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Heat Resistance of Histamine-Producing Bacteria in Irradiated Tuna Loins

ELENA ENACHE,[1]* AI KATAOKA,[1] D. GLENN BLACK,[1] LISA WEDDIG,[2] MELINDA HAYMAN,[1] AND KRISTIN BJORNSDOTTIR-BUTLER[3]

[1]*Grocery Manufacturers Association, 1350 I Street N.W., Suite 300, Washington, D.C. 20005;* [2]*National Fisheries Institute, 7918 Jones Branch Drive, Suite 700, McLean, Virginia 22102; and* [3]*U.S. Food and Drug Administration, Division of Seafood Science and Technology, Gulf Coast Seafood Laboratory, Dauphin Island, Alabama 36528, USA*

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ABSTRACT

Consumption of foods high in biogenic amines leads to an illness known as histamine, or scombrotoxin, poisoning. The illness is commonly associated with consumption of fish with high levels of histamine (≥500 ppm). The objective of this study was to determine and compare the heat resistance of five histamine-producing bacteria in irradiated albacore tuna loins. Heat-resistance parameters (*D*- and *z*-values) were determined for *Morganella morganii, Raoultella planticola, Hafnia alvei,* and *Enterobacter aerogenes*. *D*- or *z*-values were not determined for *Photobacterium damselae,* which was the most heat-sensitive organism in this study. *P. damselae* declined >5.9 log CFU/g after a heat treatment of 50°C for 10 min, 54°C for 3 min, and 56°C for 0.5 min. *M. morganii* was the most heat-resistant histamine-producing bacteria in albacore tuna loins, followed by *E. aerogenes, H. alvei,* and *R. planticola*. *M. morganii* and *E. aerogenes* had the highest $D_{50°C}$, 49.7 ± 17.57 and 51.8 ± 17.38 min, respectively. In addition, *M. morganii* had the highest *D*-values for all other temperatures (54, 56, and 58°C) tested. *D*- and z-values were also determined for *M. morganii* in skipjack tuna. While no significant ($P > 0.05$) difference was observed between $D_{54°C}$ and $D_{56°C}$ of *M. morganii* in either albacore or skipjack tuna, the $D_{58°C}$ (0.4 ± 0.17 min) was significantly lower ($P < 0.05$) in skipjack than in albacore (0.9 ± 0.24 min). The *z*-values for all organisms tested were in the range of 3.2 to 3.8°C. This study suggests that heat treatment designed to control *M. morganii* in tuna loins is sufficient for controlling histamine-producing bacteria in canned-tuna processing environments.

Scombrotoxin, or histamine, fish poisoning is an acute illness associated with the consumption of certain fish species containing high levels of histamine and/or other biogenic amines. Although symptoms are similar to those of allergic reactions, e.g., rash or hives on the upper body, headaches, dizziness, drop in blood pressure, swelling of tongue, respiratory distress, heart palpitation, nausea, vomiting, diarrhea, etc., the illness is caused by the ingestion of biogenic amines (toxins) produced by bacterial spoilage of fish. This type of poisoning is a mild but unpleasant illness of short duration (≤24 h), but can on occasion last for several days *(4, 15, 33)*. Severe symptoms (e.g., cardiac manifestations, bronchospasm, cyanosis, and blurred vision) are rare and vary with the amount of histamine consumed and individual sensitivity; therefore, scombrotoxin poisoning remains an important health risk for consumers *(13, 18, 27, 33, 41, 42)*. From 1998 to 2002, scombrotoxin fish poisoning was the most frequently reported chemical foodborne illness to the Centers for Disease Control and Prevention (CDC), resulting in 167 outbreaks and 703 cases with no deaths *(11)*. More recently, from 2009 to 2010, a total of 18 outbreaks with 76 associated illnesses were recorded by the CDC *(13)*.

