU/ml) and compared to detection by colony lift hybridization on $TSAN_2$ agars and Niven's agar.

CHAPTER 4

EFFECT OF PH ON HISTAMINE FORMATION IN FISH

4.1 INTRODUCTION

Scombrotoxic fish poisoning remains a significant health concern in spite of significant efforts by public health officials for its control. Currently, the primary control measure for prevention of histamine formation in fish is low temperature storage. The FDA implemented time and temperature guidelines for preventing histamine formation in 1997 as part of the Hazard Analysis Critical Control Points (HACCP) program *(70)*. However, rapid chilling alone does not prevent formation of high levels of histamine and other biogenic amines due to the growth of psychotropic histamine-producing bacteria *(86)*. Once histamine has formed, no method used in the preparation of fish including freezing, canning, or smoking will destroy the toxin *(65)*. Therefore there is a need to identify secondary control measures for reducing or preventing histamine-formation in susceptible fish species.

Control of scombrotoxic formation can be achieved by: 1) inhibiting growth of histamine-producing bacteria; 2) inhibiting activity of the histidine decarboxylase enzyme and/or 3) destruction or degradation of histamine. Inhibiting the growth of histamine-producing bacteria in has been achieved by strict adherence to time/temperature limits, or by high pressure processing, and/or the use of preservatives *(21, 115, 144, 207, 208)*. These

measures have been ineffective against psychrotrophic histamine-producing bacteria or have had undesirable effects of the taste or appearance of the product.

Research on histamine control by the inhibition of histidine decarboxylase enzyme activity in fish is limited. Degradation of histamine once formed occurs as part of the natural detoxification process in the gut. Although medical studies of the detoxification of histamine by endogenous diamine oxidase in the gut have been conducted *(6)*, direct use of diamine oxidase in food applications has been limited.

One promising measure for control of histamine formation in fish may is inhibiting the activity of the histidine decarboxylase enzyme. Studies on the purified histidine decarboxylase of *P. damselae, M. morganii*, *R. planticola* have optimum activity at 30-40˚C and pH 6.0-6.5 *(96)*. The enzyme activity is known to decrease above and below the optimum pH. Therefore, it may be possible to reduce enzyme activity by manipulating the muscle pH.

Trisodium phosphates (TSP) have been used for many years in the food industry, mainly for decontamination of beef and poultry carcasses by a process of dipping or spraying using 8-12% solutions. TSP is Generally Recognized as Safe (GRAS) by the FDA as a food processing aid (with no label declaration needed) *(31)*. One characteristic of TSP is its high pH in solutions (approx. 12). TSP solutions may be used to increase surface pH of fish muscle by dipping, spraying or other applications. Research has shown that dipping catfish fillets in 10% and 20% TSP increased muscle surface to pH 11-12 *(153)*. In addition to high pH, TSP solutions have been reported to have antimicrobial activities focusing on ionic

strength alteration and weakening of the bacterial cell wall of Gram-positive bacteria *(31)*. Several researchers have investigated the effect of phosphates on various bacteria from fish. Kirby et al. *(110)* found that 10% solution of TSP resulted in a 1.5 log CFU/ml decrease in a cocktail of 6 bacteria isolated from fish including, *P. phosphoreum* and *Enterobacter* spp. Some of these bacteria have been reported to produce high levels of histamine *(194)*. Similarly, Marshall et al. *(140)* reported that dipping catfish in 10 %TSP reduced APC and total coliform counts by 1.0 and 2.4 log CFU/ml. The effect of TSP on survival and growth of histamine-producing bacteria and histamine formation has not been investigated.

The purpose of this study was to investigate the affect of pH on the activity of the histidine decarboxylase enzyme in fish. Specifically, we examined the effect of pH in culture and TSP in fish muscle on growth of histamine-producing bacteria and histamine formation. First, growth of histamine-producing bacteria and histamine formation was studied in bacterial cultures at different pH conditions. Next, TSP treatment effects on mahi-mahi and tuna muscle was investigated. Finally, the application of TSP for control of histamine-producing bacteria in artificially contaminated tuna and mahi-mahi muscle was evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

The bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC, Manassas,VA, USA), Dr. Graham Fletcher (New Zealand Institute for Plant & Food Research, Christchurch, New Zealand), **),** Dr. John Kaneko

(PacMar Inc., Honolulu, HI, USA) and isolated from fish [gills, belly cavities and muscle tissues of yellowfin tuna (*Thunnus albacares*), mahi-mahi (*Coryphaena hippurus*) bluefish (*Pomatomus saltatrix*) and wahoo (*Acanthocybium solandri*)] and environmental samples collected in North Carolina as previously described in chapters 2 and 3. Specific isolates used in this study are listed in Table 4. 1.

Table 4. 1 Bacterial isolates their histamine production (ppm) and original source

Strain	**Microorganism**	**Histamine (ppm)**	**Source**
ATCC 35200	*Morganella morganii*	3750	ATCC
HPP 301	*Morganella morganii*	5526	Mahi
ATCC 43176	*Raoultella planticola*	6143	ATCC
HPP T15	*Raoultella ornithinolytica*	5789	Tuna
ATCC 13048	*Enterobacter aerogenes*	*7779*	*ATCC*
HW 18.3	*Enterobacter aerogenes*	7783	Mahi
BR 100	*Photobacterium damselae*	6157	Mahi
BR 107	*Photobacterium damselae*	2612	Mahi
ATCC 13337 (12)	*Hafnia alvei*	204	ATCC
NZ1	*Hafnia alvei*	171	New Zealand
BR 121	*Citrobacter freundii*	<50	Mahi
A 4086	*Citrobacter freundii*	262	Environment
ATCC 25922	*Enterobacter coli*	<50	ATCC
FT 534	*Shewanella putrefaciens*	<50	Tuna

*Histamine detected by HPLC after 48 h incubation in TSB supplemented 1% histidine, 2% NaCl, and 0.0005% pyridoxal-HCl (pH 6.5) at 37˚C. Data are shown as averages of three idependent replicas.

4.2.2 Bacterial growth and histamine production by impedance measurements

Bacterial isolates found in Table 4. 1 were tested for their ability to increase conductance at pH 5.5, 6.5, 7.5 and 8.5 in histidine decarboxylase broth (HDB) [Klausen and Huss *(112)* with slight modification]. Single colonies were incubated in 9 ml of TSB at 30˚C for 24 h. The 24 h culture was diluted (1:10) in TSB and 1 ml of the diluted culture

incubated in 9 ml of HDB containing 0.2% peptone, 0.1% Lab-Lemco (Oxoid, Hampshire, UK), 0.81% histidine, 0.5% NaCl and 0.0005% pyridoxal·HCl in BacTrac vials. Vials were placed in a BacTrac instrument (SY-Lab, Neupurkersdorf, Austria) and conductance measured at 30˚C for 48 h. Results were expressed graphically as an average of three independent replicates showing percent increase in media conductance (%M) vs. time. Initial bacterial levels of the 24 h culture and final counts after 48 h in the BacTrac instrument were determined by serially diluting in saline (0.85% NaCl) and spread plating on trypticase soy agar containing 2% NaCl ($TSAN_2$). Histamine levels were determined by HPLC as described in section 5.2.4.

4.2.3 Growth and histamine production in tuna fish infusion broth

Histamine-producing bacteria were inoculated in 5 ml of $TSBN_2$ and incubated at 30˚C for 24 h. Tuna fish infusion broth (TFIB) was prepared as previously described by Omura et al. *(163)*. Briefly, tuna muscle (*Thunnus albacares)* was homogenized with twice its weight in water and steamed at 100˚C for 1 hr. The tuna fish homogenate was cooled, vacuum filtered though Whatman No. 1 (Whatman Inc., Piscataway, NJ) filter paper and enriched with 1% glucose. The pH then adjusted to 5.5 and 8.5 and 30 ml aliquots sterilized at 121˚C for 15 min. The 24 h culture was serially diluted in saline and 300 µl were inoculated in 30 ml TFIB incubated at 30˚C for 72 h. Samples were taken every six hours for the first 24 h and then every 12 h for the next 48 hours and analyzed for histamine content (HPLC) and bacterial counts on $TSAN_2$ (30˚C, 24h).

4.2.4 Measurement of histamine content

Histamine content in culture broths (BacTrac and TFIB) and tissue samples was measured by high performance liquid chromatography (HPLC) using the method described by Cinquina et al. *(42)*. HPLC chemicals were obtained from J.T. Barker (Hayward, CA) unless otherwise specified. Briefly, culture broth (200µl) or tissues samples (5 g) were extracted in 800 µl or 25 ml of 1M perchloric acid (1:5). The diluted sample was vortexed for 1 min, sonicated for 15 min and centrifuged for 15 min at 4,160 *x g* at 4˚C. For the culture broth, the supernatant was filtered through a 0.45 µm PTFE (polytetrafluoroethylene, VWR, West Chester, PA) filter. The tissue samples were filtered though a Whatman 40 filter (Whatman Inc., Piscataway, NJ). The procedure was repeated twice for the tissue samples and supernatant filtered through a 0.45 µm PTFE filter. The extract was diluted 1:10 in HPLC grade water and injected onto a 4.6 x 250 mm Luna C18(2) column (Phenomenex, Torrance, CA, USA) using an Agilent 1050 HPLC and separated under isocratic conditions using 85% Eluent A (85% phosphate buffer pH 6.9 and 15% methanol) and 15% Eluent B (acetonitrile). Flow rate was set at 0.5 ml/min and detection was achieved using a diode-array detector (DAD) at 214 nm. The detection limit for this method was 50 ppm and 90 ppm for the culture and tissue samples, respectively.

4.2.5 Phosphate treatment on dipped and vacuum-packaged mahi-mahi muscle

Mahi-mahi (*Coryphaena hippurus*) fillets were obtained from a local fish market. The fillets were sliced in 5x8 cm^2 tissue blocks and the surface pH measured using a flat surface pH electrode (Pinacle; Nova Analytics Corp., Cold River, CA). For the phosphate dipped samples, the tissue blocks were dipped in 330 ml of sterile distilled water or 1%, 5% and 10% trisodium phosphate (TSP anhydrous; Prayon, Augusta, GA) at 4˚C for 1, 5 and 10 min at room temperature (RT). The tissue blocks were drained at RT for 15 min and then stored at 10˚C for 6 days. Muscle pH was measured at three random locations on day 0, 2, 4, and 6 using the flat surface pH electrode and average pH was reported. For the vacuum-packaged fish samples, mahi-mahi and tuna tissue blocks were placed in oxygen permeable bags (HP2700, polyoefin, 10,000 $cc/m^2/24h$, Cryovac, Duncan, SC) and 5, 10, 15 ml of TSP at 2%, 5% or 7% and sterile distilled water (control) added before vacuum sealing (Hollymatic Corp., La Grange, IL). The vacuum-packaged phosphate-treated fish samples were stored at 10˚C for 4 days and external and internal surface pH measured daily. The internal pH was measured by slicing the tissue blocks at three locations and the internal surface pH was measured with the flat-surface pH electrode. Measurements were taken in triplicates and the average value was reported. The pH was measured on three random locations on the external surface of each tissue sample and an average was reported.

4.2.6 Inoculated vacuum-packed fish samples

Fresh mahi-mahi and tuna fillets were sliced in 5x8 cm^2 with external surface pH measured as previously described. *M. morganii* 301, *R. planticola* 6, *E. aerogenes* 18.3, and *P. damselae* 100 were inoculated in 5 ml $TSBN_2$ at 37˚C for 24 h. A subsample (0.25 ml) of each culture was washed by centrifugation at 13,700 x *g* for 1 min with the pellet resuspended in 1 ml of saline (0.85% NaCl). A 0.1 ml aliquot of the histamine-producing bacterial cocktail was transferred to mahi-mahi and tuna samples and spread on the surface with a sterile hockey stick. The samples were left at room temperature for 20 min to allow attachment of the histamine-producing bacteria. The bacteria-laden fish muscle samples were placed in oxygen permeable bags followed by the addition of 15 ml of 7% TSP and vacuum-packing. Samples were stored at 10˚C to simulate mild temperature abuse conditions and held for 4 days. External and internal surface pH was measured daily at three random places on the tissue samples as previously described. Histamine concentration of the mahi-mahi and tuna samples were determined by HPLC as described earlier. Histamine-producing bacteria were determined by the colony lift hybridization method described in chapter 4.

4.2.7 Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and discrimination of means by Tukey Honest Significant Difference (HDS) by STATISTICA (StatSoft, Inc., Tulsa, OK). Correlation in conductance experiments was examined in SigmaPlot (Systat

Software Inc, Richmond, California). Each set of experiments was performed in three independent replicates.

4.3 RESULTS AND DISCUSSION

4.3.1 Relationship between conductance, histamine-formation and bacteria growth

Klausen and Huss *(112)* developed the potentiometric method for detection of histamine-producing bacteria based on an increase in conductance in histidine decarboxylase broth. Samples of pure or mixed bacteria cultures were incubated in histidine containing broth (pH 5.5) at 25˚C and conductance was measured for 24 h. Significant increase in conductance was evident when high histamine-producing *M. morganii* strains were tested, but no changes in conductance was observed with non-histamine producing strains of *Pseudomonas* sp. and *Alteromonas putrefaciens.* Klausen and Huss *(112)* reported good correlation between the increase in conductance and histamine content of the media after 24 hour incubation with *M. morganii*. However, they did not compare bacterial growth and histamine-production for non-histamine producing bacteria. Only the histamine content and increase in conductance of one high-histamine producing strain at 24 hour incubation was reported. To further investigate the relationship between conductance, histamine-formation and growth of bacteria in histidine broth incubated with two selected strains was investigated; using the high-histamine producing strain *R. planticola* 6 (6143 ppm; Table 4. 1) and the non-histamine producer *C. freundii* 121 (<50 ppm). Changes in conductance, histamine-formation and bacteria growth during 48 hours of incubation were monitored.

Histamine-formation of the high-histamine producer *R. planticola* 6 in the histidine broth showed good correlation (R^2 = 0.9971) with changes in conductance (Figure 4. 1). *C. freundii* 121, on the other hand, produced undetectable histamine (<50 ppm) in the same broth and therefore a correlation between histamine-production and increase in conductance was not possible for this strain. The correlation between bacterial growth of *R. planticola* 6 and *C. freundii* 121 and conductance was poor for both strains, R^2 = 0.1586 and 0.4843, respectively. Altenatively, Klausen and Huss *(112)* found good correlation (R^2 = 0.998) between histamine content and conductance when *M. morganii* was inoculated in the same histidine containing media. However, they did not examine the relationship between growth and increase in conductance. Our data show a good correlation between conductance and histamine formation irrespective of bacterial growth.

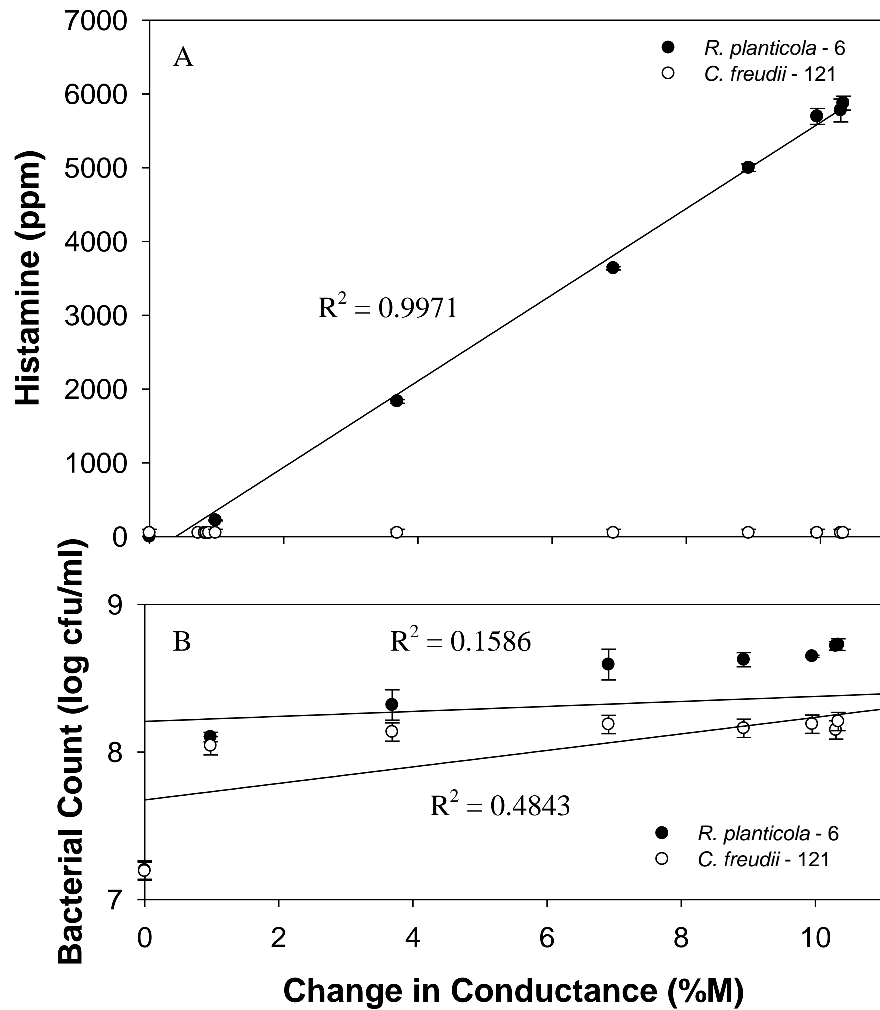


Figure 4. 1 Relationship between increase in conductance and histamine concentration (A) or growth (B) at 30˚C for *R. planticola* and *C. freundii* incubated in histidine broth.

4.3.2 Effect of pH on histamine formation

4.3.2.1 Effect of pH on histamine formation as measured by change in conductance

On the basis of HPLC analysis of histamine content, isolates were sub-classified into three groups: high histamine producers (>1000 ppm), low histamine producers (125 - 500

ppm) and non- producers (<125 ppm) after incubation in histidine containing media for 24 hours. To evaluate the effects of pH on histamine formation, histidine broth of varying pH (pH 5.5-8.5) was incubated with 8 high-histamine, 3 low-histamine and 3 non-histamine-producing bacteria. The bacterial cultures were monitored at 1 hour intervals for conductance at 30˚C for 48 h (Figure 4. 2 & Figure 4. 3). Bacterial counts were determined at the beginning and end of the 48 hours incubation and final histamine concentrations were determined as previously described (Table 4. 2 & Table 4. 3).

Increase in conductance was greater for high histamine producing strains (*M. morganii*, *Raoultella* spp., *E. aerogenes*, and *P. damselae;*Figure 4. 2) at pH 5.5. We previously showed a correlation between increase in media conductance and histamine formation when histamine-forming bacteria were incubated in histidine decarboxylase broth (section 5.3.1). Thus, higher conductance indicates increased histamine-formation. Conductance was diminished at higher pH for the high histamine-producing bacteria (*M. morganii*, *Raoultella* spp., *E. aerogenes*, and *P. damselae;*Figure 4. 2). For all high-histamine producing bacteria, conductance was lowest at pH 8.5. These results agreed with histamine analysis data where histamine concentration decreased with high initial pH of the media (Table 4. 2). The *Raoultella* spp. and *E. aerogenes* spp. produced the highest concentrations of histamine at pH 5.5, 6.5 and 8.5. *P. damselae* 100 & 107 and *M. morganii* 10 produced the lowest amount of histamine when comparing all of the high-histamine producing bacteria tested.

It is noteworthy that growth of *P. damselae* strains was poor in the TSB inoculation broth and as a result was inoculated at lower levels that the other bacteria examined (Table 4. 3). In addition, the *P. damselae* bacterial counts and histamine concentrations after 48 h incubation in histidine broth were lower than other bacteria (Table 4. 3). The reduced growth and ultimately lower histamine-production by *P. damselae* may have been caused by suboptimal growth conditions. *Photobacterium damselae* is an obligate halophilic bacteria that fails to grow at an NaCl concentration less than 0.5% or greater than 4.0%, or at a temperature below 15 or above 32.5˚C *(188)*. Optimum growth occurs at 1.0% – 2.5 % NaCl and temperature of 22.5 – 30˚C *(87)*. The incubation temperature was set at 30˚C which falls within the optimum level for these bacteria, however the TSB inoculation broth and the histidine broth contain 0.5% salt which may have resulted in reduced growth and ultimately reduced histamine formation of the two *P. damselae* strains.

Results with low and non-histamine-producing bacteria revealed inconsistent increase in conductance (Figure 4. 3). *C. freundii* 121 & 4086 and *E. coli* 25, both histamine non-producers, showed no increase in conductance and undetectable histamine production at all pH values. Both *H. alvei* strains (low producers) showed increased conductance at higher pH, although low-histamine production was only detected at pH 5.5 (Table 4. 2). Similarly, the non-histamine producer *S. putrefacience* showed increased conductance at higher pH although histamine was undetectable at all pH values.