The term ''scombroid poisoning'' was originally used to describe the illness, because fish belonging to families *Scombridae* and *Scomberesocidae* (i.e., tuna, skipjack, bonito, and mackerel) were often associated with this type of food poisoning. In 2007, the CDC reported that 42% of the scombrotoxin poisoning outbreaks reported from 1978 to 1982 were associated with mahi-mahi, which is a non-scombroid fish *(9, 12, 13)*. Other non-scombroid species including sardine, anchovy, herring, bluefish, amberjack, and marlin can also be involved. From a food safety perspective, the most important biogenic amines are histamine, putresceine, cadaverine, and tyramine, which are formed as a result of decarboxylation of the amino acids histidine, ornithine, lysine, and tyrosine, respectively.

Spoilage bacteria associated with histamine production in fish flesh are common inhabitants of seawater and naturally found in the gills, intestinal tract, and skin of saltwater fish *(31, 44, 45, 51)*. Fish can also be contaminated with these microorganisms during postharvest handling of the fish *(33, 44)*. Scombroid species have naturally high levels of histidine in their muscle tissue, which can be used by microorganisms capable of producing the enzyme

* Author for correspondence. Tel: 202-639-5969; Fax: 202-639-5991; E-mail: eenache@gmaonline.org.

histidine decarboxylase to convert histidine to histamine during growth *(5, 35, 39)*. Fresh scombrotoxin-forming fish contain negligible amounts of histamine (<1 ppm) *(18, 23, 25, 26, 48, 51)*; high levels of histamine occur when harvested fish are held at temperatures above 15°C for several hours, permitting spoilage microorganisms including species of *Morganella, Enterobacter, Hafnia, Raoultella,* and *Photobacterium* to grow *(6, 22, 28, 35)*. The most effective measure to prevent histamine formation is rapid chilling of fish to ≤0°C immediately after harvest. However, when long-line fishing methods or purse seine nets are used to catch schools of tuna, the fish are not always collected promptly and can remain in the ocean for considerable time *(10, 51)*.

The U.S. Food and Drug Administration recognizes 50 ppm of histamine in fish tissues as an indicator of decomposition and has established 500 ppm as the hazard guidance level for histamine in tuna *(47)*. In most outbreaks of scombrotoxin poisoning, histamine concentrations are greater than 500 ppm in implicated fish. However, in some cases, fish with histamine levels of 100 to 200 ppm have caused illness *(14)*. The largest scombrotoxin poisoning outbreak in the United States was related to consumption of canned tuna, where the histamine levels were 680 to 2,800 ppm *(37)*. High levels of histamine in commercially produced canned fish products have occurred worldwide and are primarily due to temperature abuse before canning *(33, 41, 43)*. Tuna for canned trade are mostly processed from fresh catch that is frozen at sea and later thawed, butchered, partially cooked (or precooked), and cooled. The industry has long been aware that the precooking step has an inhibitory effect on histamine formation during the processing of canned tuna *(38)*. The tuna canning industry supports validation of the precook step as an effective control against histamine formation during processing. To accomplish this task, *D*- and *z*-values of histamine-producing bacteria were needed *(50)*.

The purpose of this study was to determine thermal inactivation parameters of five common histamine-producing bacteria in tuna loins. The objective was to provide heat-resistance parameters (*D*- and z-values) needed by the tuna industry to better understand the impact of precooking temperatures on thermal death rates of histamine-producing bacteria.

MATERIALS AND METHODS

Frozen tuna loins were obtained from a supplier to the canned tuna industry. The frozen tuna loins were sent to Food Technology Service, Inc., an International Organization for Standardization 13485–certified company, for irradiation treatment (7 kGy per treatment) to inactivate background microflora. Tuna loin characteristics (pH, water activity, total salt, and total fat) are presented in Table 1. The pH was determined with a pH meter (Accumet Research AR 20, Fisher Scientific, Pittsburg, PA), and the water activity was measured with a water activity meter (AquaLab 4TEV, Decagon Devices, Inc., Pullman, WA). Total fat *(2)* and NaCl *(3)* were determined according AOAC International official methods by the National Food Laboratories. No attempts were made to alter the fish in an effort to mimic the conditions used in the industry for the precook step before canning.