These results indicate that at high pH, *H. alvei* and *S. putrefacience* may produce metabolic products other than histamine that may affect conductance of the media. The

increase in conductance was not detectable under normal growth conditions (pH 5.5). Similarly, Klausen and Huss *(112)* found no significant increase in conductance when *Pseudomonas* spp. were incubated in histidine media at pH 5.5. Our results confirmed that optimum histamine production occurred when histamine-producing bacteria were incubated for 48 hours at pH 5.5 in histidine containing media and but histamine production was non-detectable or low at pH 8.5.

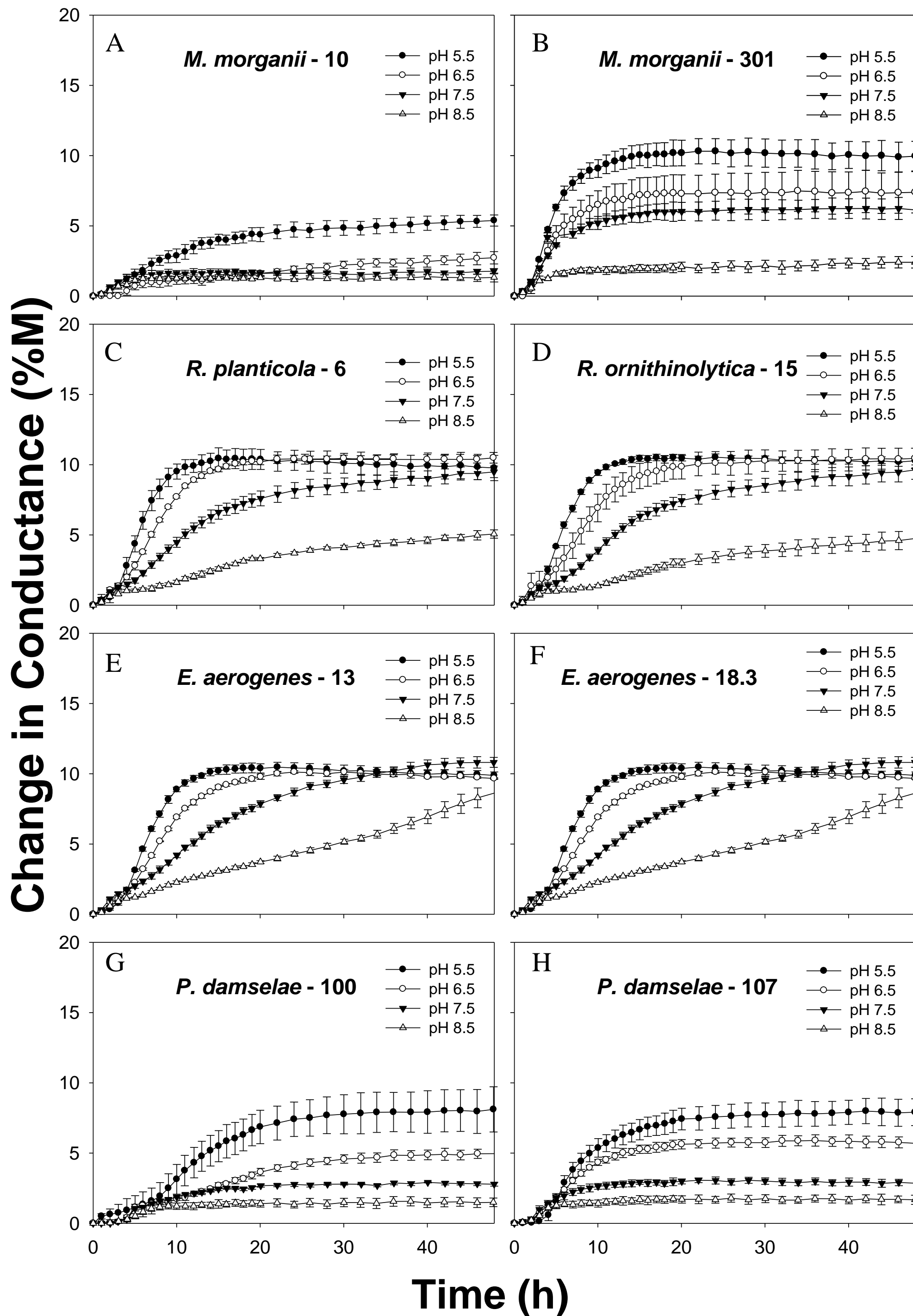


Figure 4. 2 Change in conductance over time in histidine broth at 30˚C for 48 h as measured by the BacTrac instrument at different pH's for *M. morganii* strain 10 (A) and 301 (B), *R. planticola* 6 (C), *R. ornithinolytica* 15 (D), *E. aerogenes* strain 13 (E) and 18.3 (F) and *P. damselae* strain 100 (G) and 107 (H).

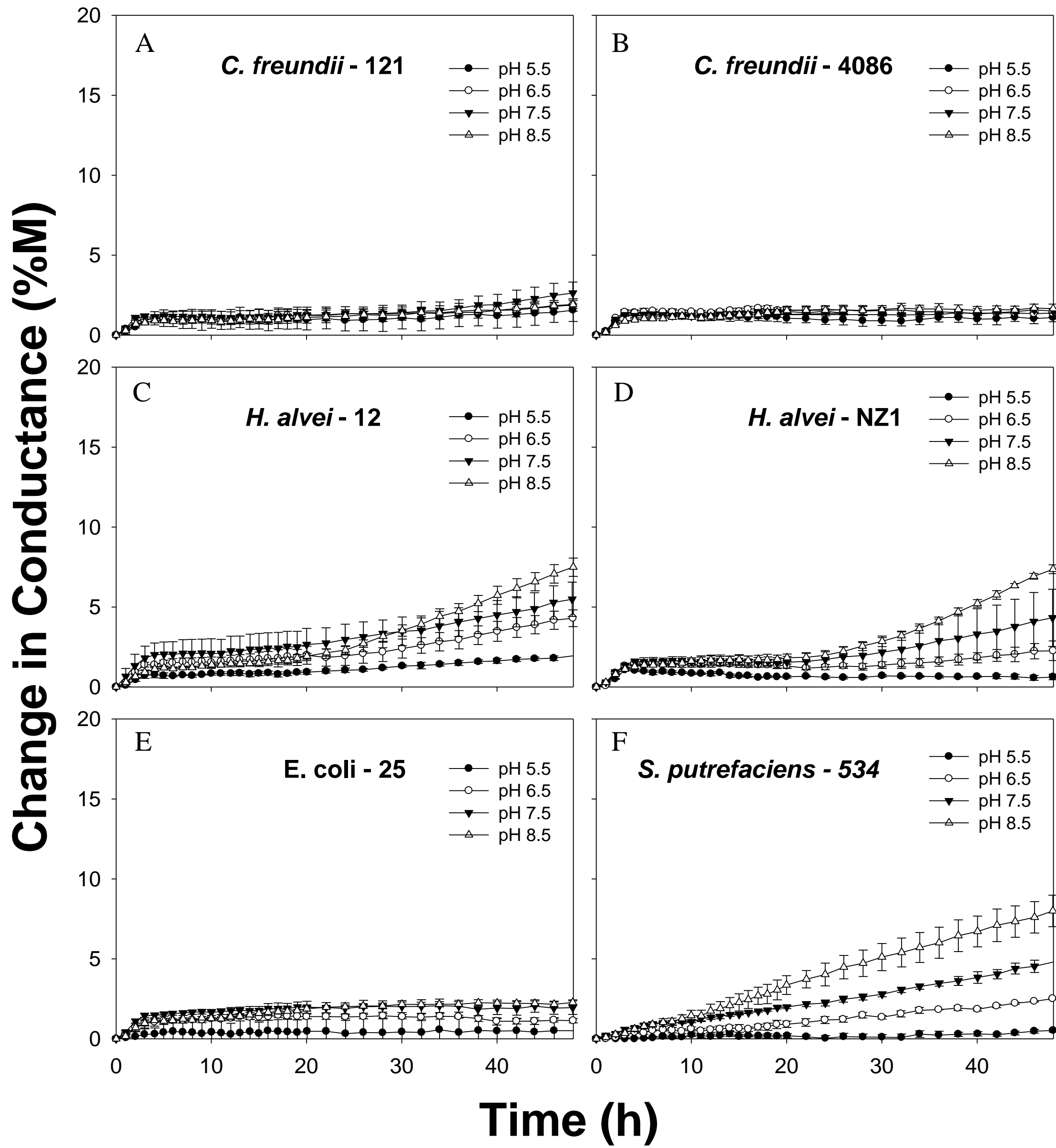


Figure 4. 3 Change in conductance over time in histidine broth at 30˚C for 48 h as measured by the BacTrac instrument at different pH's for *C. freudii* strain 121 (A) and 4086 (B), *H. alvei 12* (C) and NZ1 (D), *E. coli* 25 (E) and *S. putrefaciens* 534 (F).

Table 4. 2 Histamine concentration in histidine broth at different pH (5.5-8.5) after 48 h incubation in BacTrac instrument at 30˚C.

	Histamine (ppm)[a]			
Microorganism	**pH 5.5**	**pH 6.5**	**pH 7.5**	**pH 8.5**
M. morganii 10	2927.1 (52.9)	1364.2 (57.2)	111.0 (7.6)	<50
M. morganii 301	5621.4 (96.5)	3829.8 (48.5)	2427.7 (32.6)	442.6 (16.8)
R. planticola 6	6015.8 (55.4)	6057.2 (204.2)	4817.9 (104.2)	2262.5 (185.2)
R. ornithinolytica 15	5903.3 (77.6)	6023.3 (34.5)	5001.0 (90.7)	2093.0 (103.7)
E. aerogenes 13	5662.5 (85.1)	5561.8 (26.0)	5225.2 (46.1)	2655.7 (76.9)
E. aerogenes 18.3	5969.9 (90.1)	5817.1 (69.1)	4947.8 (112.9)	2761.8 (117.7)
P. damselae 100	4947.4 (147.7)	2817.2 (30.3)	807.3 (38.4)	52.5 (2.3)
P. damselae 107	3731.6 (120.0)	2334.6 (81.8)	851.1 (50.3)	53.2 (6.2)
H. alvei 12	86.3 (2.6)	<50	<50	<50
H. alvei NZ1	104.7 (60.8)	<50	<50	<50
C. freundii 121	<50	<50	<50	<50
C. freundii 4086	<50	<50	<50	<50
E. coli 25	<50	<50	<50	<50
S. putrefaciance 354	<50	<50	<50	<50

Table 4. 3 Inoculation and final bacterial counts of different bacteria after incubation in histidine broth in Bactrac instrument at 30˚C for 48h.

	Microbial Counts (log cfu/ml)[a]				
Microorganism	**Inoculation**	**pH 5.5**	**pH 6.5**	**pH 7.5**	**pH 8.5**
M. morganii 10	7.2 (0.1)	8.7 (0.2)	8.8 (0.1)	8.7 (0.1)	8.5 (0.1)
M. morganii 301	7.4 (0.5)	8.9 (0.1)	8.9 (0.1)	8.8 (0.1)	8.7 (0.0)
R. planticola 6	6.9 (0.0)	8.6 (0.1)	8.2 (0.2)	8.1 (0.1)	7.9 (0.2)
R. ornithinolytica 15	6.8 (0.0)	8.7 (0.3)	8.3 (0.1)	7.9 (0.1)	7.8 (0.0)
E. aerogenes 13	6.9 (0.0)	8.8 (0.0)	8.6 (0.1)	8.2 (0.1)	8.1 (0.1)
E. aerogenes 18.3	7.1 (0.0)	8.6 (0.1)	8.3 (0.1)	8.6 (0.6)	8.3 (0.2)
P. damselae 100	4.9 (0.2)	7.7 (0.1)	7.7 (0.1)	7.1 (0.2)	7.7 (0.1)
P. damselae 107	5.7 (0.0)	7.8 (0.0)	7.6 (0.1)	7.6 (0.1)	7.8 (0.0)
H. alvei 12	7.0 (0.0)	8.7 (0.1)	8.5 (0.3)	8.7 (0.1)	8.5 (0.6)
H. alvei NZ1	7.1 (0.0)	8.5 (0.0)	8.8 (0.0)	8.6 (0.1)	8.9 (0.1)
C. freundii 121	7.1 (0.0)	8.7 (0.1)	8.6 (0.0)	8.7 (0.0)	8.3 (0.3)
C. freundii 4086	6.9 (0.0)	8.5 (0.2)	8.6 (0.1)	8.6 (0.1)	8.2 (0.1)
E. coli 25	6.9 (0.1)	8.4 (0.1)	8.5 (0.1)	8.3 (0.2)	8.3 (0.3)
S. putrefaciance 354	6.7 (0.1)	7.8 (0.1)	8.2 (0.2)	8.3 (0.1)	8.0 (0.2)

[a] Data are expressed as mean of three independent replicas (n=3) and values given in parentheses are standard deviations of the three replicas

4.3.2.2 Histamine-production in tuna fish infusion broth

To further examine the effect of pH on histamine production in a more representative substrate, growth and histamine production of producing and non-producing bacteria was examined in tuna fish infusion broth (TFIB). For this experiment, four high- (*M. morganii* 301, *R. planticola* 6, *E. aerogenes* 18.3, and *P. damselae* 100) and two low-histamine producing strains (*H. alvei* 12 & *C. freudii* 4086) were tested. Histamine-producing bacteria were incubated in TFIB *(163)* at pH 5.5 and 8.5 at 30˚C for 72 hours. Samples were taken every six hours for the first 24 h and then every 12 h for the next 48 hours and analyzed for histamine content and bacterial growth.

Histamine production was significantly less (ANOVA; $p = 0.000$) at pH 8.5 compared to histamine production at pH 5.5 (Figure 4. 1). *M. morganii* 10, *R. planticola* 6 and *E. aerogenes* 18.3 produced greater histamine levels than did *P. damselae*, *H. alvei* and *C. freundii,* at both pH 5.5 and 8.5. Histamine production was similar for the three high histamine-producing bacteria at pH 5.5, but *E. aerogenes* 18.3 produced considerably higher histamine than the other two high-histamine producers at pH 8.5. *H. alvei* 12 and *C. freundii* 4086 produced low histamine (33-66 ppm) at pH 5.5 but no histamine was detectable at pH 8.5. *P. damselae* growth was poor in TFIB and as a result, no detectable histamine was produced at pH 5.5 and 8.5. The growth of all other histamine-producing bacteria increased to 7.5-8.5 log cfu/ml within 18 hours of incubation in broth at both pH values. A rate of histamine-production at pH 5.5 was most rapid as histamine-producing bacteria reached the

stationary phase in growth. It has been previously reported that histamine production occurs only after aerobic plate counts reach 7 log cfu/ml *(96, 107)*. This observation was confirmed in this study.

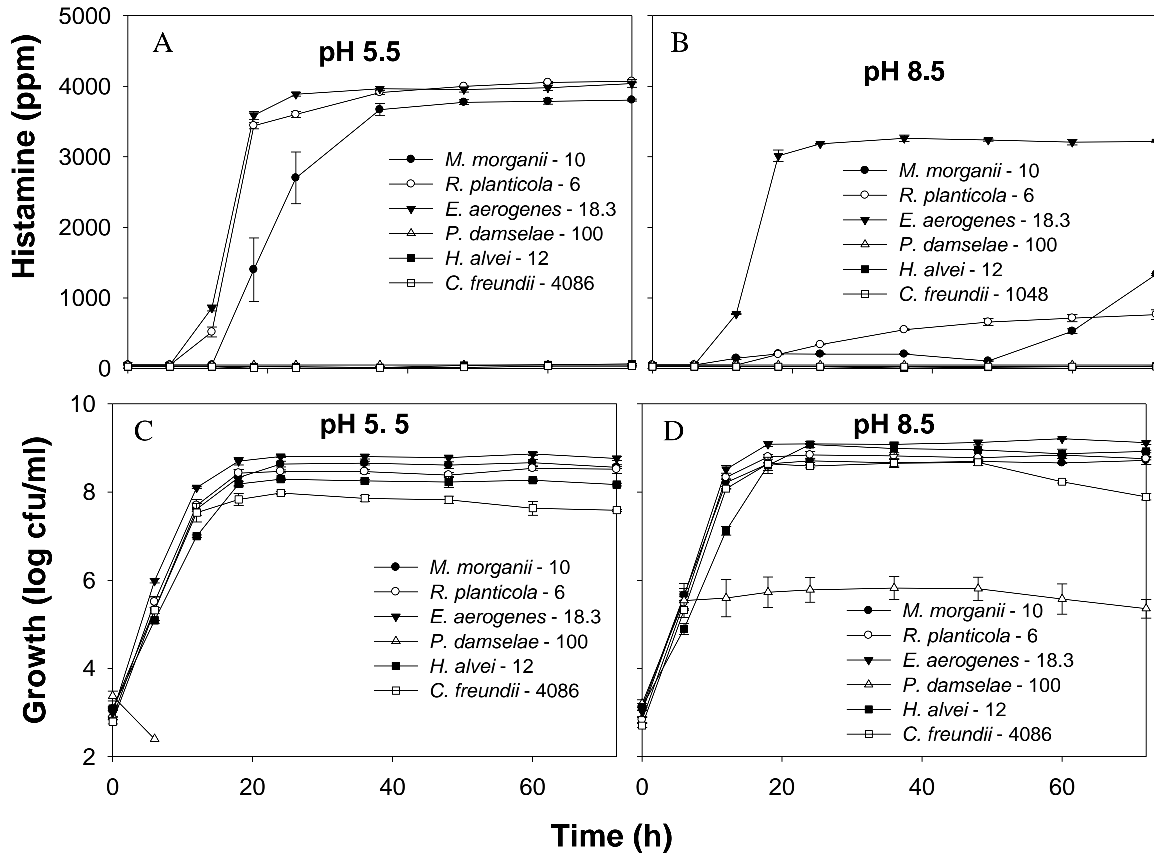


Figure 4. 4 Growth and histamine production of high- and low-histamine producing bacteria at 30˚C in TFIB at pH 5.5 (A & C) and pH 8.5 (B & D).

In this study, histamine-producing bacteria were added to TFIB at various initial pH values. During growth of these bacteria, histidine decarboxylase is formed which decarboxylases available histidine in the culture media. As CO_2 and the more basic histamine accumulate, the medium pH increases. The PLP-dependent histidine

decarboxylases from Gram-negative histamine-producing bacteria have been reported to be most active at pH 6.0-6.5 *(83, 96)*. The activity of the enzyme is greatly reduced at pH values above 8.0. Therefore, the less histamine produced at pH 8.5 may be a result of reduced activity of the histidine decarboxylase enzyme or the expression of the enzyme may have been reduced.

Histamine production was previously reported to be more pronounced at low media pH, which is in agreement with our results. Chen et al. *(35)* found that histamine production was more readily observed in media with a pH of 5.3, although maximum histamine-production was observed at pH 5.8 to 6.8. Other researchers have found that a low media pH is more effective in the detection of histamine-producing bacteria *(112, 142)*. It has been postulated that bacteria produce histamine and other biogenic amines to increase the pH of the surrounding environment as a protective mechanism against acidic environments *(113)*. Similar acid resistance systems have been reported in other important food pathogens such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Typhimurim and *Shigella flexneri (25, 45, 99, 129)*.

4.3.3 Evaluation of phosphate treatment conditions

4.3.3.1 Phosphate dipping of fish muscle

Phosphates are considered GRAS by the FDA and are widely used in the beef and poultry industry to reduce contamination levels of carcasses *(31)*. Trisodium phosphate (TSP) solutions have a pH ca. 12 which suggests that they may be used to alter the surface

pH of fish muscle. TSP was applied to mahi-mahi and tuna to determine the effect on surface pH. First, mahi-mahi samples (5x8 cm) were dipped in 0, 1%, 5%, and 10% TSP solutions for 1, 5, and 10 min at 4˚C and incubated for 6 days at 10˚C (Figure 4. 5). Storage temperature of 10˚C was used to simulate abusive conditions. Surface pH was measured at 2 day intervals with a flat surface pH electrode.