TABLE 1. *Characteristics of tuna loins used in this study*[a]

Tuna sp.	pH	Water activity	Total salt (%)	Total fat (%)
Albacore	6.3 ± 0.16	0.98	0.31	0.71 ± 0.15
Skipjack	5.9 ± 0.07	0.98	0.66	0.76 ± 0.19

[a] $n = 3$.

Bacterial strains and culture conditions. Five histamine-producing bacteria, *Morganella morganii, Raoultella planticola, Hafnia alvei, Enterobacter aerogenes,* and *Photobacterium damselae,* were used in this study, with three strains of each organism (Table 2). All strains were obtained from the American Type Culture Collection (ATCC), and the presence of the histidine decarboxylase gene (*hdc*) of the high-histamine-producing bacteria was analyzed according to the method of Bjornsdottir-Butler et al. *(6)*.

Prior to inoculation, two consecutive loop transfers of each strain in 10 ml of tryptic soy broth (TSB) were grown aerobically at 30°C overnight, except for *P. damselae,* which was inoculated into TSB with 1.5% NaCl and incubated at 26°C for 48 h. From these individual cultures, a three-strain composite of each organism was used to inoculate the fish samples. Individual strains were quantified on tryptic soy agar (TSA; with 1.5% NaCl for *P. damselae*) before compositing to ensure approximately equal numbers of cells for each strain. The composite inocula were also quantified before and after inoculation of the product.

Preparation and inoculation of samples. The bacterial composite was prepared in a Pyrex brand crystallizing dish (740-ml capacity, diameter of 125 mm) held in an iced water bath during the inoculation. Tuna (loin) samples (5 g each) were submerged into one bacterial composite at a time (three strains per organism) for 30 min to allow the bacterial cells to adhere to the tuna sample and achieve at least 1×10^6 CFU/g. After inoculation, samples were aseptically transferred to sterile plastic pouches with an inner filter (65 by 120 mm; Whirl Pak, Nasco, Fort Atkins, WI), vacuum sealed with a vacuum sealer chamber (VacMaster Commercial Vacuum Sealer VP-321, Pleasant Hill Grain, NE), and heat treated as described below.

Thermal death time studies. Thermal inactivation parameters of *R. planticola, H. alvei, E. aerogenes,* and *M. morganii* were determined at 50 to 60°C by using thermal death time tests. Three temperatures (50, 54, and 56°C) were tested for *R. planticola* and *H. alvei,* four temperatures for *E. aerogenes* (50, 54, 56, and 58°C) and five temperatures for *M. morganii* (50, 54, 56, 58, and 60°C). For each thermal treatment, eight inoculated and two uninoculated pouches were vacuum sealed and then transferred into the preheated, circulating water bath (model W45, Thermo Haake, Karlsruhe, Germany), while two other inoculated pouches were diluted and plated without undergoing the heat treatment to evaluate the initial inoculum level on tuna loins. Thermocouples were placed in the center of each of the two uninoculated tuna samples in order to monitor the temperature profile. The heating time started when duplicate pouches reached the test temperature ±0.1°C. During the heat treatment, the eight inoculated pouches were removed at four predetermined time intervals (duplicate pouches per heating time), which was based on the organism tested and test temperature. For example, the heating times for *M. morganii* were between 20 and 50 min at 54°C, and between 1 and 3.5 min at 58°C; for *R. planticola,* they were in the range of 5 to 14 min at 54°C, and 1 to 2.5 min at 56°C. To enumerate the

TABLE 2. *Growth, histamine production capabilities, and* hdc *gene detection of histamine-producing bacteria used in the present study*