Phosphate treatment had a significant effect (ANOVA; $p < 0.000$) on surface pH of the mahi-mahi fillets. However, only the 5% and 10% TSP solutions increased muscle pH above 8.0 on day 1. Dipping in 5% and 10% TSP for 10 min produced an initial pH of 10.2 and 10.6, compared with 9.3/9.9 and 8.0/ 8.9 for 1 and 5 min dips at the same TSP concentration. The pH shifted to that of the control sample at day 2 and was not considerably different from the control for the remainder of the incubation period. Mu et al. *(96)* reported a significant increase in surface pH for 3 and 6 days for trout inoculated with *Listeria monocytogenes* and dipped in 10% and 20% TSP. Trout tissue samples were dipped for 10 min and stored for 9 days at 4˚C. Our results indicated that surface pH of the mahi-mahi fillets increased only for < 2 days of storage when dipped in 5 and 10% TSP. The difference in TSP concentrations between the two studies may be due to the type of TSP used. For our experiment, trisodiumphosphate anhydrous was used. We found that 10% of the anhydrous form precipitated during storage at 4˚C. Mu et al.*(153)* did not indicate the specific form of TSP used. With further experiments, we found that solubility of TSP anhydrous at 4˚C was between 7-8%. Therefore, based on these results, the maximum of 7% TSP was used in our experiments.

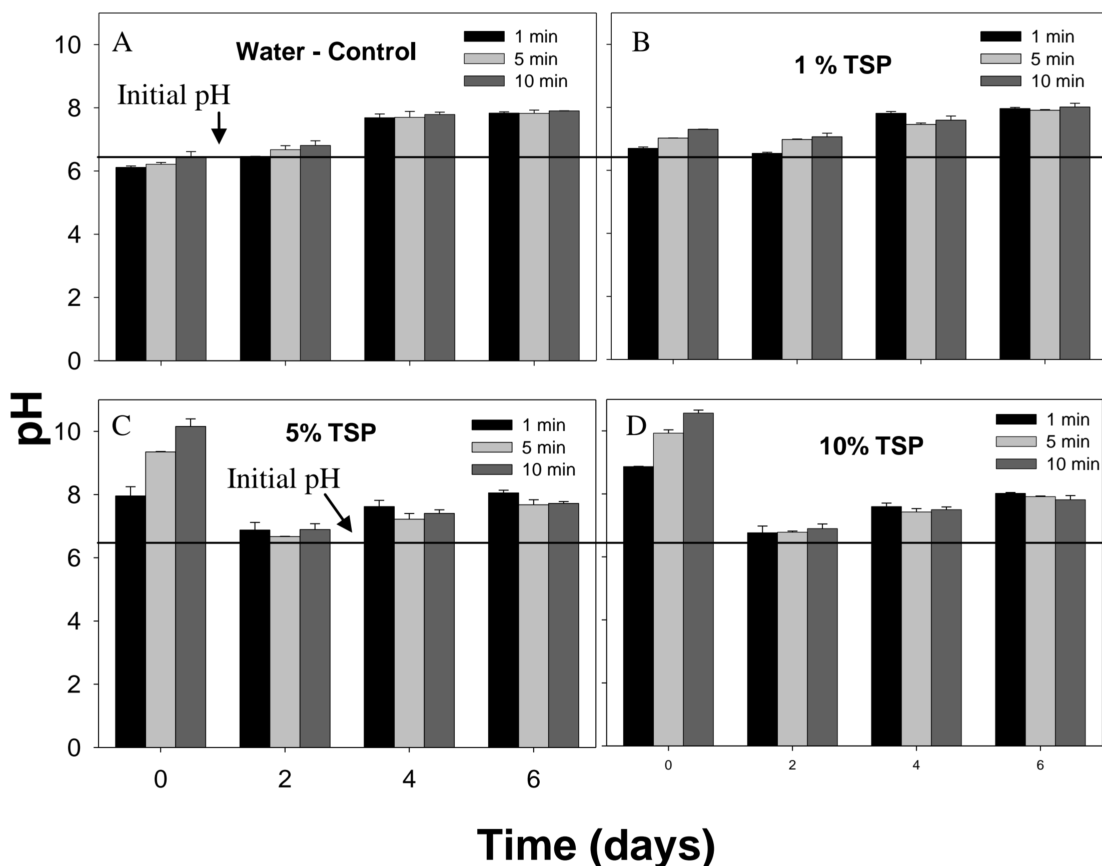


Figure 4. 5 Effect of water (A), 1% (B), 5% (C) and 10% TSP (D) dip on surface pH of mahi at different dip times (1-10 min).

4.3.3.2 Phosphate treatment in vacuum-packed fish muscle

The addition of phosphate to vacuum-packed mahi-mahi and tuna was evaluated to determine the effect on muscle pH during the storage period. First, we evaluated the effect of TSP concentrations (0, 2%, 5%, and 7% TSP) added to mahi-mahi and tuna samples (5x8 cm) vacuum-packed in oxygen permeable bags (10,000 OTR) and incubated at 10˚C for 4 days. Next we examined the effect of 7% TSP treatment by volume (5, 10, ad 15 ml) of

solution added to the vacuum-packed fish samples. Storage time of fish samples was reduced to avoid spoilage within 4 days of storage. Results showed that 15 ml of 7% TSP significantly increased surface pH during the 4 day period (data not shown). These conditions were used in subsequent experiments in which bacterial suspension were inoculated onto tissue.

4.3.4 Phosphate treatment of inoculated fish samples

4.3.4.1 Effect of phosphate treatment on surface pH

To determine the effect of phosphate on growth of histamine-producing bacteria and histamine formation, mahi-mahi and tuna were sliced into 5x8 cm samples, inoculated with a cocktail of histamine-producing bacteria and vacuum-packed in oxygen permeable bags containing 15 ml of 7% TSP or sterile deionized water. Fish samples were tested daily for surface pH (internal and external), excess liquid in bags (drip), histamine-producing bacteria and histamine formation.

External surface pH in both phosphate treated mahi-mahi and tuna samples was significantly (ANOVA; $p = 0.000$, $p = 0.000$) higher than control samples during the four days of storage (Figure 4. 6A&C). The external surface pH of the phosphate treated mahi-mahi samples did not change significantly during storage and was between pH 9.3-9.6 on day 4. The external surface pH of tuna, on the other hand, decreased significantly with storage from pH of 9.6 at day 0 to pH 7.3-7.8 at day 4, although it was significantly higher ($p = 0.047$; appendix B Table 7. 3) than the control at the end of the storage period. External pH

of the water treated mahi-mahi and tuna control samples did not change significantly and was between pH 6.1-6.7 and 5.9-6.4 throughout the storage period, respectively.

Internal surface pH of phosphate-treated mahi-mahi and tuna were significantly higher than the water treated control (ANOVA; $p = 0.000$, $p = 0.000$) during the storage period (Figure 4. 6B,D). For mahi-mahi internal pH was significantly higher than the control at day 2 and 3 (pH 7.5 and 7.6) but showed no difference at day 4 ($p = 0.008$, $p = 0.021$, $p = 0.148$; appendix B Table 7. 2). On the other hand, internal pH for phosphate-treated tuna was significantly higher than the water control at day 3 and 4 ($p = 0.001$, $p = 0.017$; appendix B Table 7. 4). Internal pH of the mahi-mahi and tuna controls did not change significantly during the storage period and were between 6.1-6.7 and 5.8-6.2 throughout (appendix Table 7. 2 & Table 7. 4). These results suggested that phosphate is taken up by the fish tissue and that not only is the external surface pH increased, but also the internal surface pH to a lesser degree.

Excess liquid (drip) in inoculated vacuum-packed fish samples was examined during storage to determine if phosphate treatment would cause increased liquid uptake. Both mahi-mahi and tuna had significantly less (ANOVA; $p = 0.000$, $p = 0.000$) drip in phosphate-treated samples than the water-treated samples during the storage period (Figure 4. 7A-B). Drip in phosphate-treated mahi-mahi was higher than in phosphate-treated tuna at day 4. For mahi-mahi, drip in phosphate-treated samples was not significantly different from the water treated control at the end of the storage period ($p = 0.424$, Appendix B Table 7. 5), whereas the phosphate-treated tuna had significantly less drip than the control ($p = 0.000$,

Appendix B Table 7. 6) at day 4. The drip in water-treated mahi-mahi also decreased significantly during the storage period, but the drip in water-treated tuna did not change significantly. The results suggest that phosphate solutions are taken up by the fish muscle which may cause increase in surface pH of the fish tissue. Phosphate had more effect on liquid uptake in the tuna muscle than in mahi-mahi.

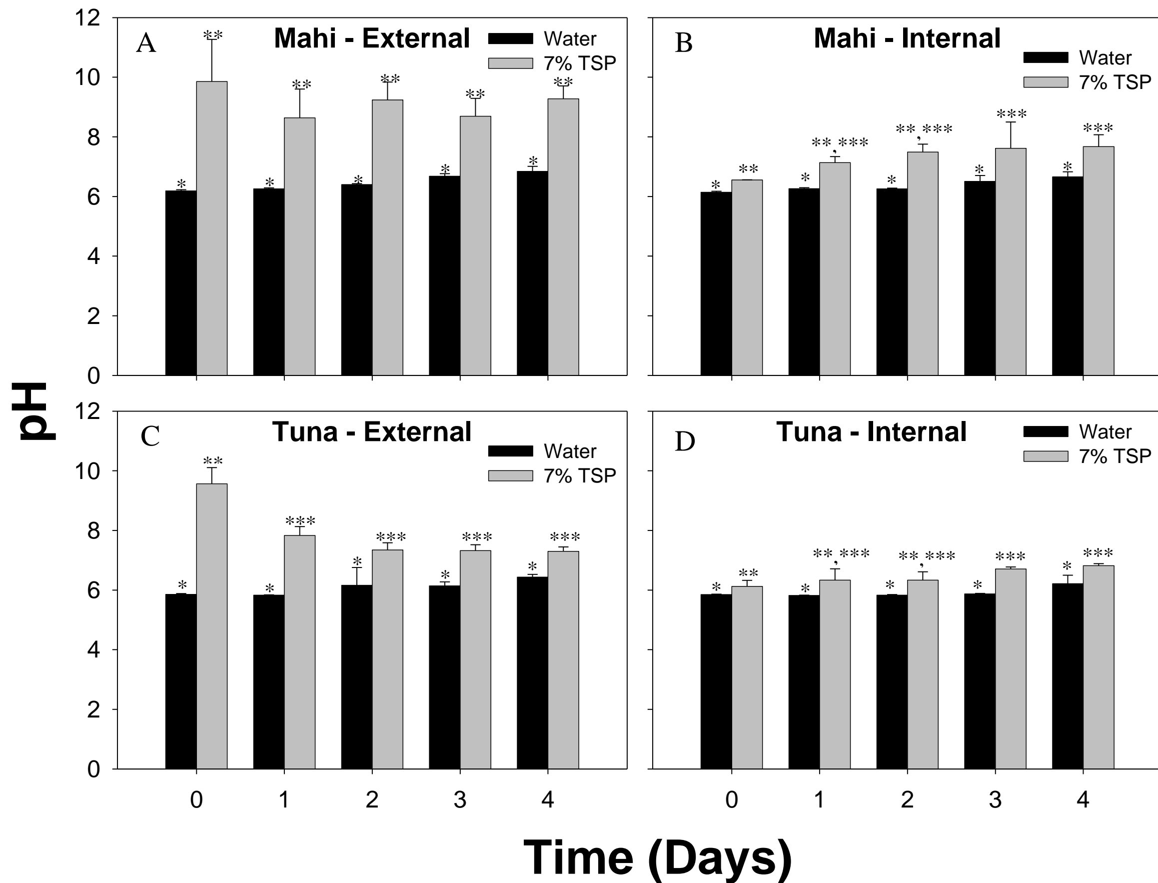


Figure 4. 6 Effect of phosphate on external and internal surface pH on vacuum-packed mahi (A & B) and tuna (C & D) inoculated with histamine-producing bacteria and stored at 10˚C. *, **, and *** indicate significant different within treatment (7% TSP or water) further statistical comparison can been seen in appendix

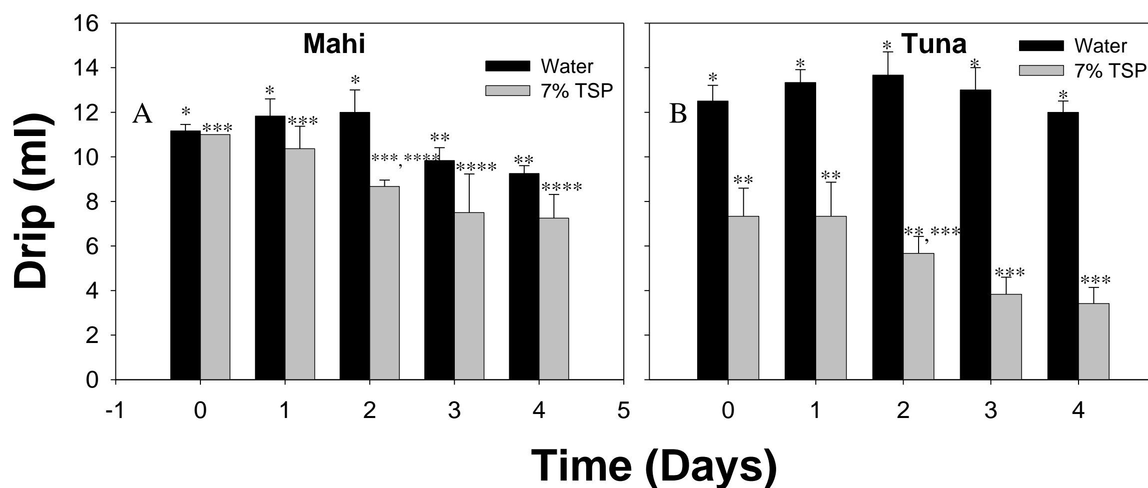


Figure 4. 7 Effect of phosphate on drip in vacuum packed mahi (A) and tuna (C) inoculated with histamine-producing bacteria and stored at 10˚C. *, **, and *** indicate significant different within treatment (7% TSP or water) further statistical comparison can been seen in appendix B

4.3.4.2 Effect of phosphate treatment on histamine content and growth of HPB

Histamine-producing bacteria produced significantly lower (ANOVA; $p = 0.001$, $p = 0.007$) histamine in phosphate-treated mahi-mahi and tuna than in the water-treated controls during storage at 10˚C for 4 days (Figure 4. 8). In addition, the histamine content of tuna samples in both water and 7% TSP was lower than the mahi-mahi samples. For mahi-mahi, histamine was detected at day 2 and increased to 166 and 609 ppm on day 4 of storage, for the phosphate-treated and water-treated samples, respectively. For tuna, on the other hand, histamine was detected at day 3 of storage but increased more rapidly to 2,205 and 3,306 ppm after 4 days of storage in phosphate-treated and water-treated samples.

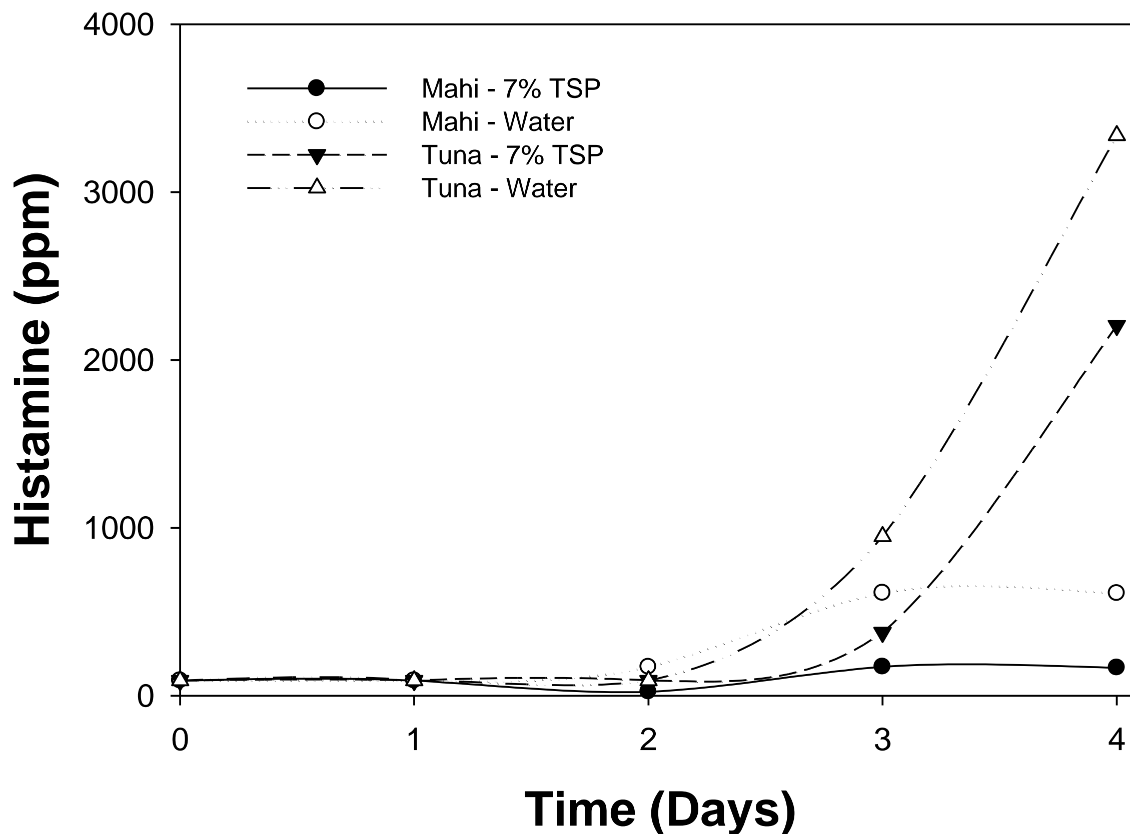


Figure 4. 8 Histamine-production in vacuum-packed mahi and tuna tissues samples with 15 ml of water or 7% TSP inoculated with histamine-producing bacteria cocktail and stored at 10˚C for 4 days.

Histamine formation is affected by the amount of free histidine in the muscle, which is a substrate for the histidine decarboxylase enzyme. Other factors that may promote histamine formation in fish muscle are autolysis or bacterial proteolysis that accelerates release of histidine from muscle tissue *(107)*. In addition, free histidine content in fish muscle varies considerably due to the differences in feeding, season, sex, and stage of maturity. Mahi-mahi contains between 4,900-9,400 ppm of free histidine *(17)*. The free

histidine in the dark of tuna muscle is reported to be 3,980-7,980 ppm and between 4,820-5,960 ppm in the white muscle *(9)*. In another study, histidine was distributed uniformly at 5,780 ppm throughout the tuna fish muscle *(213)*. In our study histamine-production was lower in mahi-mahi than tuna at the end of 4 days of storage at 10˚C. Similar to our findings, Kim et al. *(107)* found that histamine produced in mahi-mahi inoculated with *M. morganii* was lower than that of tuna and mackerel. The lower histamine produced in mahi-mahi may be related to amount of free histidine available or to some other unknown factor yet to be determined.

Growth of histamine-producing bacteria was not significantly different (ANOVA; p = 0.153) between phosphate- and water-treated mahi-mahi and tuna samples during 4 days of storage at 10˚C (Figure 4. 9). Histamine-producing bacteria increased from 5.1-5.4 log cfu/ml to 8.3-8.8 during the 4-day storage period.

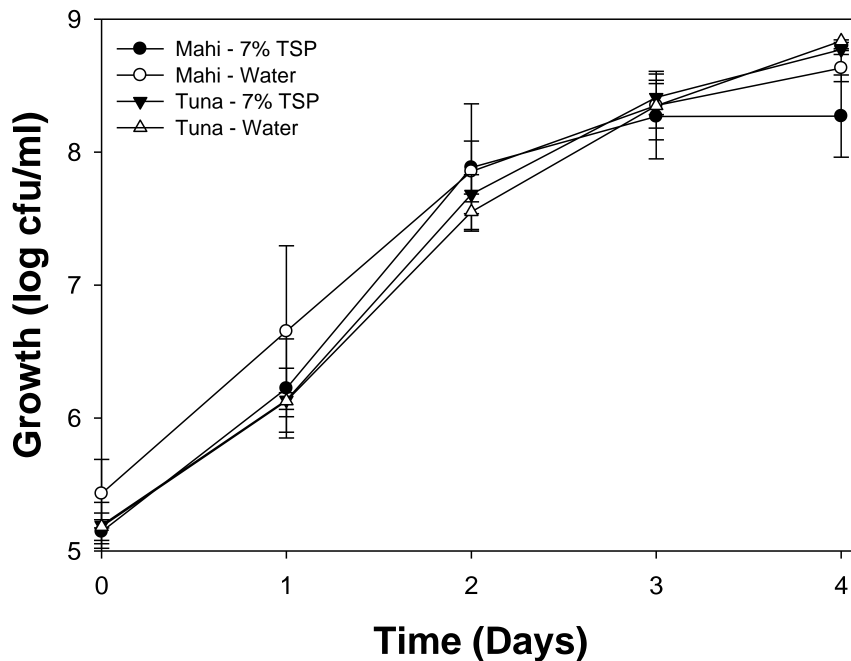


Figure 4. 9 Growth of histamine-producing bacteria inoculated on vacuum-packed mahi-mahi and tuna tissues samples with 15 ml of water or 7% TSP and stored at 10˚C for 4 days.

Phosphates are known to have antimicrobial activity against various bacteria. For instance, dipping trout fillets in 20% TSP significantly reduced psychrotrophic bacteria and *L. monocytogenes* counts throughout 6 days of storage at 4˚C *(153)*. In another study, *S.* Typhimurium was reduced by 1.5 to 2.3 log on chicken skins sprayed with 10% TSP *(203)*. In contrast to previous reported studies, we did not find a significant reduction in the number of histamine-producing bacteria after TSP treatment of mahi-mahi and tuna muscle tissues.

To our knowledge, this is the first study of its kind to investigate the relationship between on phosphate treatment and histamine formation in fish. We have shown that histamine-formation was reduced by phosphate treatment in vacuum-packed mahi-mahi and

tuna samples. This effect is most likely due to an increase in muscle pH which reduced the activity of histidine decarboxylase enzymes. Reduced histamine formation may also occur due to inhibition of the expression of histidine decarboxylase enzyme. However, the specific mechanism for reducing histamine formation needs to be studied further. Since vacuum-packing is widely used in the fish industry as a means of increasing storage life of the product, the addition of phosphate solutions to vacuum-packed mahi-mahi or tuna may help control histamine formation and increase the safety of the product. The use of pH as a control measure could be an additional hurdle to help reduce histamine-formation during chill storage of fish.

4.4 CONCLUSION

The effect of pH in fish muscle is important for bacteria growth and histamine formation. High muscle pH can significantly reduce histamine formation. This study shows that phosphate treatment of mahi-mahi and tuna fish muscle resulted in significantly reduced histamine formation over 4 days of storage at 10˚C although histamine formation was not eliminated. Phosphate treatment of fish prone to histamine formation has the potential of being used as a secondary control measure, in addition to temperature. This hurdle effect may be more effective than just temperature alone for the reduction histamine formation in scombroid fish, although this combined effect was not examined in this study.

CHAPTER 5

FUTURE RESEARCH NEEDS

Histamine fish poisoning continues to be a human health challenge despite recent FDA efforts for its control. The high incidence of histamine fish poisoning can in part be attributed to poor detection methods and lack of adequate control measures. In order to evaluate potential control strategies, rapid and reliable techniques are needed for detection and quantification of histamine-producing bacteria. Available culture-based detection techniques for histamine-producing bacteria are often slow and unreliable. Molecular-based techniques are more rapid and reliable but still have some challenges associated with their use.

Polymerase chain reaction (PCR) has become an important method for rapid, sensitive and specific detection of target genes. Several oligonucleotide primers have been developed for detection of Gram-negative histamine-producing bacteria capable of producing biogenic amines *(98, 119, 176, 194)*. However, researchers have reported variability in detection of low-histamine producing bacteria by PCR *(194)*. The variability in detection of low-histamine producing bacteria can partly be attributed to lack of histidine decarboxylase gene sequence information available for these bacteria. There is a need for better understanding of the genetic differences between high- and low-histamine producing bacteria in order to develop more universal primers. Differences in the mechanism of histamine production, gene sequence, or both needs to be investigated.

To quantitatively measure the effect of control strategies on histamine-producing bacteria, a rapid, reliable and specific enumeration technique is needed. In this study, a colony-lift hybridization method was developed for quantitative detection of high histamine-producing bacteria using combination of culture and molecular techniques (Chapter 4). This is the first attempt to quantify Gram-negative histamine-producing bacteria on the molecular level. However, probes used in this study were able to detect bacteria producing only high levels of histamine. There is a need for a universal probe that is able to detect both high and low-histamine producing bacteria.

Recently, real-time PCR technology was developed for quantitative detection of microorganisms. In these assays, fluorescent signals are generated as the PCR takes place and thus real-time monitoring during amplification is possible *(187)*. Real-time PCR methods have been developed for quantitative detection of foodborne pathogens including *Salmonella*, *Campylobacter* and Gram-positive histamine-producing bacteria *(24, 38, 117, 156)*. Quantification of Gram-negative histamine-producing bacteria by real-time PCR however, has not been developed. Development of real-time PCR for Gram-negative histamine-producing bacteria may allow quantitative detection in a few hours. The rapid detection of histamine-producing bacteria is of great importance not only for its potential health benefits, but also from an economic point of view since products exceeding recommended limits can be refused in commercial transactions.

The primary control measure for prevention of histamine fish poisoning is temperature. However, rapid chilling alone may not prevent the formation of high levels of

biogenic amines due to the growth of psychrophic histamine-producing bacteria *(86)*. Therefore, there is a need to identify hurdles other than temperature to prevent histamine formation in fish. Control of histamine formation can be achieved by: 1) inhibiting growth of histamine-producing bacteria; 2) inhibiting activity of the histidine decarboxylase enzyme; and/or 3) destruction or degradation of formed histamine.

Inhibition of histamine-forming bacteria by antimicrobials/preservatives, modified atmosphere, high pressure and irradiation has been studied. Effects of clove, sodium chloride, essential oils and chlorine on histamine formation in fish has been effective, but these substances can have undesirable effects on the appearance and taste of the product *(144, 207)*. The effect of high pressure processing (HPP) on histamine-producing bacteria was recently studied in our laboratory and we found that HPP treatment can reduce the presence of Gram-negative histamine-producing bacteria and histamine formation in pressurized fish. However, pressure treatment had some undesirable effect on the appearance and texture of the product. Irradiation and certain combinations of modified atmosphere have also been found to reduce histamine formation by reducing the levels of histamine-producing bacteria *(63, 131, 145, 146)*. Additional research is needed on these potential control strategies that may be applied alone or in combination with temperature for the control of histamine-producing bacteria.

Histidine decarboxylase enzyme activity is another area that needs further study. In, particularly, food grade inhibitors of the histidine decarboxylase enzyme are needed. Ethanol extract of cinnamon, allspice and sage can reduce the activity of Gram-negative PLP-

dependent histidine decarboxylase *(144)*. Soyuflavones, citric acid and lactic acid have been shown to inhibit the action of histidine decarboxylase from Gram-positive bacteria *(109)*. However, these substances have undesirable effects on flavor and taste of the fish. In our study we found that addition of phosphate to vacuum-packed fish significantly reduced histamine formation in tuna and mahi-mahi. This reduced histamine formation may be due to the increase in surface pH which may have affected the activity of the histidine decarboxylase enzyme. The need for food grade inhibitors of the histidine decarboxylase enzyme that does not alter taste and appearance of the fish is needed.

Degradation of histamine is yet another area that has potential for use in controlling histamine fish poisoning. Histamine can be degraded by the enzyme diamine oxidase and starter cultures containing this enzyme have been isolated. Further research on the use of diamine oxidase for use in the control of histamine in fish is needed, either by microorganisms producing the diamine oxidase enzyme or the enzyme alone.

Development of more rapid and reliable detection methods and the study of secondary control measures for histamine-producing bacteria are two important areas of future research. Future investigations should consider the use of two or more control measures in combination to reduce the risk of scombroid fish poisoning. This type of hurdle technology may prove more powerful than temperature alone in reducing the risk of fish poisoning.

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APPENDICES

APPENDIX A

DNA PROBE DEVELOPMENT

6.1 INTRODUCTION

The dominant histamine-producing bacteria in fish belong to Gram-negative enteric and marine bacteria *(119, 194)*. These bacteria produce the enzyme histidine decarboxylase which catalyses the decarboxylation of free histidine in the muscle of some fish creating toxic levels (>500 ppm) of histamine. Since histamine is generally not distributed uniformly in fish, the U.S. Food and Drug Administration (FDA) established histamine a Defect Action Level (DAL) of 50 ppm. In the European Union, this action level was set at a maximum average content of 200 ppm for fresh and canned products *(122)*. There are two distinct classes of histidine decarboxylase (HDC) enzymes: those found in Gram-negative bacteria and eukaryotic cells that require a pyridoxal-5'-phosphate (PLP) as a cofactor and those found Gram-positive cells that usually require pyruvoyl group as a prosthetic group *(194)*.

The *hdc* genes from several Gram-negative histamine-producing bacteria have been sequenced. The *Photobacterium phosphoreum hdc* gene is 1,137 – 1,140 bp in size and produces a protein product consisting of 379-380 amino acid residues *(96, 152)*, while those from *Morganella morganii*, *Raoultella planticola, Enterobacter aerogenes* and *Photobacterium damselae* are 1,131 bp and produce a protein of 377 amino acid residues *(96, 201)*. Nucleotide sequences for *M. morganii*, *E. aerogenes*, *P. damselae* and *P. phosphoreum* have been reported to show between 67% to 74% sequence similarities *(96)*.

The PLP-histidine decarboxylase enzyme has a relative molecular mass (Mr) of ~170,000 and contains four identical subunits of Mr ~43,000 *(119)*.

Several researchers have developed primers to amplify fragments of the Gram-negative PLP-dependent *hdc* gene. Kanki et al. *(98)* designed a primer set, KPF2/KPR4 based on the *Raoultella planticola* HDC protein to amplify a 724 bp fragment from the *hdc* gene. Positive PCR results were obtained from all histamine-producing *R. planticola* and *R. ornithinolytia* strains. However, these primers were not tested on other important histamine-producers such as *Morganella morganii* or *Enterobacter aerogenes*. Takahashi et al. *(194)* developed a PCR assay for the amplification of a 709 bp fragment of the *hdc* gene from Gram-negative histamine-producing bacteria. Out of 36 HPB that were tested, all but one *Citrobacter brakii* strain produced PCR product. De Las Rivas et al. *(175)* later designed a primer set that successfully amplified a 534 bp fragment of the *hdc* gene from *M. morganii*, *Proteus vulgaris*, *R. planticola* and *Photobacterium phosphoreum*. Interestingly, the amplification of the histidine decarboxylase gene from Gram-negative low histamine-producing bacteria (those producing less than <500 ppm of histidine) such as *Citrobacter freundii*, *Hafnia alvei* or *Vibrio alginolyticus* has been relatively unexplored. In fact, the decarboxylase gene from these bacteria has not been sequenced.

The purpose of this study was to develop DNA-based probes for the detection of Gram-negative histamine-producing bacteria from fish. Specifically, dioxigenin (DIG) labeled degenerate probes and PCR DIG-labeled probes were derived based on histidine

decarboxylase gene (*hdc*) sequence and examined for the detection of histamine and non-histamine producing bacteria by dot-blot hybridization.

6.2 MATERIALS and METHODS

6.2.1 Bacterial strains

The bacterial strains used in this study were obtained from multiple sources: (i) the American Type Culture Collection (ATCC, Manassas, VA, USA); (ii) isolation from fish [gills, belly cavities and muscle tissues of yellowfin tuna (*Thunnus albacares*), mahi-mahi (*Coryphaena hippurus*)]; and (iii) from environmental samples collected in North Carolina.

Media components were obtained from Becton Dickinson (BD; Franklin Lakes, NJ) unless otherwise specified. Surface swabs (32 cm^2) or tissue samples (5g) were enriched in 1% bacto proteose peptone, 0.3 % yeast extract (EMD, Gibbstown, NJ), 1.5% NaCl (EMD, Gibbstown, NJ) and 0.5% histidine (L-histidine hydrochloride monohydrate; Acros, Morris Plains, NJ) at 37˚C for 24 h. Enrichment cultures were incubated (1ml) in 9 ml trypticase soy broth (TSB) containing 2% histidine, 2% NaCl and 0.0005% pyridoxal-HCl (TSB+; Alexis, Plymouth Meeting, PA; pH 5.8) for 24 h at 37˚C. Histamine formation was confirmed using Neogen's Veratox histamine test kit (Neogen, Lansing, MI). Positive samples for histamine production were serially diluted in saline (0.85% NaCl) and spread plated on trypticase soy agar containing 2% NaCl ($TSAN_2$). Ten representative colonies were streaked on Niven's agar *(158)* with modifications suggested by Mavromatis and Quanticket *(142)* and incubated at 30˚C for 48 hours. Niven's positive strains were inoculated in 9 ml TSB+ for 48 h at 37˚C and histamine formation confirmed with Neogen's

Veratox histamine test kit. Histamine-positive isolates were identified based on cell morphology, gram staining, and by the Enteric and Nonfermenter BBL crystal identification test kith (Becton Dickinson, Franklin Lakes, NJ). Bacterial identities were confirmed by 16S ribosomal DNA sequencing (Davis, Carlsbad, CA).

6.2.2 DNA extraction

Culture strains were inoculated in 5 ml $TSAN_2$ for 24-48 hours at 37˚C and DNA isolated using UltraClean™ Microbial DNA Isolation Kit (MoBio, CA, USA) and/or CTAB with isopropanol precipitation *(209)*. Absorbance was recorded at 260 and 280 nm (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) in a 96-well quartz plate and stock solutions diluted to 10 ng/µl in Tris-EDTA (TE) buffer (pH 8.0). DNA concentration and purity was calculated using standard formulae.

6.2.3 Identification by 16S rDNA sequencing

A 1.5 Kb fragment was amplified from the 16S rDNA gene using the universal primers 16S39F (5'–TGGCTCAGRWYGAACGCTRG-3') and 16S1389R (5'–ACGGGCGGTGTGTACAAG-3'). Amplification was performed in 50 µl reactions that included: 2 U Taq polymerase (Fisher, Pittsburg, PA), 2.5 µM each primer (Sigma-Genosys, St. Louis, MA), 3.5 µM $MgCl_2$, 200 µM each nucleotide (Fisher, Pittsburg, PA), 10 µl 10x reaction buffer (500 mM KCL, 100 mM Tris-HCl, pH 9.0) and 2 µl sample DNA. Amplifications were carried out for 25 cycles (94˚C for 1 min, 59˚C for 1 min, and 72˚C for

1 min) in a GTC-2 thermal cycler (Precision Scientific, Chicago, IL). PCR products were purified using PCR Purification Kit (Qiagen, Valencia , CA) and separated on a 1% agarose gel at 86V in 1x TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) for 1 h. Gels were stained with ethidium bromide (0.3 µg/ml) and visualized using a UV transilluminator (UVP, Upland, CA). Product size and concentration was confirmed using Low DNA Mass Ladder (Invitrogen, Carlsbad, CA). Purified PCR products were diluted in sterile distilled water as to obtain 280 ng/µl. Sequencing of the amplified 16S rDNA fragment was performed by Davis sequencing (Davis, CA, USA). Both forward and reveser strands were sequenced using the16S39F and 16S1389R primers. Identification was performed by comparing the sequences to known sequences by BLAST search in GeneBank (http://www.ncbi.nlm.nih.gov/).

6.2.4 Amplification of the *hdc* gene

Two fragments, 709 bp and 249 bp, of the *hdc* gene were amplified using three sets of primers (Table 6. 1). The 709 fragment corresponding to the base pair position 249 to 958 of the *hdc* gene from *M. morganii* (GenBank accession number J02577) was amplified using primers developed by Takahashi et al. *(194)*, *hdc*-f (5'-TCH ATY ARY AAC TGY GGT GAC TGG RG-3') and *hdc*-r (5'-CCC ACA KCA TBA RWG GDG TRT GRC C-3'). The 249 bp fragment corresponding to base pair position 709 to 958 was amplified using the *hdc*-r primer developed by Takahashi et al. *(194)* along with *hdc*2-f (5'-AYG CBG AYG CSG

CDC TRA GYG GHA TGA-3') primer developed by alignment of the 17 *hdc* genes cloned from *M. morganii*, *P. damselae*, *E. aerogenes*, and *Raoultella spp.*. (section 3.2.4).

Amplifications were performed in 50 µl reactions that included: 25 µl PCR master mix (50 units/ml Taq DNA polymerase, 400µM of each of the four deoxynucleoside triphosphate, reaction buffers, 3 mM $MgCl_2$, pH 8.5 (all provided by Promega Corp., Madison, WI)), 75 pmol of each primer and 20 ng DNA template. Amplifications were carried out for 40 cycles (94˚C for 1 min, 52˚C or 54˚C for 1 min, and 72˚C for 1 min) in a GTC-2 thermal cycler (Precision Scientific, Chicago, IL). PCR products were separated on a 1% agarose gel at 86V in 1x TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) for 1 h. Gels were stained with ethidium bromide (0.3 µg/ml) and visualized using a UV transilluminator (UVP, Upland, CA). Product size was confirmed using 100 bp molecular weight markers (Invitrogen, Carlsbad, CA).

Table 6. 1 Strains cloned and target amplification products of the *hdc* gene.

		Target amplification product			
Strain	**Species**	**249 bp**	**709 bp**	**Cloned**	**Source**
10	*Morganella morganii*	x	x	x	ATCC35200
11	*Morganella morganii*		x	x	ATCC25830
BR-D5-119	*Morganella morganii*		x	x	Mahi Gills/Gut
BO-S5-255	*Morganella morganii*		x	x	Mahi Gills/Gut
BO-S4-249	*Morganella morganii*		x	x	Mahi Gills/Gut
HPP-S12-301	*Morganella morganii*		x	x	Mahi Gills/Gut
HPP-S13-309	*Morganella morganii*		x	x	Mahi Gills/Gut
6	*Raoultella planticola*	x	x	x	ATCC43174
HPP-T15	*Raoultella ornithinolytica*		x	x	Tuna Muscle
HPP-T19	*Raoultella ornithinolytica*		x	x	Tuna Muscle
13	*Enterobacter aerogenes*	x	x	x	ATCC13048
HPP-T14	*Enterobacter aerogenes*		x	x	Tuna Muscle

Table 6.1 continued

BR-D1-100	*Photobacterium damselae*	x	x	x	Mahi Gills/Gut
BR-D2-107	*Photobacterium damselae*		x	x	Mahi Gills/Gut
BR-D6-132	*Photobacterium damselae*		x	x	Mahi Gills/Gut
BR-D9-147	*Photobacterium damselae*		x	x	Mahi Gills/Gut
BR-T1-165	*Photobacterium damselae*		x	x	Tuna Gills/Gut
FT 534	*Shewanella putrefaciens*	Neg. control			Tuna Gills

6.2.5 *hdc* cloning and sequencing

Cloning of the 709 bp PCR fragment of the *hdc* gene was performed using pCR2.1 TA cloning kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. From each cloned library 10 representative colonies from X-Gal plates were examined for inserts by restriction analysis and by PCR. Plasmids were purified using PureLinke HQ Mini Plasmid Purification kit (Invitrogen, Carlsbad, CA). Restriction digest was performed on plasmids using the restriction enzyme *Eco*RI (Invitrogen, Carlsbad, CA). The reaction mixture consisted of 5 µl plasmid, 1 µl 10X reaction buffer (Invitrogen, Carlsbad, CA) and 3 µl of sterile deionized water. Restriction endonuclease (1 µl) was added to the reaction mixture following incubation for 1 h at 37˚C. The reaction was stopped by addition of 2.5 µl 10X loading buffer. The restriction digest was separated with 100 bp molecular marker (Invitrogen, Carlsbad, CA) in 1% agarose gel electrophoresis at 86V in 1x TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) for 1 hour. Amplification of the *hdc* from the plasmid was performed as described earlier using primers *hdc*-f and *hdc*-r developed by Takashahi et al. *(194)*. Purified plasmids were sequenced in both directions by Davis Sequencing utilizing the inbuilt M13 priming sites by the reverse primer M13R (5'-TCA CAC AGG AAA CAG CTA TGA C-3') and forward primer M13 (-20; 5'-TGT AAA ACG ACG GCC AGT-3').

Clones were incubated in LB containing 50 µl/ml kanamycin for 24 hours. The inoculated clones (0.85 ml) were mixed with 0.15 ml glycerol and stored at -80˚C.

6.2.6 Restriction digest and southern blots

PCR products of *M. morganii* (ATCC 35200), *R. planticola* (ATCC 43176), *E. aerogenes* (ATCC 13048) and *P. damselae* (BR 100) were digested with KpnI and SauA3I (Invitrogen, Carlsbad, CA). The reaction mixture consisted of 5 µl of PCR product, 1 µl of 10X reaction buffer (Invitrogen, Carlsbad, CA) and 3 µl of sterile deionized water. Restriction endonuclease (1 µl) was added to the reaction mixture and incubated for 1 h at 37˚C. The reaction was stopped by adding 2.5 µl of 10X loading buffer and loaded on 1.75% agarose gel. Restriction digests reactions were separated using 100 bp molecular weight markers (Invitrogen, Carlsbad, CA) by gel electrophoresis at 86V in 1x TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) for 1 h. After electronic separation the DNA was denatured in 500 ml solution A (1.5 M NaCl, 0.5M NaOH) twice for 30 min with periodic agitation. The gel was neutralized twice in solution B (2M ammonium acetate, 0.4M NaOH) for 30 min with periodical agitation. The digested PCR product was transferred by capillary action with solution B to a positive charged nylon membrane. Hybridization of the southern blot was performed as described below.