Organism	Strain	Histamine level (ppm)[a]	Count (log CFU/ml)	*hdc* gene	
				Total	Spp. specific
Morganella morganii	ATCC 79	5,391 (47)	9.3	+	*M. morganii*
	ATCC 9237	5,347 (86)	9.5	+	*M. morganii*
	ATCC 9916	4,457 (317)	9.1	+	*M. morganii*
Raoultella planticola	ATCC 21609	5,319 (86)	9.1	+	*R. planticola*
	ATCC 31900	4,767 (396)	8.9	+	*R. planticola*
	ATCC 33531	<DL[b]	8.5	−	−
Hafnia alvei	ATCC 13337	213 (0)	9.0	NA[c]	NA
	ATCC 25927	<DL	9.1	NA	NA
	ATCC 29926	313 (2)	9.0	NA	NA
Enterobacter aerogenes	ATCC 13048	5,124 (346)	9.0	+	*E. aerogenes*
	ATCC 15038	5,399 (62)	9.0	+	*E. aerogenes*
	ATCC 29007	5,430 (65)	8.9	+	*E. aerogenes*
Photobacterium damselae	ATCC 29688	29 (8)	6.4	−	−
	ATCC 29689	17 (2)	5.8	−	−
	ATCC 29690	<DL	5.2	−	−

[a] Histamine levels are represented as averages with standard deviations in parentheses.
[b] DL, detection limit, 5 ppm.
[c] NA, not applicable.

recovered cells, 45 ml of sterile 0.1% peptone water was added (1:10) to each pouch, and the sample was stomached for 2 min; further 10-fold dilutions were made in 9.9 ml of peptone water before spiral plating (Autoplate 4000, Spiral Biotech, Norwood, MA) onto TSA (with 1.5% NaCl for *P. damselae*). Depending on the organism tested, the plates were incubated for 48 to 72 h at 26 to 30°C before enumeration with a Q-Counter (Spiral Biotech) of colonies. *D*-values were calculated with the GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool) xla program *(24).*

Histamine production of strains. To confirm histamine production, each strain was inoculated into duplicate tubes of 10 ml of TSB containing 1.5% NaCl, 1% histidine hydrochloride monohydrate (MP Biomedical, Solon, OH), and 0.00005% pyridoxal phosphate (TSB+, Sigma, St. Louis, MO) and incubated for 48 h at 30°C. Final counts were determined after incubation by serially diluting in alkaline peptone water and spread plating on TSA with 1% NaCl. Plates were incubated at 30°C for 24 h for all strains except *P. damselae,* which was incubated for 48 h. Histamine concentrations were determined with a modification of the AOAC International fluorometric method *(1).* In brief, histamine was extracted from 1 ml of TSB+ cultures, with 9 ml of 75% methanol (Fisher Chemical, Pittsburg, PA) and centrifuged (centrifuge 5804R, Eppendorf, Hauppauge, NY) for 10 min at 5,000 × *g*. The supernatant was poured into a separate vial, and a 1-ml aliquot (either undiluted 1:10 or 1:100) of each extract was loaded onto an ion-exchange column (80 by 5 mm) of AG 1X-8 resin (Bio-Rad Laboratories, Hercules, CA) in hydroxide form. The column was eluted with 35 ml of deionized water, and each eluate was collected in a 50-ml conical tube containing 5 ml of 1 N HCl (Fisher Scientific). The final volume of each tube was brought up to 50 ml with deionized water. Five milliliters of the extract and histamine standards (50, 100, and 150 ppm of histamine free base; histamine dihydrochloride, Acros Organics, Geel, Belgium) were added individually to 50-ml conical tubes. Ten milliliters of 0.1 N HCl, 3 ml of 1 N NaOH, and 1 ml of 0.1% *o*-phtalaldehyde (Alfa Aesar, Ward Hill, MA) were added to the extracts or standards and mixed. Four minutes after the addition of the *o*-phtalaldehyde, the reactions were stopped by the addition of 3 ml of 3.57 N H_3PO_4 (Sigma-Aldrich). The amount of derivatized histamine in each tube was determined fluorometrically in dark-bottom 96-well plates by using a SpectraFluor Plus fluorometer (Tecan, Männedorf, Switzerland) at an excitation of 360 nm and emission of 465 nm.

Statistics. Means and standard deviations of the *D*-values for at least three independent experiments for each organism at each temperature were determined and compared; their variances were analyzed with Minitab Release 14 software (one-way analysis of variance, Tukey's 95% simultaneous confidence intervals—all pairwise comparisons).

RESULTS

The three *M. morganii* and *E. aerogenes* strains produced between 4,457 to 5,391 and 5,124 to 5,430 ppm of histamine after 48 h of incubation in histidine broth at 30°C (Table 2). Two of the *R. planticola* strains produced between 4,767 and 5,319 ppm of histamine, but one strain (ATCC 33531) produced no histamine (<5 ppm), despite reaching a density of 9.1 log CFU/ml at the end of the incubation period. This strain was also negative for the presence of the *hdc* gene. Two of the *P. damselae* strains produced low (17 to 29 ppm) and one strain produced no (<5 ppm) histamine, but these strains grew poorly (<6.5 log CFU/ml) under the experimental conditions. Two *H. alvei* strains produced low histamine (213 to 313 ppm), and one strain (ATCC 25927) produced no histamine, despite growing to a density of 9.1 log CFU/ml. All strains that produced toxic levels of histamine (>500 ppm) were positive for total and species-specific *hdc* gene (Table 2).

The *D*- and *z*-values of *M. morganii, E. aerogenes, H. alvei,* and *R. planticola* are presented in Table 3. A three-strain composite for each organism tested was used to determine the thermal parameters of histamine-producing bacteria in irradiated albacore tuna loins. For *M. morganii,* the most heat-resistant organism tested in this study, *D*- and *z*-values in skipjack tuna were also determined (Table 3). The results

TABLE 3. D- *and* z-*values of histamine-producing bacteria in irradiated albacore tuna loins*[a]

Organism	Mean *D*-value ± SD (min) at temp (°C) of:					*z*-value (°C)
	50	54	56	58	60	
Morganella morganii	49.7 ± 17.57	8.2 ± 1.7 A[b]	2.8 ± 1.06 A	0.9 ± 0.24 A	0.1 ± 0.08	3.8
	ND[c]	6.6 ± 1.52 A[d]	2.3 ± 0.28 A[d]	0.4 ± 0.17 B[d]	ND	3.3
Raoultella planticola	40.0 ± 4.95	2.3 ± 0.24 C	0.5 ± 0.18 B	ND	ND	3.2
Hafnia alvei	42.6 ± 1.90	2.6 ± 0.48 C	0.9 ± 0.34 B	ND	ND	3.7
Enterobacter aerogenes	51.8 ± 17.38	5.2 ± 1.26 B	0.8 ± 0.08 B	0.4 ± 0.13 B	ND	3.6

[a] $n = 3$. Means and standard deviation were calculated from enumeration data obtained from three independent experiments, except if otherwise noted.
[b] Capital letters designate significant ($P < 0.05$) differences of the *D*-values within the same column.
[c] ND, not determined.
[d] Skipjack tuna.

demonstrate that *M. morganii* was the most heat-resistant bacteria in tuna loins, followed by *E. aerogenes, H. alvei,* and *R. planticola* (Table 3). Preliminary studies showed that *P. damselae* was extremely heat sensitive compared with the other four organisms at all temperatures tested. A >5.9-log reduction of *P. damselae* occurred within 10 min at 50°C, while for all other organisms tested, a heat treatment of ≥40 min was needed to achieve only 1-log reduction at 50°C (Table 3). In general, *M. morganii* had significantly higher ($P < 0.05$) *D*-values at all temperatures tested in albacore tuna when compared with *E. aerogenes, H. alvei,* and *R. planticola,* except at 50°C, where *D*-values were not statistically different for all four of these organisms (Table 3). Significantly higher *D*-values ($P < 0.05$) for *M. morganii* were observed in albacore than in skipjack tuna at 58°C, but not at 54 and 56°C (Table 3). Thermal inactivation curves for average *D*-values for *M. morganii, E. aerogenes, H. alvei,* and *R. planticola* were determined (Fig. 1), as well as thermal death curves for maximum *D*-values for *M. morganii, E. aerogenes, H. alvei,* and *R. planticola* (Fig. 2).