6.2.7 DNA dot-blot

DNA dot-blots were prepared using a 96-well Bio-Dot Microfiltration apparatus (Biorad, Hercules, CA). A positively charged nylon membrane (Roche, Indianapolis, IN) was equilibrated in sterile distilled water for 10 min before being applied to the dot-blot apparatus. The membrane in the dot-blot apparatus was pre-washed with 500 µl of sterile distilled water by vacuum filtration prior to DNA addition. Aliquots (2 µl) of purified DNA at a standardized concentration of 10 ng/µl were denatured by the addition of 1M NaOH and 200 mM EDTA (pH 8.2) solution to a final concentration of 0.4 M NaOH/10 mM and heated in at 100˚C for 10 min. Denatured DNA solutions were briefly centrifuged and placed on ice until application. The 200 µl denatured DNA sample was applied to the positively charged nylon membrane in the dot-blot apparatus by vacuum filtration and then washed with 500 µl 0.4 M NaOH by vacuum filtration. The DNA dot-blot membrane was then removed from the dot-blot apparatus, rinsed in 2x SSC, air dried and cross-linked using a UV-crosslinker (UVP, Upland, CA).

6.2.8 Hybridization

Membranes were prehybridized in Dioxigenin (DIG) Easy Hyb solution (Roche, Indianapolis, IN) at 40˚C for 1-2 hours. DNA probes (709 bp and 249 bp) were dioxigenin (DIG)-labeled using the PCR DIG Probe Synthesis Kit (Roche, Indianapolis, IN) and PCR conditions described above. Labeled probes derived from *M. morganii* (ATCC 35200), *R. planticola* (ATCC 43176), *E. aerogenes* (ATCC 13048), and *P. damselae* (BR 100) were

produced separately. Hybridization were done using both individual probes and an equal mixture (1:1:1:1) of the probes. Probes were obtained from Integrated DNA technologies (Coralville, IA) and labeled with DIG Oligonucleotide 3´ End Labeling Kit or DIG Oligonucleotide Tailing Kit (Roche, Indianapolis, IN). The prehybridization solution was replaced with hybridization solution (DIG Easy Hyb) containing 0.5, 1, or 2 µl/ml DIG PCR-labeled or 5, 15 or 18 pmol/ml degenerate *hdc* probe and the membranes incubated overnight at 37-42˚C. The membranes were washed twice in 2X SSC/0.1% SDS at room temperature (RT) for 5 min (first wash) and twice again in 0.1X - 0.5X SSC/0.1% SDS at 55-80˚C for 15 min for the PCR labeled probes; this second wash was done twice in 3.0 M Me_4NCl (Sigma) for 15 min for the degenerate probes. After a brief wash in washing buffer (Roche, Indianapolis, IN), the membranes were blocked in maleic acid buffer (100 mM Maleic acid/150 mM NaCl, pH 7.5) containing 1X blocking reagent (Roche, Indianapolis, IN) for 30 min at RT and in the same buffer containing 1:5000 of anti-DIG-alkaline phosphatase Fab fragments (Roche) for 30 min. After washing twice in washing buffer (Roche) for 15 min, membranes were developed in 20 µl/ml of NBT/BCIP (Roche, Indianapolis, IN) in detection buffer (0.1 M Tris-HCl/0.1M NaCl, pH 9.5).

6.3 RESULTS AND DISCUSSION

6.3.1 16S rDNA identification

Histamine-producing bacteria isolated from fish were identified using the BBL Crystal identification system. Results from the BBL Crystal ID system were then verified

by sequencing of the 16S ribosomal gene (Table 6. 2). Identification by the BBL crystal identification system agreed with the 16S rDNA sequences for 5 *Morganella morganii*, 1 *Enterobacter aerogenes*, 5 *Photobacterium damselae* and 8 *Citrobacter freundii* strains. However, two histamine-producing strains (HPP-T15 and HPP-T19) identified as *Klebsiella oxytoca* by the BBL crystal identification system were identified as *Raoultella ornithinolytica* by 16S rDNA sequencing.

Raoultella planticola was previously reported to be misidentified as *Klebsiella oxytoca* by conventional methods used for identification of histamine-producing bacteria (HPB). Kanki et al. *(98)* reported that 48 strains of *R. planticola* were misidentified as *K. pneumonia* or *K. oxytoca* by the API 20E system. In fact, the same researchers found that 18 strains of *K. oxytoca* produced no histamine when incubated in TSB containing 1% histidine (pH 5.8) at 30˚C for 18 h. In addition, the same strains did not generate a positive PCR product for the histidine decarboxylase gene. *R. planticola* cannot easily be distinguished from *K. oxytoca*, because *R. planticola* is not included in the database of the identification systems. In fact, *R. planticola* is not present in the data base of the BBL Crystal Identification system.

These results indicated that identification of *M. morganii*, *E. aerogenes* and *P. damselae* by the BBL system is equal to identification by the 16S rDNA sequence. However, identification of *K. oxytoca* must be confirmed by sequencing of the 16S rDNA or other equivalent methods.

Table 6. 2 Identification of histamine-producing bacteria by BBL crystal identification system and by 16Sr DNA sequencing.

Strain	BBL Crystal (Confidence factor)	16S rDNA sequencing (% Identity)	Source
BR-D5-119	*Morganella morganii (0.8969)*	*Morganella morganii* (100%)	Mahi Gills/Gut
BO-S5-255	*Morganella morganii* (0.9955)	*Morganella morganii* (99%)	Mahi Gills/Gut
BO-S4-249	*Morganella morganii* (0.9955)	*Morganella morganii* (99%)	Mahi Gills/Gut
HPP-S12-301	*Morganella morganii (0.8969)*	*Morganella morganii* (99%)	Mahi Gills/Gut
HPP-S13-309	*Morganella morganii* (0.9955)	*Morganella morganii* (99%)	Mahi Gills/Gut
HPP-T15	*Klebsiella oxytoca* (0.9715)	*Raoultella ornithinolytica* (99%)	Tuna Muscle
HPP-T19	*Klebsiella oxytoca* (0.9715)	*Raoultella ornithinolytica* (100%)	Tuna Muscle
HPP-T14	*Enterobacter aerogenes* (0.9783)	*Enterobacter aerogenes* (100%)	Tuna Muscle
BR-D1-100	*Photobacterium damselae* (0.999)	*Photobacterium damselae* (99%)	Mahi Gills/Gut
BR-D2-107	*Photobacterium damselae* (0.9956)	*Photobacterium damselae* (99%)	Mahi Gills/Gut
BR-D6-132	*Photobacterium damselae* (0.9986)	*Photobacterium damselae* (100%)	Mahi Gills/Gut
BR-D9-147	*Photobacterium damselae* (0.9956)	*Photobacterium damselae* (100%)	Mahi Gills/Gut
BR-T1-165	*Photobacterium damselae* (0.9998)	*Photobacterium damselae* (100%)	Tuna Gills/Gut
BR-D5-121	*Citrobacter freundii* (0.9986)	*Citrobacter freundii* (100%)	Mahi Gills/Gut
BR-D6-129	*Citrobacter freundii* (0.9985)	*Citrobacter freundii* (100%)	Mahi Gills/Gut
BO-S3-240	*Citrobacter freundii* (0.9933)	*Citrobacter freundii* (99%)	Mahi Gills/Gut
BO-S5-251	*Citrobacter freundii* (0.9988)	*Citrobacter freundii* (99%)	Mahi Gills/Gut
BO-S10-201	*Citrobacter freundii* (0.991)	*Citrobacter freundii* (90%)	Mahi Gills/Gut
BO-F1-222	*Citrobacter freundii* (0.9944)	*Citrobacter freundii* (99%)	Mahi Gills/Gut
BO-F4-216	*Citrobacter freundii* (0.991)	*Citrobacter freundii* (99%)	Mahi Gills/Gut
HPP-S12-304	*Citrobacter freundii* (0.9997)	*Citrobacter freundii* (99%)	Mahi Gills/Gut

6.3.2 *hdc* gene nucleoide sequences

Partial *hdc* genes from 17 high-histamine producing bacteria were cloned, sequenced and submitted to GenBank under the following accession nutmbers: *M. morganii* strain ATCC 35200 (10), FJ469557; *M. morganii* strain ATCC 25830 (11), FJ469558; *M. morganii* strain BR119, FJ469559; *M. morganii* strain BO249, FJ469560; *M. morganii* strain BO255, FJ469561; *M. morganii* strain HPP301, FJ469562; *M. morganii* strain

HPP309, FJ469563; *R. planticola* strain ATCC 43176 (6), FJ469564; *R. ornithinolytica* strain HPP15, FJ469565; *R. ornithinolytica* strain HPP19, FJ469566; *E. aerogenes* strain ATCC 13048 (13), FJ469567; *E. aerogenes* strain HPP14, FJ469568; *P. damselae* strain BR100, FJ469569; *P. damselae* strain 107, FJ469570; *P. damselae* strain BR132, FJ469571; *P. damselae* strain BR147, FJ469572; *P. damselae* strain BR165, FJ469573.

The *hdc* gene from the seven *M. morganii* strains exhibited between 96-99% sequences similarity. The three *Raoultella spp*. and the two *E. aerogenes hdc* genes exhibited between 87-99% and 98% sequence similarity, respectively. Finally, the five *P. damselae hdc* genes exhibited 99% sequence identity. When comparing the nucleic acid sequence identities between the four HPB species (*M. morganii*, *R. planticola*, *E. aerogenes* and *P. damselae*), similarities ranged from 73-78%. Similarly, Takahashi et al. *(194)* reported between 74-99% sequence similarity for the 709-bp *hdc* fragments of *M. morganii*, *R. planticola*, *P. vulgaris*, *Erwinia* sp. *Photobacterium damselae*, and *P. phosphoreum*. In addition Kanki et al. *(96)* found that the nucleic acid sequence similarities from three high-HPB, *M. morganii, R. planticola* and *P. damselae*, ranged from 67%-74%.

The high sequence similarity of the *hdc* genes far exceeds the accepted relatedness among genera of the *Enterobacteriaceae*. While *Enterobacter* and *Raoultella* have 30-60% DNA relatedness, *Morganella* is more distantly related. Assuming a common parental gene for these *hdc* genes, there appears to have been few additions or deletions of nucleotides during evolution *(119)*. Despite the high degree of sequence similarity, the corresponding enzymes from the native hosts differ sufficiently enough such that polyclonal antisera against

the *M. morganii* HDC protein does not cross-react with the corresponding enzymes from *E. aerogenes* and *R. planticola (95, 119).*

6.3.3 Southern blot of restriction digests

In order to identify a location on the *hdc* gene that would be suitable for probe development Southern blots were performed on restriction digests of 709 bp amplification products of the *hd* gene using the restriction enzymes KpnI and Sau3AI. The PCR amplification products from *M. morganii* 10, *R. planticola* 6, *E. aerogenes* 13 and *P. damselae* 100 were digested with KpnI and/or SauA3I, separated by gel electrophoresis and Southern blots were subsequently performed using DIG DNA probes generated from each strain.

Restriction map analysis showed that KpnI cut the 709 bp *hdc* gene fragment from *M. morganii* 10 at two sides (141 bp and 369 bp) and Sau3A1 at three (309, 350 and 485 bp; Figure 6. 1). *R. planticola hdc* partial gene was cut by KpnI once at the 141 bp and by Sau3A1 three time at 485, 618, 623 bp. The *hdc* partial genes from *E. aerogenes* 13 and *P. damselae* 100 were not digested with KpnI; restriction digestion from the *hdc* gene of these two strains was thus not performed. The *hdc* gene from these two strains were cut at six (314, 434, 485, 504, 566 and 635 bp) and four (309, 323, 414, and 623 bp) sites by the restriction enzyme Sau3A1.

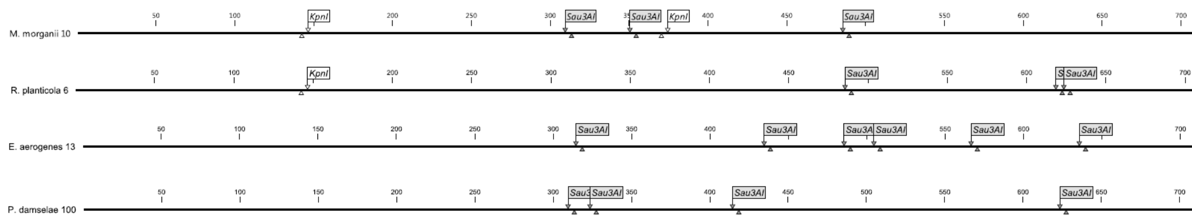

Figure 6. 1 Restriction digest map of KpnI and SauA31 for the *hdc* gene of *M. morganii* 10, *R. planticola* 6, *E. aerogenes* 13 and *P. damselae* 100. Map obtained from CLC Sequence Viewer (Massachusetts, USA)

When the *hdc* gene fragment from *R. planticola* 6 was cut with KpnI, all three PCR DIG-labeled probes from *M. morganii* 10, *R. planticola* 6 and *E. aerogenes* 13 detected the two restriction fragments (Figure 6. 2, Figure 6. 3, & Figure 6. 4). Strong signals were obtained for the three *hdc* fragments from the *M. morganii hdc* partial gene digested with KpnI and hybridized with the *hdc*-probe generated from *M. morganii* 10 (Figure 6. 2). However, when these fragments were hybridized with *hdc*-probes generated from *R. planticola* and *E. aerogenes*, the strongest signal was obtained from the 369 bp fragment at the end of the gene (Figure 6. 3 & Figure 6. 4). The 141 bp fragment at the beginning of the 709 bp partial *hdc* from the restriction digest of KpnI on *M. morganii* 10 *hdc* gene also gave strong signal when hybridized with the *hdc* probe generated from *E. aerogenes* 13 (Figure 6. 4).

When Sau3AI restriction digest of *R. planticola* were hybridized with the *hdc*-probe from *M. morganii* 10, strong hybridization singals were obtained for all 4 fragments, the strongest being that associated with the 138 bp fragment at the end of the gene. When the same restriction digest was hybridized with the *hdc*-probe from *E. aerogenes* the strongest signal was observed for a 485 bp fragment at the beginning of the gene. However, when

hdc-probe from *P. damselae* was used the whole lane was detected indicating that either too much probe or DNA was used. When the Sau3AI digest of *hdc* gene from *M. morganii* was hybridized with *hdc*-probe generated from *M. morganii* signals were strong for all fragments (Figure 6. 2). However, when the same digest was hybridized with *hdc*-probes generated from *E. aerogenes* 13 and *R. planticola* 6, two restriction fragments gave strong detection signals, i.e., a 309 bp fragment at the beginning of the gene and a 224 bp fragment at the end of the gene (Figure 6. 3, Figure 6. 4). Hybridization signals for 7 fragments from restriction digestion of the *hdc* gene from *E. aerogenes* was strong when probes from *E. aerogenes* and *R. planticola* were used (Figure 6. 3, Figure 6. 4), but somewhat weaker when the probe from *M. morganii* was used. The strongest signal was observed for a 314 bp fragment at the beginning of the partial gene and a smaller fragment (<200 bp) at the end of the gene. Hybridization of the Sau3AI restriction digest of the *hdc* from *P. damselae* was weak when probes from *M. morganii* and *E. aerogenes* (Figure 6. 2, Figure 6. 3) were used but strong when probe from *R. planticola* was used (Figure 6. 4). The hybridization signal was strongest for a small fragment at the beginning of the gene (<200 bp) when probe from *R. planticola* was used (Figure 6. 4)

The results from southern blots on restriction digests of the partial *hdc* gene using the enzymes KpnI and SauA3I did not give a clear indication of a specific area of sequence similarity for use in designing a specific probe. However, some southern blots showed stronger signals at either the beginning or the end of the 709 bp *hdc* partial gene.

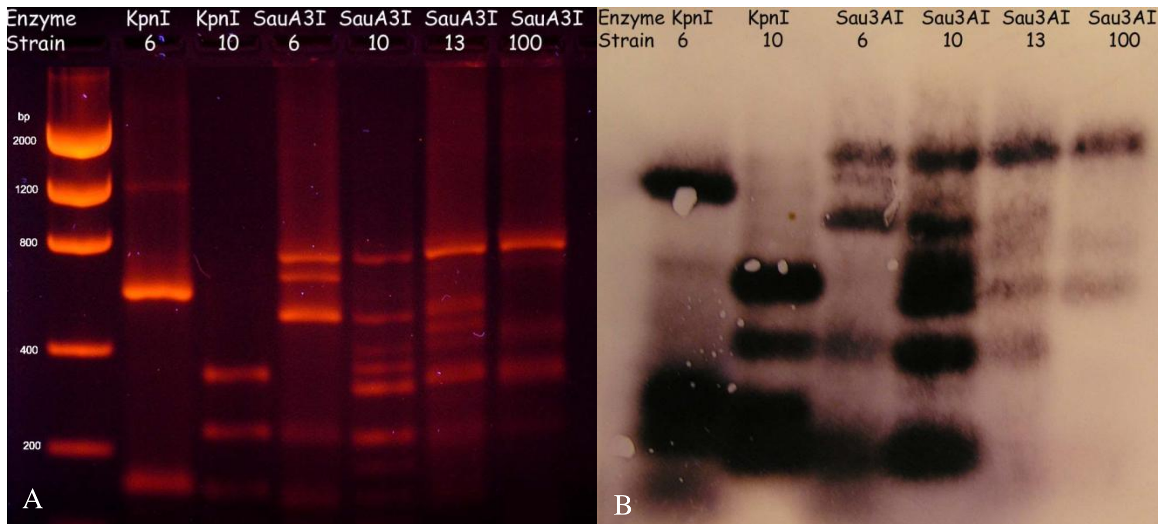

Figure 6. 2 Restriction digest (A) and southern blot (B) from restriction digest using PCR labeled DIG probe from *M. morganii* 10.

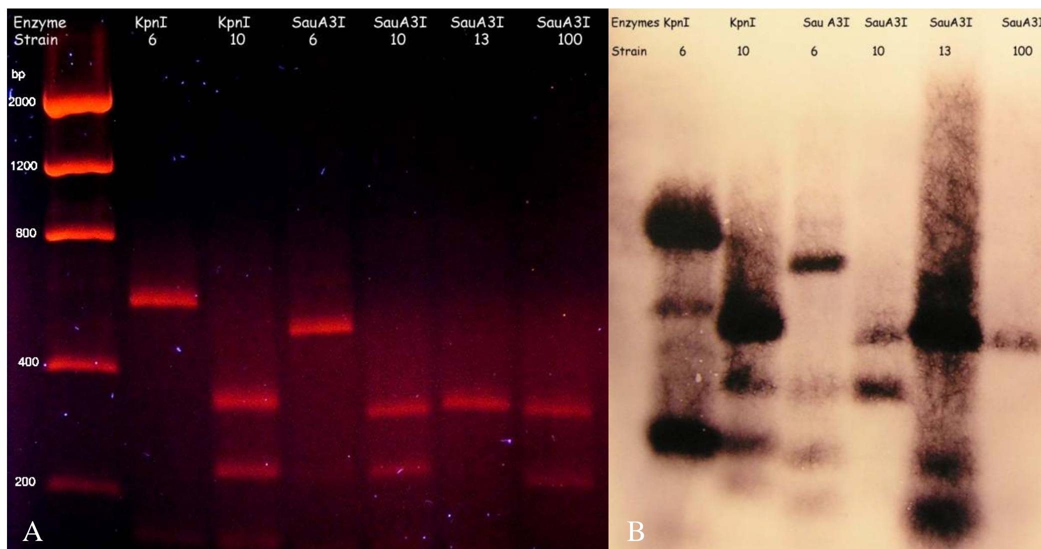

Figure 6. 3 Restriction digest (A) and southern blot (B) from restriction digest using PCR labeled DIG probe from *E. aerogenes* 13.