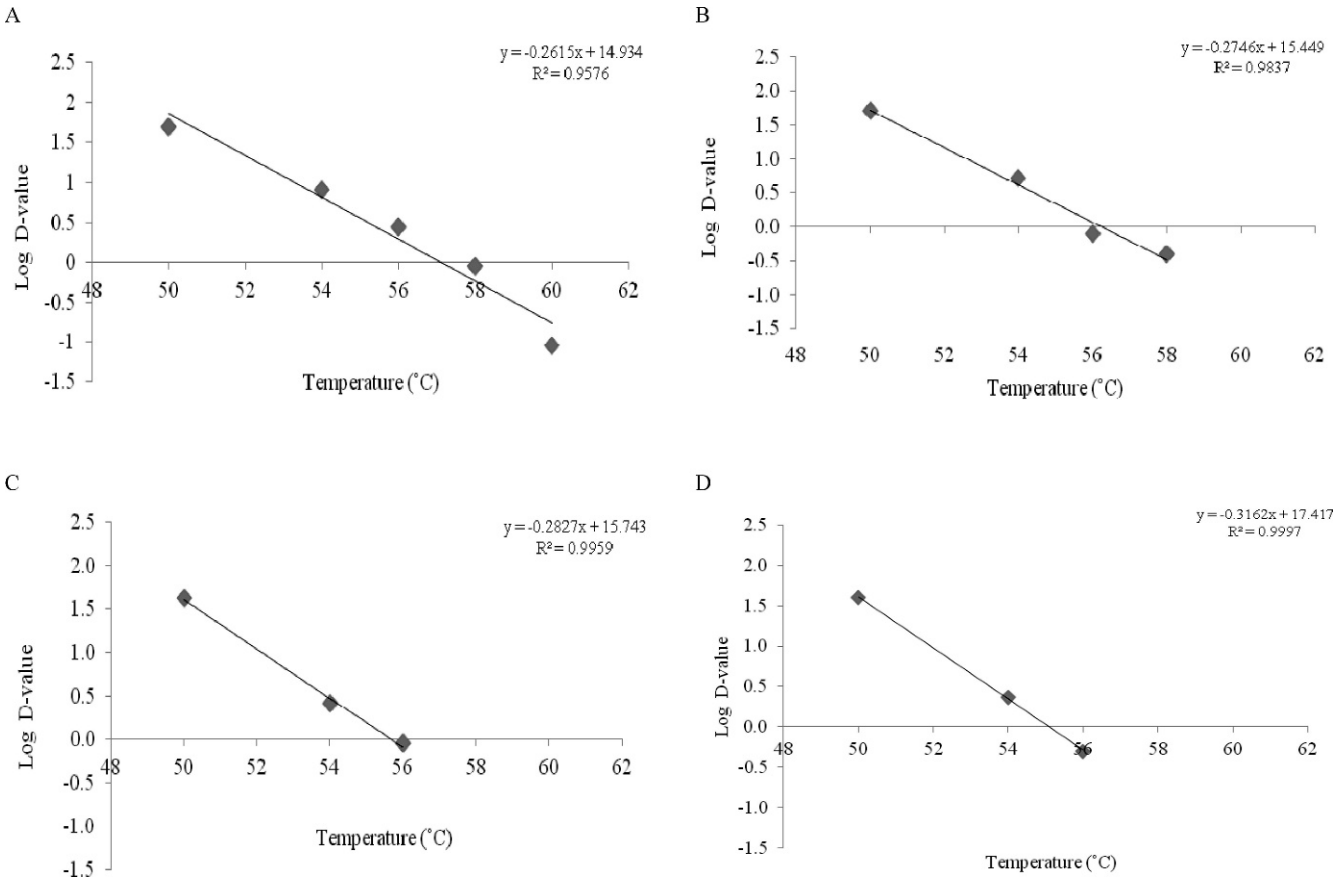

FIGURE 1. *Thermal death curves for the average* D-*values of* Morganella morganii *(A),* Enterobacter aerogenes *(B),* Hafnia alvei *(C), and* Raoultella planticola *(D) across the temperatures tested* (z-*values presented in Table 3).*

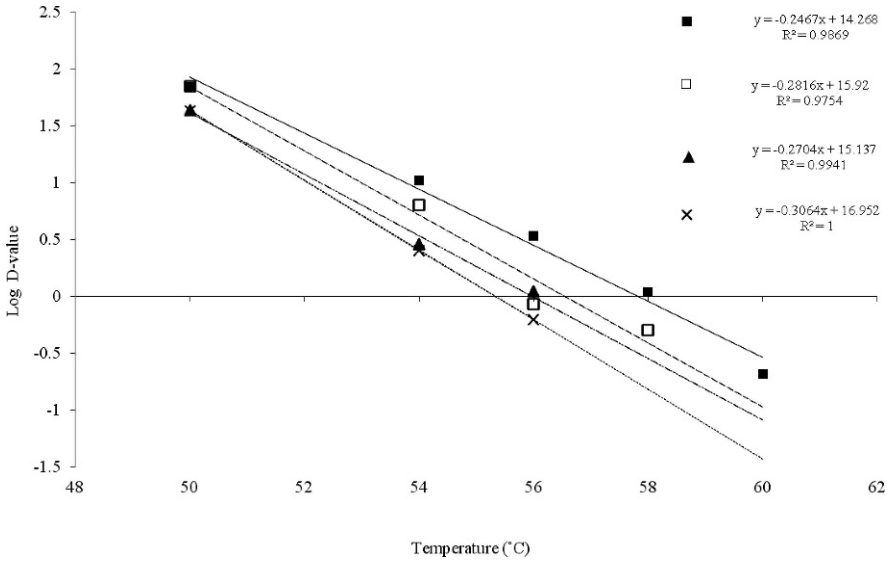

FIGURE 2. *Thermal death curves for maximum* D*-values of Morganella morganii (■),* Enterobacter aerogenes *(□),* Hafnia alvei *(▲), and* Raoultella planticola *(×), with z-values of 4.1, 3.6, 3.7, and 3.3°C, respectively.*

DISCUSSION

In this study, we determined and compared the thermal resistance of five bacterial species in inoculated tuna loins. This study demonstrated that *M. morganii* was the most heat-resistant bacteria of those tested. This information is important for the seafood industry, because *M. morganii* has been identified as the most prolific and prevalent histamine-producing bacteria in fish *(7, 16, 19, 22, 29–31, 33, 40).* To date, only a few studies have characterized the thermal resistance of histamine-producing bacteria in fish products *(8, 16, 40).* The *D*- and *z*-values for *M. morganii* obtained in our study were considerably different from those reported in laboratory media by Emborg and Dalgaard *(16).* In the latter study, the authors reported a $D_{50°C}$ of 13.1 min for *M. morganii* inoculated in Luria-Bertani broth supplemented with amino acids and a *z*-value of 7.2°C in the temperature range of 50 to 60°C. In contrast, we found a more conservative $D_{50°C}$ for the same organism in tuna loin, 49.7 min, with a *z*-value of 3.8°C. It has been reported that experimental factors including heating menstruum could have an influence on heat-resistance parameters. For example, Bremer et al. *(8)* reported *D*-values of 0.63, 0.36, 0.20, 0.11, and 0.006 min at 54, 55, 56, 57, and 58°C, respectively, with a corresponding *z*-value of 4.14°C for *H. alvei* in a model suspension of 0.1% peptone water. The *D*-values of the same organism were about two times greater (1.42, 0.74, 0.38, and 0.20 min at 54, 55, 56, and 57°C, respectively) in hot-smoked kahawai, with a *z*-value of 3.85°C *(8).* The *D*-values of *H. alvei* observed in tuna loins in the present study were higher, 42.6 ± 1.9, 2.6 ± 0.5, and 0.9 ± 0.3 min at 50, 54, and 56°C, respectively, with a *z*-value of 3.7°C. All organisms tested, except for *P. damselae,* had $D_{50°C}$ of ≥40 min, and *z*-values in the range of 3.2 to 3.8°C. Similar *z*-values have been reported for *H. alvei* and *M. morganii* in hot-smoked kahawai *(8, 40).*