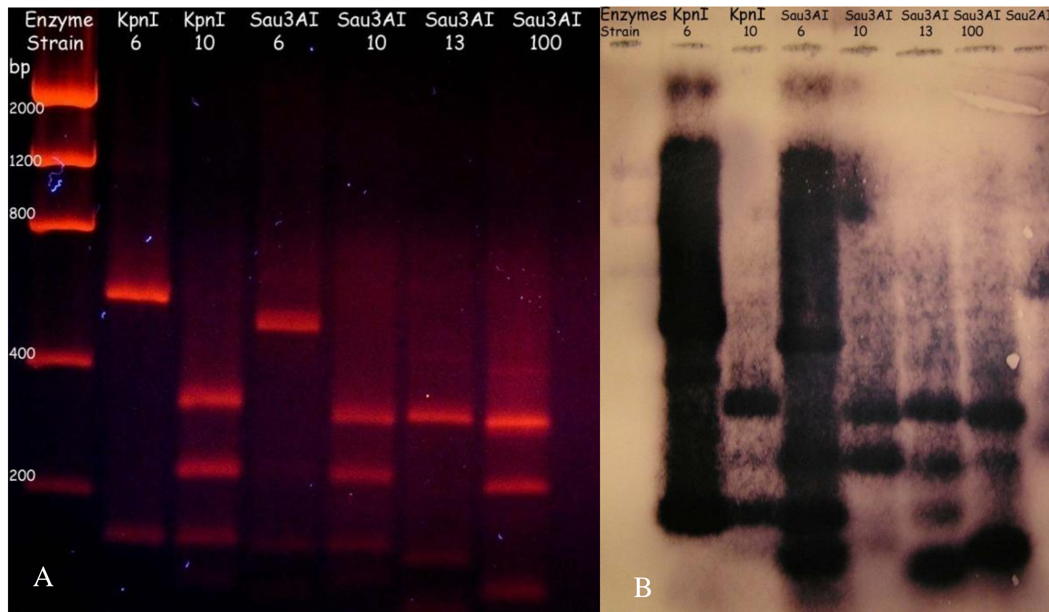

Figure 6. 4 Restriction digest (A) and southern blot (B) from restriction digest using PCR labeled DIG probe from *R. planticola 6*.

6.3.4 PCR labeled DIG probes

Ideally, one DNA probe for the histidine decarobyxlase (*hdc*) gene would be used for the detection of all histamine-producing bacteria. Our first approach was to see if a dioxigenine (DIG)-labeled DNA probe from *M. morganii* could be used to detect all histamine-producing bacteria. *Morganella morganii* has been reported to be the main contributor to histamine accumulation in fish because of its capability to form high levels of histamine *(104, 106)*. In addition, the nucleotide sequence from the *hdc* gene of *M. morganii* had 67% to 75% similarity to other high-histamine producing bacteria *(96, 119)*. DNA dot-blots were performed using DNA from various histamine and non-histamine

producing bacteria (Table 6. 3) and PCR DIG-labeled probe from *M. morganii* 10 was examined for the detection of these bacteria under various wash and hybridization conditions.

Table 6. 3 Strains and their histamine-production

Strain ID	Strain	Histamine (ppm)*
10	*Morganella morganii*	3750
11	*Morganella morganii*	5807
255	*Morganella morganii*	4890
6	*Raoultella planticola*	6143
15	*Raoultella ornithinolytica*	5789
13	*Enterobacter aerogenes*	7779
14	*Enterobacter aerogenes*	6743
100	*Photobacterium damselae*	6157
107	*Photobacterium damselae*	2612
12	*Hafnia alvei*	*204*
9	*Vibrio alginolyticus*	*<125*
113	*Citrobacter freundii*	*<125*
121	*Citrobacter freundii*	*<125*
129	*Citrobacter freundii*	*<125*
EC 25	*Escherichia coli*	*<125*
EC 96	*Escherichia coli*	*<125*
PA	*Pseudomonas aeruginosa*	*<125*
3T5005	*Enterococcus casseliflavus*	*<125*
2M12005	*Empedobacter brevis*	*<125*

*Histamine detected by HPLC after 48 h incubation in TSB supplemented 1% histidine, 2% NaCl, and 0.0005% pyridoxal-HCl (pH 6.5) at 37˚C.

The DNA probe from *M. morganii* 10 was initially hybridized to the DNA dot-blot at 40˚C and 42˚C (Figure 6. 5 & Figure 6. 6) with a second wash of 0.1X SSC at 65˚C. Under these conditions, the probe clearly detected *M. morganii* strains (ID 255, 11 and 10; Figure 6. 5 & Figure 6. 6), but weak signals were obtained for *R. planticola* (ID 6), R. *ornithinolytica* (ID 15) and *E. aerogenes* (ID 14) strains.

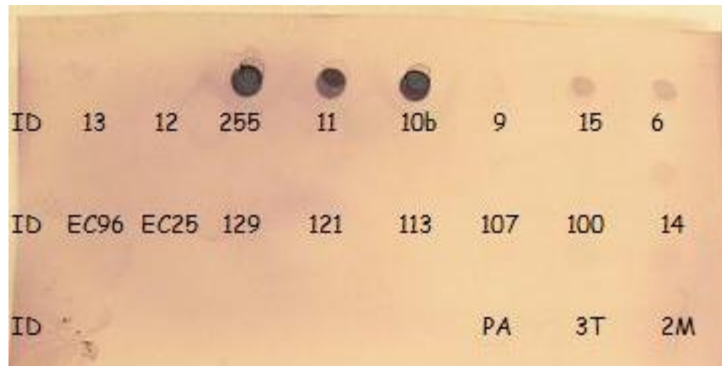

Figure 6. 5 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 40˚C and wash B (0.1X SSC/0.1% SDS) at 65˚C using 1 ul/ml probe and 50 ng DNA.

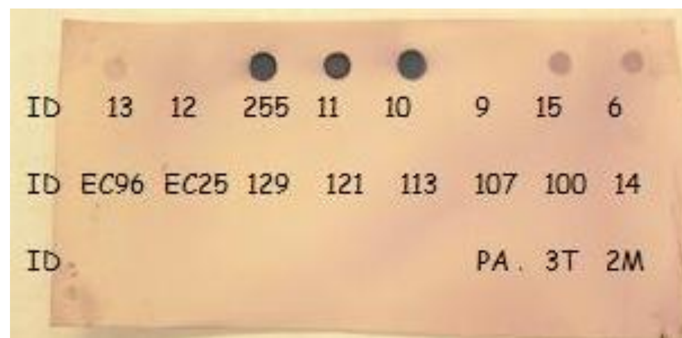

Figure 6. 6 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 42˚C and wash B (0.1X SSC/0.1% SDS) at 65˚C using 1 ul/ml probe and 50 ng DNA.

Several approaches were taken to see if stronger signals could be obtained for other histamine-producing isolates. First, DNA concentrations were increased from 50 ng to 100 ng at two hybridization temperatures under the same washing conditions as previously used (Figure 6. 7 & Figure 6. 8). Increasing the DNA concentration did not result in substantial improvement in signal strength for other high-histamine producing bacteria. Strong signals

were still obtained for *M. morganii* (ID 255, 11, and 10) and weak signals for the two *Raoultella* spp. (ID 15 and 6). However, hybridization at 40˚C seemed to give slightly stronger signal strength than at 42˚C (Figure 6. 7 & Figure 6. 8).

Next the salt concentration of the second wash was increased from 0.1X to 0.5X SSC while keeping temperature constant (65˚C). However, increasing the salt concentration of the second wash did not increase signal strengths further for the other histamine-producing bacteria (Figure 6. 9).

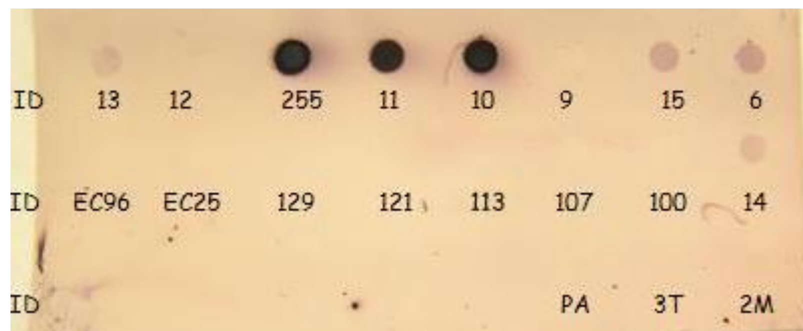

Figure 6. 7 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 40˚C and wash B (0.1X SSC/0.1% SDS) at 65˚C using 1 ul/ml probe and 100 ng DNA.

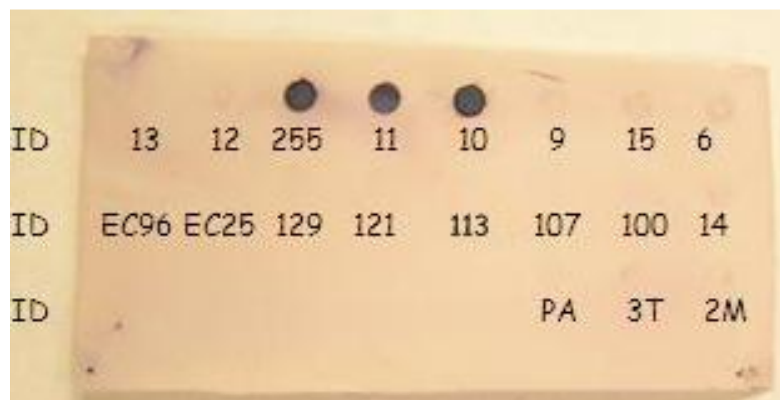

Figure 6. 8 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 42˚C and wash B (0.1X SSC/0.1% SDS) at 65˚C using 1 ul/ml probe and 100 ng DNA.

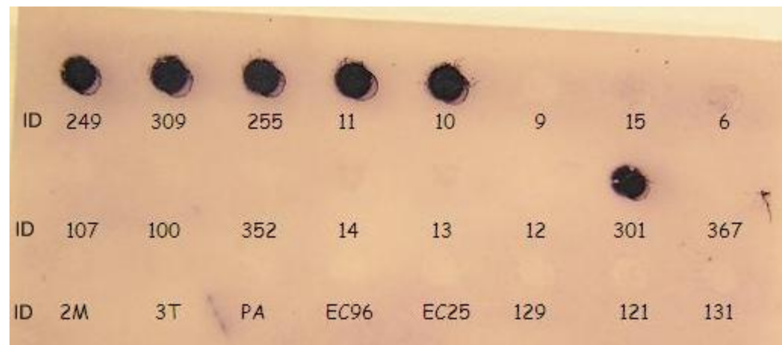

Figure 6. 9 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 65˚C using 1 ul/ml probe and 100 ng DNA.

Finally, decreasing the washing temperatures for the second wash from 60 to 50˚C using 0.5X SSC, increased signal strengths of other high histamine-producing bacteria (Figure 6. 10, Figure 6. 11 & Figure 6. 12). Washing at 60˚C resulted in signal intensity similar to that observed when washing at 65˚C, where only signals from *M. morganii* were obtained (ID 249, 309, 255, 11, 10, and 301). However, at 55˚C weak signals were obtained from another high-histamine producer, *Raoultella* spp. (ID 6 & 15) and *E. aerogenes* strains (ID 13 & 14), although no signals were obtained from *P. damselae* (100 & 107) strains at this washing temperature. Washing at 50˚C, on the other hand, resulted in more non-specific hybridization. These results indicate that a second wash at 55˚C using 0.5X SSC provided the strongest signal intensity with the lowest non-specific binding (Figure 6. 11). These conditions were thus used in further experiments.

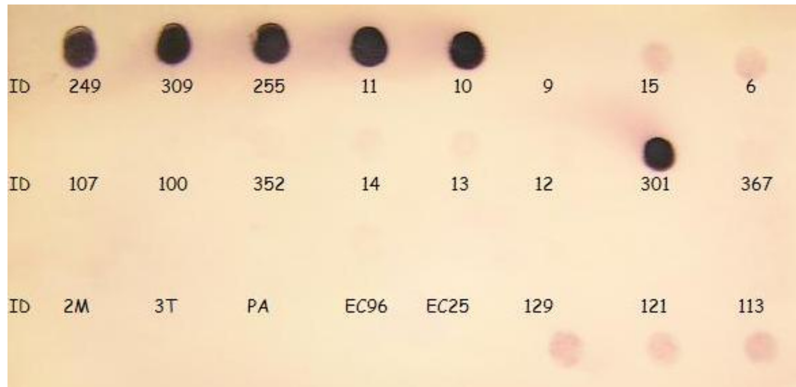

Figure 6. 10 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 60˚C using 1 ul/ml probe and 100 ng DNA.

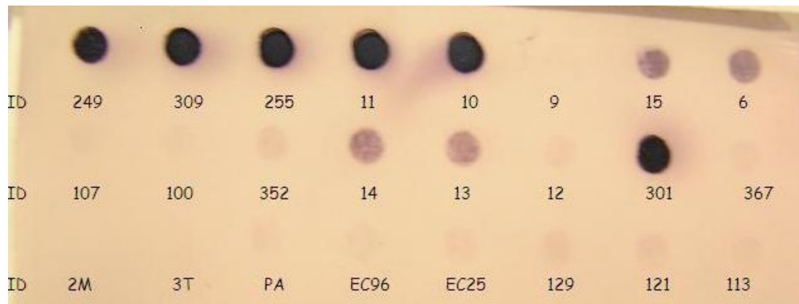

Figure 6. 11 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 55˚C using 1 ul/ml probe and 100 ng DNA.

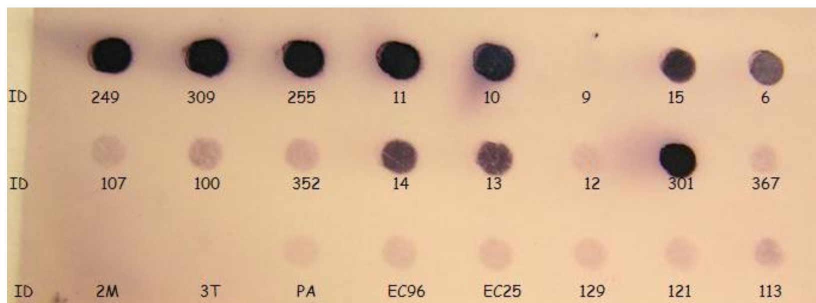

Figure 6. 12 Hybridization using DNA probe from *M. morganii* 10. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 50˚C using 1 ul/ml probe and 100 ng DNA.

We decided to examine DIG labeled PCR probes generated from the *hdc* gene of other high-histamine producing bacteria (*R. planticola* 6, *E. aerogenes* 13 and *P. damselae* 100) to investigate how DNA probes from these bacteria compared to probes generated from *M. morganii* 10 (Figure 6. 13, Figure 6. 14 & Figure 6. 15). DNA probes generated from *R. planticola* and *E. aerogenes* showed similar signals to those obtained when probe from *M. morganii* strains was used (Figure 6. 13 & Figure 6. 14). All high histamine-producers (ID 14, 13, 15, 6, 249, 10) except *P. damselae* strains (ID 100, 107) were detected using the probes from these two bacteria. However, when probe from *P. damselae* was hybridized, the two *P. damselae* strains were detected as well as one *R. ornithinolytica* and *E. coli* strains. Other high histamine-producing bacteria were not detected using the probe derived from *P. damselae*. Signals were observed from *E. coli* 25 using all probes, even though this strain did not produce detectable amounts of histamine (Table 6. 3). For these DNA dot-blots, plasmids from *E. coli* were used as a template in the PCR labeling and we suspect that some of the plasmid DNA was labeled in the process resulting in hybridization signals for *E. coli*. However, when chromosomal DNA was used as a template, no signals were obtained from 16 *E. coli* strains (Chapter 3).

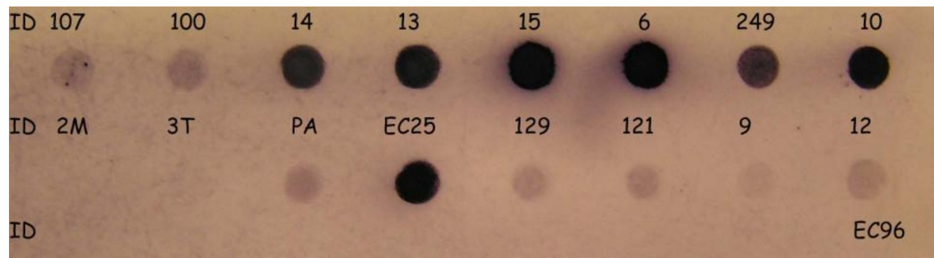

Figure 6. 13 Hybridization using DNA probe from *R. planticola* 6. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 55˚C using 1 ul/ml probe and 100 ng DNA.

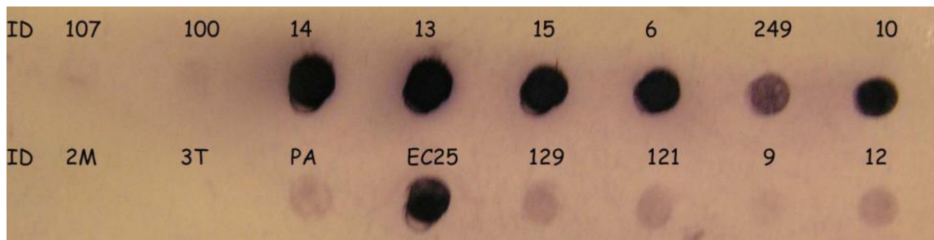

Figure 6. 14 Hybridization using DNA probe from *E. aerogenes* 6. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 55˚C using 1 ul/ml probe and 100 ng DNA.

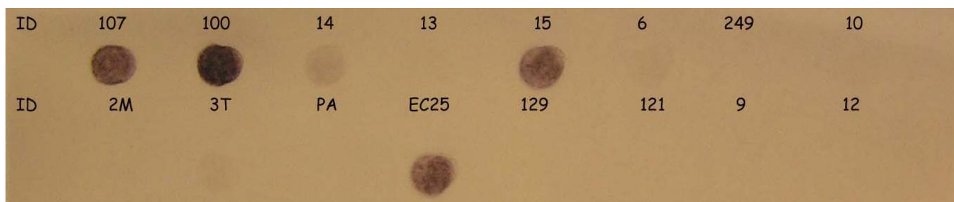

Figure 6. 15 Hybridization using DNA probe from *Photobacterium damselae 100*. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 55˚C using 1 ul/ml probe and 100 ng DNA.

A sequence identity matrix constructed based on the *hdc* gene sequences of the high-histamine producing bacteria used in the dot-blot hybridization experiments showed slightly higher similarity for *M. morganii*, *Raoultella spp.* and *E. aerogenes,* compared to the two *P.*

damselae strains (Table 6. 4). The sequence similarities among *M. morganii*, *Raoultella* spp. and *E. aerogenes* were between 76-78% for these species, but 73-75% for *P. damselae*. This slightly lower identity may be a reason for the relative poor performance of the *P. damselae* probe.

Table 6. 4 Identity matrix of the *hdc* gene from high-histamine producing bacteria used in the dot-blot experiments.

Bacteria	ID	10	11	6	15	13	14	100	107
Morganella morganii	**10**	-	0.98	0.76	0.76	0.78	0.78	0.74	0.75
Morganella morganii	**11**	0.98	-	0.76	0.76	0.78	0.78	0.75	0.75
Raoultella planticola	**6**	0.76	0.76	-	0.99	0.76	0.77	0.75	0.75
Raoultella ornithinolytica	**15**	0.76	0.76	0.99	-	0.77	0.77	0.75	0.75
Enterobacter aerogenes	**13**	0.78	0.78	0.76	0.77	-	0.98	0.73	0.73
Enterobacter aerogenes	**14**	0.78	0.78	0.77	0.77	0.98	-	0.74	0.74
Photobacterium damselae	**100**	0.74	0.75	0.75	0.75	0.73	0.74	-	0.99
Photobacterium damselae	**107**	0.75	0.75	0.75	0.75	0.73	0.74	0.99	-

Our experiments indicated that the 709 bp DIG labeled PCR probe was only able to detect high-histamine producing-bacteria. In an effort to improve the detection of low histamine-producing bacteria or to find one probe that detected all high-histamine producing bacteria the 709 bp fragment of the *hdc* gene was cut into three fragments (146 bp, 189 bp and 335 bp) using KpnI applied to the *hdc* of *M. morganii* (Figure 6. 16). Each fragment was BLAST searched against nucleotide sequences on NCBI and primers were designed to amplify the segment that gave the least amount of hits for non-histamine producing bacteria.

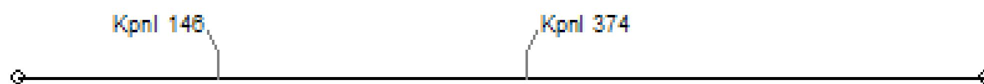

Figure 6. 16 Restriction digest pattern from a709 bp fragment of the *hdc* from *Morganella morganii* by KpnI

Results from BLAST search were compared to results previously obtained in Southern blots of restriction digests. The 335 bp fragment at the end of the 709 bp *hdc* gene fragment both gave the least amount of non-specific hits and gave strong signals in Southern blots. Ten degenerate primers were designed to amplify this fragment using FastPCR software (University of Helinksi, Helsinki, Finland). Four primers were then selected based on length and GC% content (Table 6. 5). These four primers were examined for their ability to amplify 249, 244, 242 and 239 bp fragments at the end of the 709 bp fragment in combination with the reverse primer (*hdc*-r) developed by Takahashi et al. *(194)*.