Inactivation of histamine-producing spoilage bacteria including *M. morganii* occurs during proper canning or thorough cooking. However, histamine is heat resistant and not destroyed during the canning process; therefore, if histamine is produced in the product before retort canning, it can cause illness if present in the product at toxic concentrations. A study by Kim et al. *(29)* showed that toxic levels of histamine (≥500 ppm) are produced by *M. morganii* at temperatures between 15 and 37°C, with the highest levels (5,253 ppm) of histamine produced when *M. morganii* was incubated at 25°C for 48 h. Lower histamine levels were detected after incubation at 37°C for 24 h (1,949 ppm), or at 15°C, where 3 days (2,769 ppm) were needed for *M. morganii* to reach the stationary phase. Furthermore, rapid histamine formation was reported in yellow fin tuna fillets inoculated with *M. morganii* after storage at 24 and 30°C, reaching toxic levels at both storage temperatures, whereas low enzyme activity was detected after storage at 15°C *(33).* Toxic levels of histamine were also reported in fillets, minced meat, and liver samples of mackerel after 18 days of storage at 0°C *(20, 21).*

Klausen and Huss *(32)* reported that histamine levels in histidine-containing broth and mackerel samples inoculated with *M. morganii* and preincubated at 25°C before storage at 0 to 5°C exceeded the levels in samples stored at 25°C. This information demonstrates that prolonged time at ambient temperature between refrigeration and the precook steps can contribute to accumulation of histamine in fish prior to processing. In order to prevent histamine formation in fish, it is essential to chill the fish immediately after harvest (i.e., hold fish in refrigerated seawater, ice slurry, or brine at ≤4.4°C) *(49).* Rapid chilling is essential, especially for fish exposed to warm environments (water or air); for tuna, it is even more critical, because these species are able to generate heat in their tissues after harvest *(46).* It is noteworthy to mention that although fresh fish is virtually free of histamine, in practice, acceptable fish products can contain traces of histamine at levels approaching 50 ppm in the United States or 100 to 200 ppm in Europe and New Zealand *(22, 27, 46).*

The *D*-values determined in this study could be useful to the tuna industry for assessing the impact of precooking temperatures on thermal death rates for the five histamine-producing bacteria evaluated in the study. The thermal death curve for the maximum *D*-values of *M. morganii,*

represented by the highest value obtained of the three individual experiments conducted at each temperature, was above all the other thermal death curves determined in this study (Fig. 2), with a calculated z-value of 4.1°C. Using the maximum $D_{60°C}$ of *M. morganii* (0.26 min) and z-value of 4.1°C (Fig. 2) to calculate cumulative lethality during the precook step is a more conservative approach than using the average D-values at the same temperature and the corresponding z-value of 3.8°C (Fig. 1) *(17, 36)*.

In conclusion, a heat treatment designed to control *M. morganii* in tuna loins will similarly control the other histamine-producing bacteria tested in this study.

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