Table 6. 5 Nucleotide sequences of forward primers designed and their properties

Primer	Sequence	Tm	%GC	Length (bp)	PCR Product (bp)
3	AYGCBGAYGCSGCDCTRAGYGGHATGA	59	61	27	249
7	CBGAYGCSGCDCTRAGYGGHATGATYCT	58	58	28	244
8	GAYGCSGCDCTRAGYGGHATGA	55	60	22	242
9	GCSGCDCTRAGYGGHATCATYCTRCC	58	60	26	239

The four primers were examined at two different annealing temperatures, 52˚C and 54˚C, for amplification of the *hdc* gene from four high-histamine producing bacteria (*M.*

morganii 10, *R. planticola* 6, *E. aerogenes* 13, and *P. damselae* 100) and one non-histamine producer as a control (*Shewanella putrefacience*). PCR products were obtained using all four forward primers (data not shown) at both annealing temperatures. Primer 3 was the only primer out of the four that resulted in no unspecific amplification of the negative control at annealing temperature of 52˚C. Therefore Primer 3 was used in subsequent experiments. This primer amplified a 249 bp fragment at the end of the 709 bp partial *hdc* gene.

Primer 3 was used in combination with *hdc*-r primer to DIG PCR-label 249 bp probes from *M. morganii* 10, *R. planticola* 5, *E. aerogenes* 13 and *P. damselae* 100. These probes were examined for detection of histamine and non-histamine producing bacteria in DNA dot-blot hybridizations. The 249 bp DIG-labeled DNA probes generated from *M. morganii*, *R. planticola*, and *E. aerogenes* all detected high histamine producing (ID 14, 13, 15, 6, 11, 10) bacteria except *P. damselae* (ID 100, 107; Figure 6. 17A-C). Non-specific binding was obtained from *E. coli* as previously described. Plasmid from *E. coli* containing the 709 bp *hdc* gene was used to DIG-label the probes during PCR amplification. The 249 bp probe from *P. damselae* gave high unspecific binding (Figure 6. 17 D).

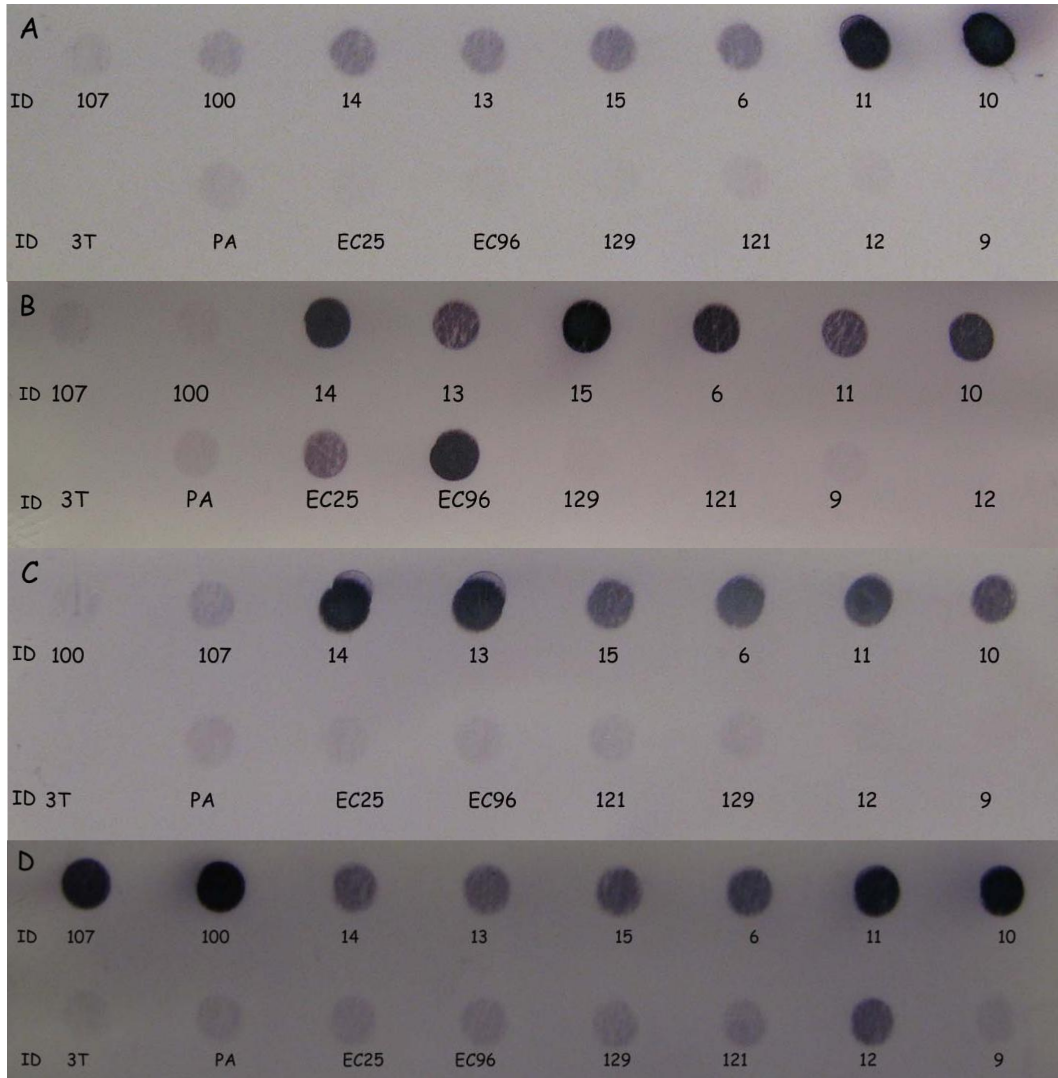

Figure 6. 17 Hybridization using DNA probe from *M. morganii* 10 (A), *R. planticola* 6 (B), *E. aerogenes* 13 (C) and *P. damselae* 100 (D). Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 55˚C using 1 ul/ml probe and 100 ng DNA

None of the probes alone could be used for the detection of the *hdc* gene of all high histamine-producing strains. It was hypothesized that using these probes in equal mix might improve assay inclusivity. To optimize DNA and probe concentrations, DNA dot-blots were

performed using 50, 100, and 200 ng of DNA and probe concentrations of 0.5, 1, and 2 µl/ml (Figure 6. 18 A-C). DNA concentrations of 50 ng gave satisfactory signals and probe concentration of 0.5 µl/ml gave the least background or unspecific binding. These concentrations were used in subsequent experiments.

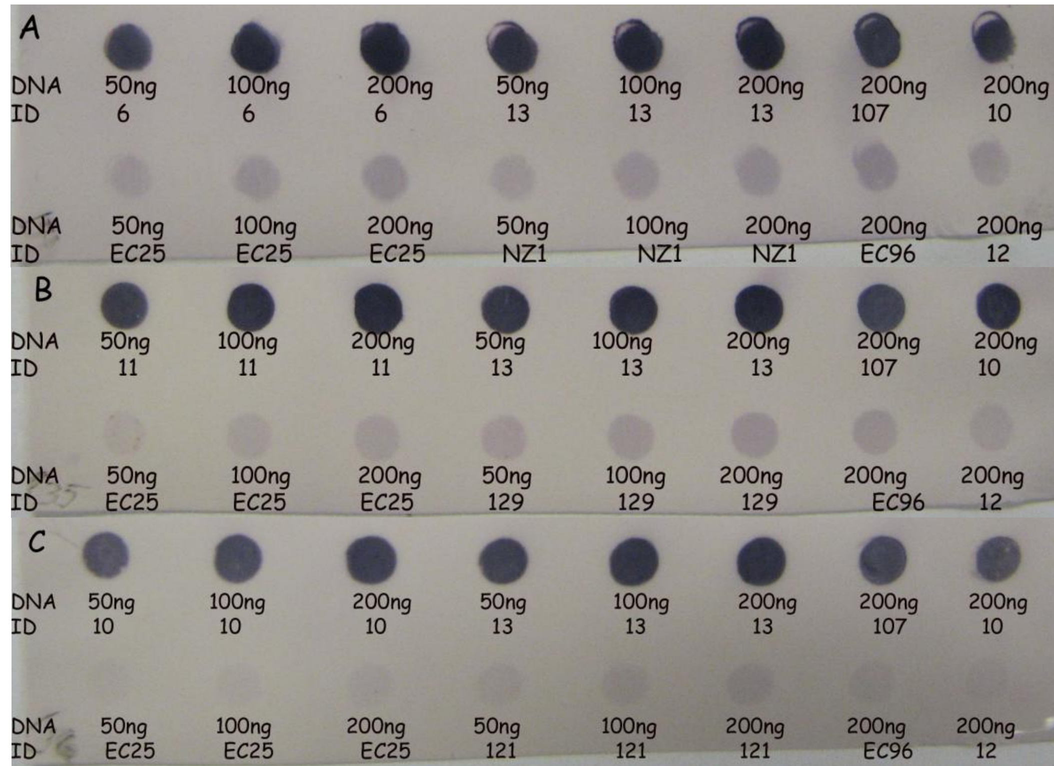

Figure 6. 18 Hybridization using 709 bp DNA probe mix from *M. morganii* 10, *R. planticola* 6, *E. aerogenes* 13 and *P. damselae 100*. Hybridization was performed at 40˚C and wash B (0.5X SSC/0.1% SDS) at 55˚C using 2 (A), 1 (B), and 0.5 (C) ul/ml probe mix (1:1:1:1) and different
DNA concentrations

DNA dot-blots were then performed and hybridized to both the 709 bp and the 249 bp probe mixes (1:1:1:1; total 0.5 µl/ml), generated from the four high-histamine-producing bacteria (*M. morganii*, *R. planticola*, *E. aerogenes*, and *P. damselae*). When DNA dot-blots were given a second wash at 55˚C using 0.5X SSC, high histamine-producing bacteria were detected using both probes but some non-specific binding occurred from the low and non-detectable histamine-producing bacteria (PA, EC25, EC96, 129, 121, NZ1, 12; Figure 6. 19A, Figure 6. 20A). In an effort to reduce the non-specific binding, the second wash temperature was increased to 60 and 65˚C while the salt concentration was held constant (Figure 6. 19B-C, Figure 6. 20B-C). Non-specific binding decreased with increasing wash temperature; the least non-specific binding occurred with a second wash temperature of 65˚C. Under these conditions, only the high histamine-producing bacteria *M. morganii* (11, 10), *Raoultella spp.* (15, 6), *E. aerogenes* (14, 13), and *P. damselae* (100, 107) were detected. However, the low histamine-producing bacteria remained negative. An equal mix of DIG PCR-labeled probes, either 709 bp or 249 bp, facilitated the detection of all high histamine-producing bacteria examined in this study.

Other researchers have also reported variation on responses from low-histamine producing bacteria. For instance, Takahashi et al. *(194)* was not able to amplify the *hdc* gene in *Citrobacter braakii* despite the strains capability of production of low levels of histamine. Interestingly, the *hdc* gene sequence from these low histamine-producing bacteria has not been reported. Therefore it is possible that these strains do not possess the gene or it may be vastly different in sequence from that of the high-histamine producing bacteria.

High histamine-producing bacteria are more likely to produce toxic levels of histamine if temperature abuse occurs than are low histamine-producers. Thus, it can be postulated that the detection of high histamine-producing bacteria suggests a higher risk of histamine fish poisoning. Therefore detection of high-histamine producing bacteria may be sufficient for the purpose of evaluating control strategies for histamine formation in temperature-abused fish.

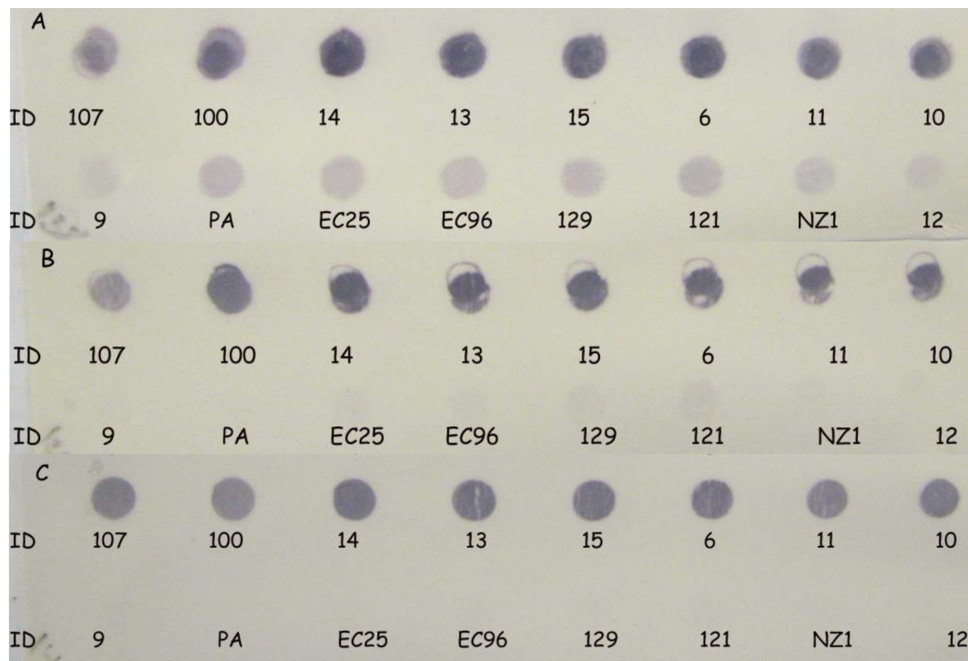

Figure 6. 19 Hybridization using 709 bp DNA probe mix from *M. morganii* 10, *R. planticola* 6, *E. aerogenes* 13 and *P. damselae 100*. Hybridization was performed at 40˚C and wash B with 0.5X SSC/0.1% SDS (A and B) and 0.1X SSC/0.1% SDS at 55˚C (A), 60˚C (B) and 65˚C (C) using 0.5 ul/ml probe mix (1:1:1:1) and 50 ng DNA.

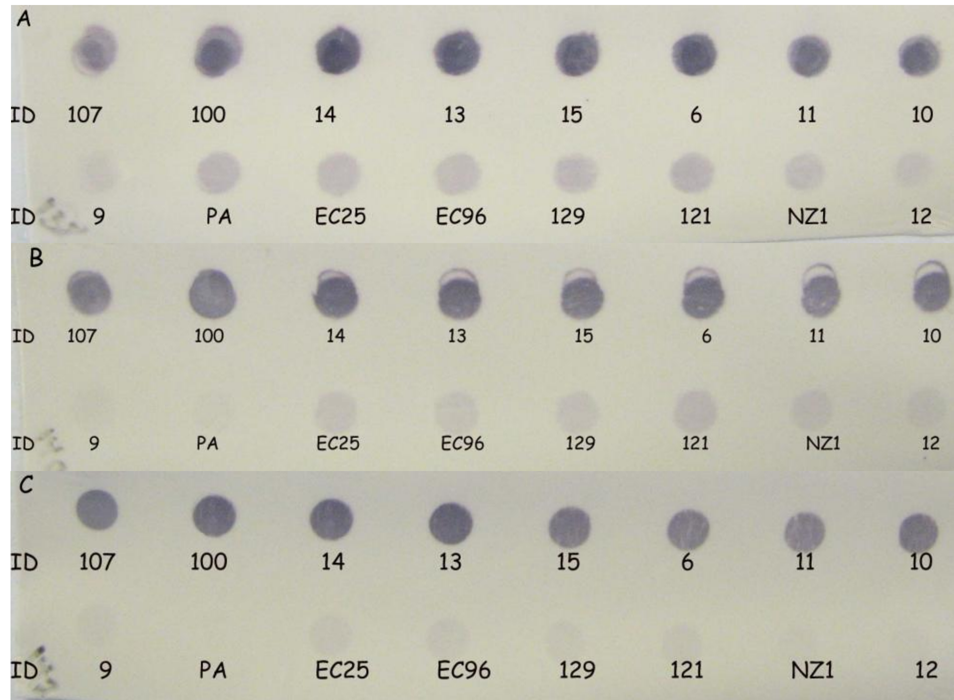

Figure 6. 20 Hybridization using 350 bp DNA probe mix from *M. morganii* 10, *R. planticola* 6, *E. aerogenes* 13 and *P. damselae 100.* Hybridization was performed at 40C and wash B with 0.5X SSC/0.1% SDS (A and B) and 0.1X SSC/0.1% SDS at 55˚C (A) and 65˚C (B and C) using 0.5 (C) ul/ml probe mix (1:1:1:1) and 50 ng DNA.

6.3.5 Degenerate probe

The DIG-labeled PCR probes previously tested, were not able to detect low histamine-producing bacteria (<300 ppm). In an effort to detect low histamine-producing bacteria and to examine if oligonucleotide probes could be used for the detection of histamine-producing bacteria, the application of degenerate oligonucleotide probes was investigated. Comparison of the 17 *hdc* gene sequences previously cloned and sequenced from *M. morganii*, *R. planticola*, *E. aerogenes* and *P. damselae* did not reveal a continuous region of complete homology suitable for probe choise. However, the HDC protein amino acid sequences showed higher sequence similarity at 82-88% compared to 73-78% for the

hdc gene. Longer homologous regions in the HDC protein sequences were therefore more easily identified. From these homologous regions, six degenerate probes were developed from three different locations in the *hdc* gene (Table 6. 6). Three degenerate probes were developed based on length and %GC content by sight (E-3, E-7, & E-8) and the other three (P-3/2, P-7/8, & P-9/6) with the aid of FastPCR software (University of Helsinki, Helsinki, Finland).

Table 6. 6 Degenerate probes and their properties

Nr	Sequence	Tm avg	%GC	Length (bp)	Location in *hdc* *(bp)**
E-3	TGGGGBTATGTVACYAAYGGYGGYACHGAA	59	52	30	283-313
E-7	GCBGAYGCSGCDCTRAGYGGHATG	58	66	24	625-649
E-8	GGHCAYAAAATGATYGGYTCNCCRATYCCWTGCGG	62	52	35	718-753
P-3/2	TGGGGBTATGTVACYAAYGGYGGYAC	58	55	26	284-310
P-7/8	GCSGCDCTRAGYGGHATGATYYTRCC	57	58	26	631-657
P-9/6	GGBTCDCGYAAYGGYCAYACHCCWCTV	58	59	27	835-862

*Location based on *Morganella morganii* JCM 1672 complete cds *hdc* gene (GI:95113538), total length 1,169 bp

DNA dot-blots of high-, low- and non-detectable histamine-producers, as previously described, were first hybridized at 37˚C with the six degenerate probes and second wash with 0.1X SSC at 37 and 42˚C. Results showed inconsistent binding of the degenerate probe when a 0.1X SSC solutions was used for the second wash (data not shown).

Binding of oligonucleotides depends on two factors: (i) the length of the hybrid formed and (ii) the GC content of the probe. Melting temperature (Td) of a given probe can be calculated empirically based on these two properties. Even though the length of the degenerate probe is constant, individual probes in the pool have different GC content,

making it hard to select appropriate stringency conditions. Wood et al. *(210)* reported that supplementation with tetramethylammonium chloride (Me_4NCl) of degenerate probe mixes can eliminate the dependence of Td on the GC content of the probes. Me_4NCl binds selectively to AT base pairs, thus displacing the dissociation equilibrium and raising the melting temperature. At 3.0 M Me_4NCl, this displacement is sufficient to shift the melting temperatures of AT bp to that of GC bp.

We examined the use of Me_4NCl solution in the second wash after degenerate probe hybridization. DNA dot-blots were hybridized with degenerate probes in DIG EasyHyb hybridization solution at 37˚C and washed in second wash with 3.0M Me_4NCl at 40, 55, 57 and 67˚C. When DNA dot-blots were hybridized with 10 pmol/ml of the degenerate probes E-3, E-7, P-3/2, and P7/8 and washed at 57˚C with 3.0M Me_4NCl solution, weak signals were obtained from high histamine producing bacteria (ID 100, 107, 14, 13, 15, 6, 11, 10) and no signals for low or non histamine-producers (3T, PA, EC25, EC96, 121, 129, 9, 12; Figure 6. 21). Probe E-8 and P-9/6 gave more non-specific binding than the other probes. In addition, washing at 40 and 55˚C resulted in non-specific binding, and no signals were observed at 67˚C (data not shown).

Degenerate probes E-3, E-7 and P3/2 were examined further for their ability to detect high-histamine producing bacteria. Attempts were made to increase signal strength by increasing the probe and DNA concentrations. Degenerate probe concentration in the hybridization solution was first increased from 10 pmol/ml to 18 pmol/ml (Figure 6. 22). Increasing the probe concentration did not result in considerable higher signal strength but

unspecific binding from *H. alvei* strain 12, *C. freundii* strain 121 and *E. coli* strain EC96 appeared. These bacteria all produced either low (<300 ppm) or undetectable histamine (<125 ppm).

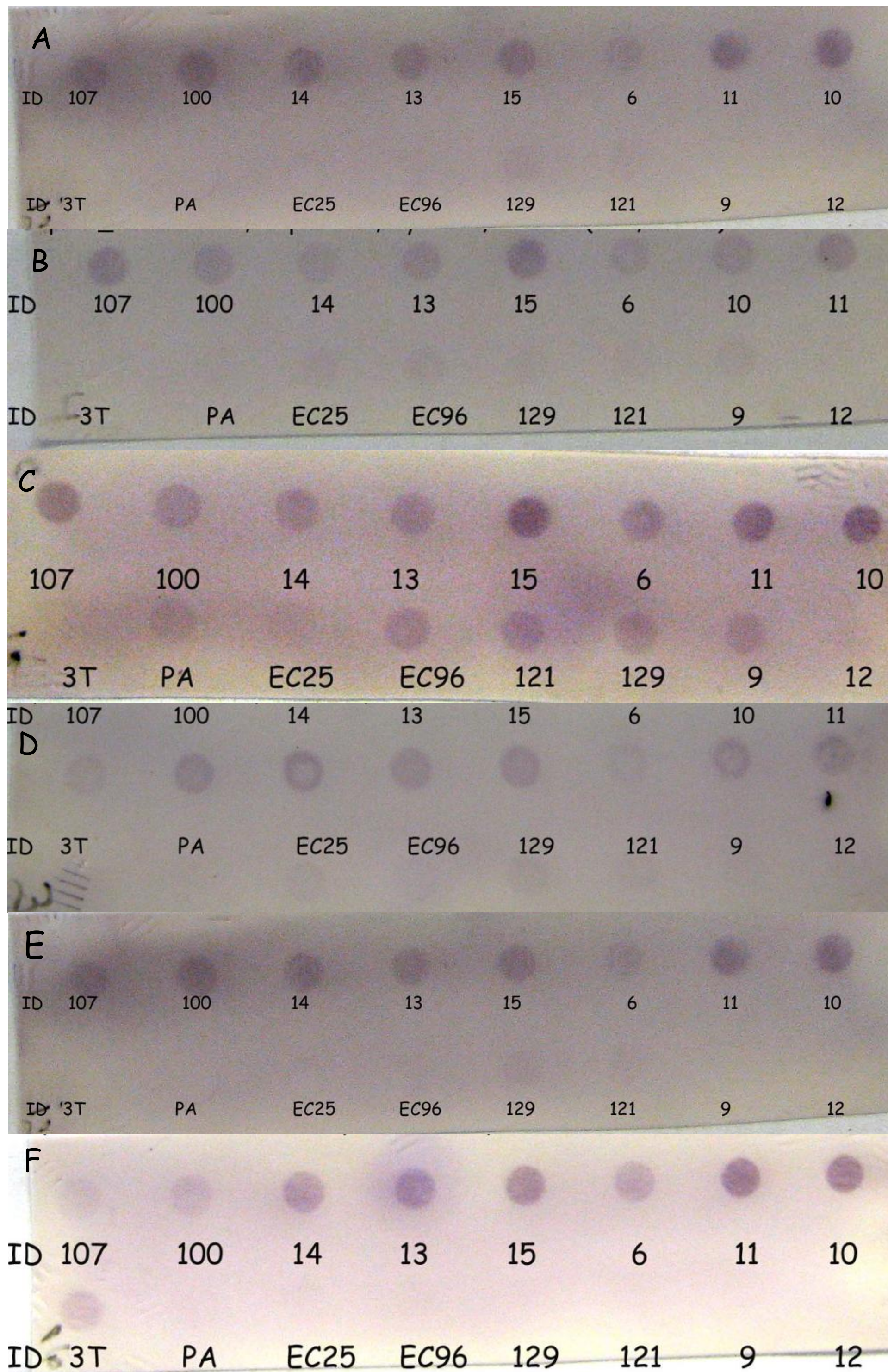

Figure 6. 21 Hybridization using 10 pmol/ml degenerate probe E-3 (A), E-7 (B), E8 (C), P 3/2 (D), P 7/8 (E), and P 9/6 (F) hybridized at 37˚C and second wash using Me_4NCl at 57˚C.

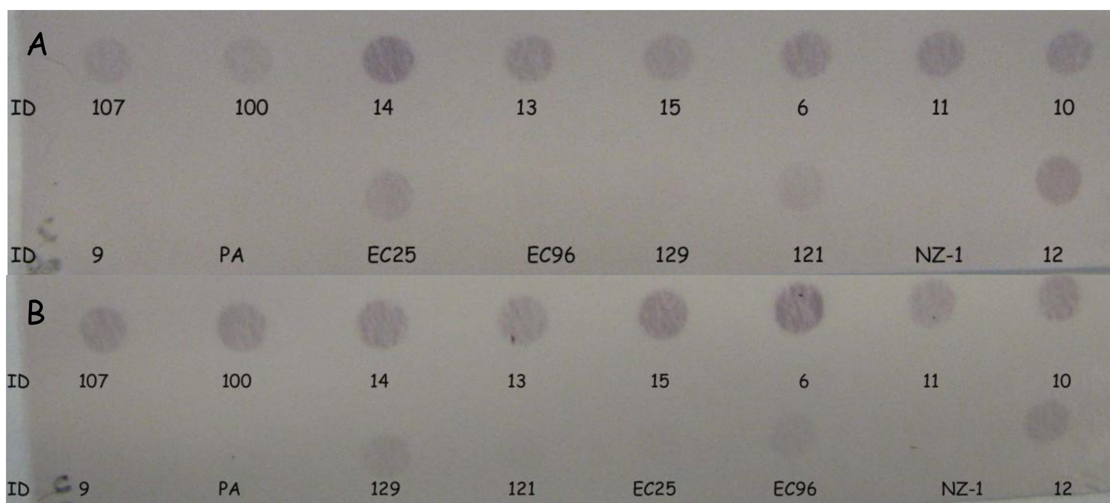

Figure 6. 22 Hybridization using 18 pmol/ml degenerate probe P 3/2 (A) and P 7/8 (B) hybridized at 37˚C and second wash using Me_4NCl at 57˚C.

DNA concentrations were next varied from 0.75 ug to 1.25 ug to see if increased DNA concentration would increase signal strength (Figure 6. 23). Signal strength increased with increased DNA concentration but non-specific binding also occurred.

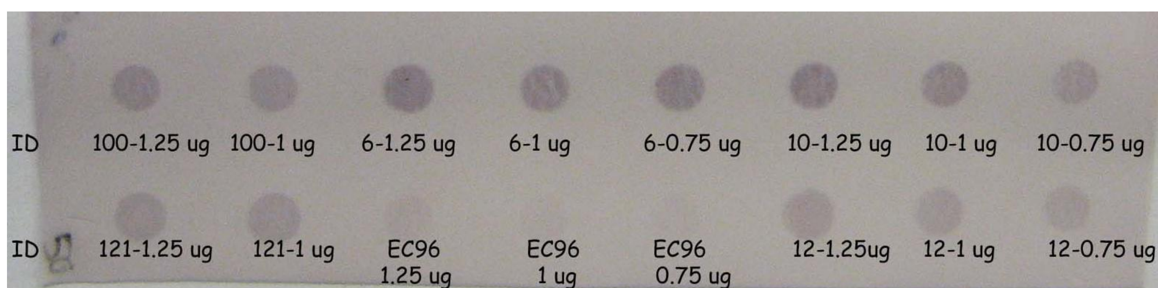

Figure 6. 23 Hybridization using 15 pmol/ml degenerate probe P 7/8 (E) hybridized at 37˚C and second wash using Me4NCl at 55˚C

Finally, DNA dot blots using 15 pmol/ml degenerate probes and 1 μg DNA at higher wash temperatures (60 and 65˚C) were tested to see if non-specific binding could be reduced. At the higher wash temperatures, only high histamine producing bacteria were detected. Non-specific binding was reduced but unfortunately, signal strength continued to be very weak (Figure 6. 24).

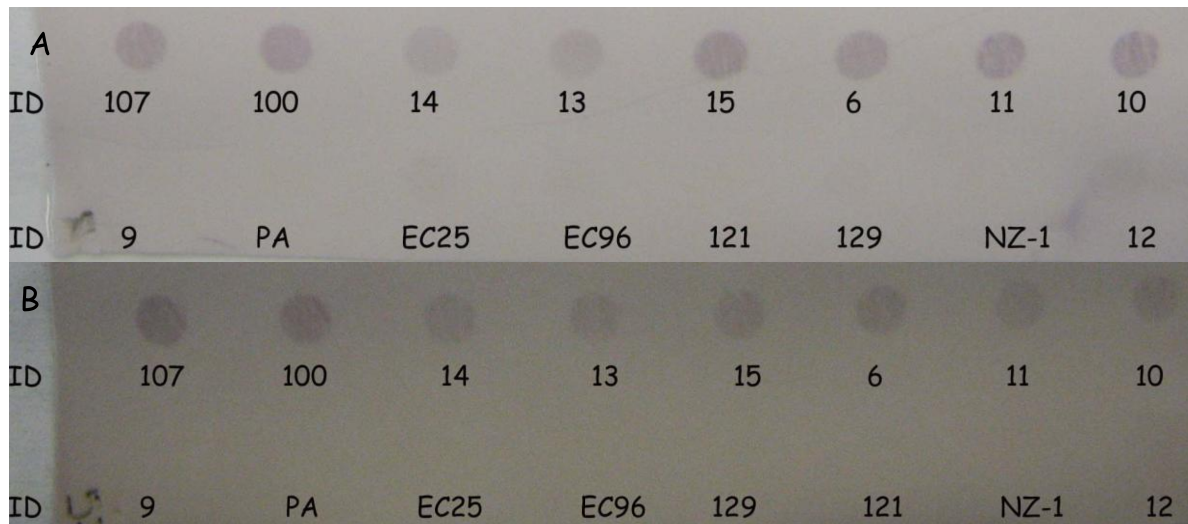

Figure 6. 24 Hybridization using 15 pmol/ml degenerate probe P 7/8 hybridized at 37˚C and second wash using Me4NCl at 60˚C (A) and 65˚C (B).

In a final effort to increase signal strengths, degenerate probes were labeled with a dideoxyuridine-triphopaste (DIG-ddUTP) tail using a DIG Oligonucleotide Tailing Kit from Roche (Indianapolis, IN). For the generation of oligonucleotide probes, a mixture of deoxynucleotide-triphopates (dNTP) and DIG-dUTP were used to generate DIG probes of various tail lengths. The type of dNTP used determines the average tail length. The

degenerate probes (P-3/2, P7/8 and E8) were first labeled using dATP in the labeling mix which will result in average tail length of 50 nucleotides. Tailed probes were hybridized using DNA dot-blots of low- and high-histamine producing bacteria and washed with Me_4NCl at 65 – 85˚C in 5˚C increments (data not shown). Very strong signal strengths were obtained from both high, low and non-detectable histamine producing bacteria at all wash temperatures. We suspected that the long tail of the probe may have caused unspecific binding or the tail of the probe got tangled in the DNA on the membranes. The degenerate probe tail was reduced by half to an average length of 25 nucleotides by the use of dCTP in the labeling reaction. However, the same results were obtained with very high signal strength, i.e., but also non-specific binding for non-detectable histamine-producing bacteria.

6.4 CONCLUSION

We examined PCR DIG-labeled DNA probes and DIG-labeled degenerated probes for the detection of histamine-producing bacteria in DNA dot-blot hybridizations. Three degenerate probes gave weak detection of high-histamine producing bacteria. An effort to increase the detection signal was unsuccessful. Detection of high-histamine producing bacteria by dot-blot hybridization was successful with an equal mixture (1:1:1:1) of PCR DIG-labeled probes generated from *M. morganii*, *R. planticola*, *E. aerogenes* and *P. damselae*. This probe mix may be applied in molecular methods for the detection and quantification of high-histamine-producing bacteria.

APPENDIX B: STATISTICAL COMPARISONS

Table 7. 1 Overall statistical comparison of treatment on external pH of mahi-mahi as determined by Tukey honest significance of difference (HDS) test (Figure 4. 6A)

			p – values									
	Time (h)	**% TSP**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0		0.000229	1.000000	0.005975	0.999990	0.000712	0.992892	0.004895	0.976808	0.001859
2	0	7	0.000229		0.000249	0.429466	0.000293	0.968235	0.000504	0.485574	0.002393	0.989903
3	1	0	1.000000	0.000249		0.007884	1.000000	0.000889	0.997770	0.006450	0.989219	0.002350
4	1	7	0.005975	0.429466	0.007884		0.013686	0.973478	0.039185	1.000000	0.138143	0.980547
5	2	0	0.999990	0.000293	1.000000	0.013686		0.001437	0.999919	0.011196	0.998622	0.003791
6	2	7	0.000712	0.968235	0.000889	0.973478	0.001437		0.003967	0.985246	0.019624	1.000000
7	3	0	0.992892	0.000504	0.997770	0.039185	0.999919	0.003967		0.032219	1.000000	0.009758
8	3	7	0.004895	0.485574	0.006450	1.000000	0.011196	0.985246	0.032219		0.118030	0.988938
9	4	0	0.976808	0.002393	0.989219	0.138143	0.998622	0.019624	1.000000	0.118030		0.035301
10	4	7	0.001859	0.989903	0.002350	0.980547	0.003791	1.000000	0.009758	0.988938	0.035301	

Table 7. 2 Overall statistical comparison of treatment on internal pH of mahi-mahi as determined by Tukey honest significance of difference (HDS) test (Figure 4. 6)

			p - values									
	Time (h)	**% TSP**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0		0.875477	0.999985	0.048335	0.999988	0.003654	0.937257	0.001534	0.795149	0.003155
2	0	7	0.875477		0.981763	0.547347	0.980393	0.073072	1.000000	0.030595	0.999998	0.046822
3	1	0	0.999985	0.981763		0.108611	1.000000	0.008658	0.995043	0.003517	0.943193	0.006809
4	1	7	0.048335	0.547347	0.108611		0.106252	0.944830	0.439622	0.765074	0.855682	0.761669
5	2	0	0.999988	0.980393	1.000000	0.106252		0.008449	0.994540	0.003434	0.940472	0.006662
6	2	7	0.003654	0.073072	0.008658	0.944830	0.008449		0.051220	0.999979	0.245147	0.999789
7	3	0	0.937257	1.000000	0.995043	0.439622	0.994540	0.051220		0.021104	0.999946	0.033789
8	3	7	0.001534	0.030595	0.003517	0.765074	0.003434	0.999979	0.021104		0.124874	1.000000
9	4	0	0.795149	0.999998	0.943193	0.855682	0.940472	0.245147	0.999946	0.124874		0.148197
10	4	7	0.003155	0.046822	0.006809	0.761669	0.006662	0.999789	0.033789	1.000000	0.148197	

Table 7. 3 Overall statistical comparison of treatment on external pH of tuna as determined by Tukey honest significance of difference (HDS) test (Figure 4. 6C)

			p - values									
	Time (h)	**% TSP**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0		0.000179	1.000000	0.000186	0.951023	0.000324	0.968066	0.000360	0.376063	0.000410
2	0	7	0.000179		0.000179	0.000197	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
3	1	0	1.000000	0.000179		0.000186	0.920339	0.000291	0.944544	0.000318	0.318679	0.000356
4	1	7	0.000186	0.000197	0.000186		0.000209	0.614954	0.000204	0.555881	0.000551	0.492262
5	2	0	0.951023	0.000179	0.920339	0.000209		0.002782	1.000000	0.003415	0.974442	0.004269
6	2	7	0.000324	0.000179	0.000291	0.614954	0.002782		0.002321	1.000000	0.031142	1.000000
7	3	0	0.968066	0.000179	0.944544	0.000204	1.000000	0.002321		0.002836	0.959731	0.003550
8	3	7	0.000360	0.000179	0.000318	0.555881	0.003415	1.000000	0.002836		0.038033	1.000000
9	4	0	0.376063	0.000179	0.318679	0.000551	0.974442	0.031142	0.959731	0.038033		0.047211
10	4	7	0.000410	0.000179	0.000356	0.492262	0.004269	1.000000	0.003550	1.000000	0.047211	

Table 7. 4 Overall statistical comparison of treatment on internal pH of tuna as determined by Tukey honest significance of difference (HDS) test (Figure 4. 6D)

			***p* - values**									
	Time (h)	**% TSP**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0		0.743980	1.000000	0.108386	1.000000	0.106923	1.000000	0.000711	0.385876	0.000257
2	0	7	0.743980		0.637212	0.921580	0.676570	0.919343	0.820274	0.027389	0.999744	0.004570
3	1	0	1.000000	0.637212		0.076641	1.000000	0.075566	0.999999	0.000530	0.296605	0.000231
4	1	7	0.108386	0.921580	0.076641		0.086960	1.000000	0.141526	0.344881	0.998405	0.081084
5	2	0	1.000000	0.676570	1.000000	0.086960		0.085753	1.000000	0.000587	0.327149	0.000239
6	2	7	0.106923	0.919343	0.075566	1.000000	0.085753		0.139685	0.348496	0.998271	0.082229
7	3	0	1.000000	0.820274	0.999999	0.141526	1.000000	0.139685		0.000923	0.466754	0.000287
8	3	7	0.000711	0.027389	0.000530	0.344881	0.000587	0.348496	0.000923		0.093213	0.996821
9	4	0	0.385876	0.999744	0.296605	0.998405	0.327149	0.998271	0.466754	0.093213		0.016796
10	4	7	0.000257	0.004570	0.000231	0.081084	0.000239	0.082229	0.000287	0.996821	0.016796	

Table 7. 5 Overall statistical comparison of treatment on drip of mahi-mahi as determined by Tukey honest significance of difference (HDS) test (Figure 4. 7A)

			***p* - values**									
	Time (h)	**% TSP**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0		1.000000	0.992164	0.973760	0.966260	0.054077	0.674785	0.001986	0.363725	0.003114
2	0	7	1.000000		0.966260	0.994549	0.905981	0.084804	0.805613	0.003142	0.478607	0.004750
3	1	0	0.992164	0.966260		0.561136	1.000000	0.008128	0.195775	0.000428	0.090038	0.000675
4	1	7	0.973760	0.994549	0.561136		0.424137	0.374032	0.998490	0.019278	0.906571	0.024159
5	2	0	0.966260	0.905981	1.000000	0.424137		0.005039	0.130408	0.000334	0.060379	0.000498
6	2	7	0.054077	0.084804	0.008128	0.374032	0.005039		0.805613	0.805613	0.998723	0.729285
7	3	0	0.674785	0.805613	0.195775	0.998490	0.130408	0.805613		0.084804	0.998723	0.090038
8	3	7	0.001986	0.003142	0.000428	0.019278	0.000334	0.805613	0.084804		0.478607	0.999999
9	4	0	0.363725	0.478607	0.090038	0.906571	0.060379	0.998723	0.998723	0.478607		0.424401
10	4	7	0.003114	0.004750	0.000675	0.024159	0.000498	0.729285	0.090038	0.999999	0.424401	

Table 7. 6 Overall statistical comparison of treatment on drip of tuna as determined by Tukey honest significance of difference (HDS) test (Figure 4. 7B)

			***p* - values**									
	Time (h)	**% TSP**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0		0.000462	0.991207	0.000462	0.928373	0.000187	0.999824	0.000184	0.999824	0.000184
2	0	7	0.000462		0.000188	1.000000	0.000185	0.515023	0.000196	0.006858	0.000428	0.002266
3	1	0	0.991207	0.000188		0.000188	0.999985	0.000184	0.999985	0.000184	0.769965	0.000184
4	1	7	0.000462	1.000000	0.000188		0.000185	0.515023	0.000196	0.006858	0.000428	0.002266
5	2	0	0.928373	0.000185	0.999985	0.000185		0.000184	0.996042	0.000184	0.515023	0.000184
6	2	7	0.000187	0.515023	0.000184	0.515023	0.000184		0.000184	0.392674	0.000185	0.169815
7	3	0	0.999824	0.000196	0.999985	0.000196	0.996042	0.000184		0.000184	0.943471	0.000184
8	3	7	0.000184	0.006858	0.000184	0.006858	0.000184	0.392674	0.000184		0.000184	0.999902
9	4	0	0.999824	0.000428	0.769965	0.000428	0.515023	0.000185	0.943471	0.000184		0.000184
10	4	7	0.000184	0.002266	0.000184	0.002266	0.000184	0.169815	0.000184	0.999902	0.000184	