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Formation of Histamine and Biogenic Amines in Cold-Smoked Tuna: An Investigation of Psychrotolerant Bacteria from Samples Implicated in Cases of Histamine Fish Poisoning

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ABSTRACT

Two outbreaks and a single case of histamine fish poisoning associated with cold-smoked tuna (CST) were reported in Denmark during 2004. The bacteria most likely responsible for histamine formation in CST implicated in histamine fish poisoning was identified for the first time in this study. Product characteristics and profiles of biogenic amines in the implicated products were also recorded. In the single poisoning case, psychrotolerant *Morganella morganii*–like bacteria most likely was responsible for the histamine production in CST with 2.2% ± 0.6% NaCl in the water phase (WPS). In outbreak 1, *Photobacterium phosphoreum* most likely formed the histamine in CST with 1.3% ± 0.1% WPS. In outbreak 2, which involved 10 persons, the bacteria responsible for histamine formation could not be determined. The measured concentrations of WPS were very low compared with those of randomly collected commercial samples of CST and cold-smoked blue marlin (4.1 to 12.7% WPS). Challenge tests at 5°C with psychrotolerant *M. morganii* and *P. phosphoreum* in CST with 4.4% WPS revealed growth and toxic histamine formation by the psychrotolerant *M. morganii*–like bacteria but not by *P. phosphoreum.* In a storage trial with naturally contaminated CST containing 6.9% WPS, lactic acid bacteria dominated the microbiota, and no significant histamine formation was observed during the shelf life of about 40 days at 5°C and of about 16 days at 10°C. To prevent toxic histamine formation, CST should be produced with >5% WPS and distributed with a declared 5°C shelf life of 3 to 4 weeks or less.

Histamine fish poisoning (HFP) is a foodborne chemical intoxication primarily caused by ingestion of fish muscle tissue containing a high concentration of free histidine. During storage, the histidine can be converted into histamine by bacterial decarboxylation. HFP is a relatively mild disease with allergy-like symptoms, including rash, nausea, headache, and sometimes diarrhea. HFP is associated with seafood containing histamine at greater than 500 to 1,000 mg/kg and is a common form of fish poisoning, but many HFP incidents probably go unreported because of the mildness of the disease and misdiagnosis *(33, 51).*

In the United States, about 200 HFP outbreaks with more than 1,000 cases were recorded between 1990 and 2003, corresponding to more than 40% of all finfish-associated human disease outbreaks *(5).* It is not clear if any of these outbreaks were caused by smoked fish. However, in New Zealand hot-smoked kahawai (*Arripis trutta*) with histamine at as much as 2,000 mg/kg has caused several HFP outbreaks *(18, 41).* Hot-smoked mackerel has also been implicated in many HFP outbreaks, e.g., in Britain during the mid-1970s coincident with a change in fish consumption from herring to mackerel *(33, 51).* We have found no previous reports of cold-smoked fish causing HFP. However, a single case and two outbreaks of HFP associated with cold-smoked tuna (CST) occurred in Denmark during 2004.

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Published information about CST is sparse, but according to Paleari and Soncini *(46)* and Nicolaides and Fuchs *(42)* tuna loins are salted to 3 to 4.5% and then smoked. This process can result in a product with a shelf life of 60 days when stored at 5°C *(46).* Thus, CST processing is similar to that of cold-smoked salmon, in which both processing and product characteristics have been extensively studied. For sliced and vacuum-packed cold-smoked salmon, shelf life depends on factors other than just the storage temperature. Thus, at 5°C, shelf life can be as short as 2 weeks or as long as 12 weeks depending on the concentration of NaCl and smoke components in the product *(19, 20, 35, 37).* Temperature, NaCl, and smoke components most likely have a similar effect on shelf life of CST, although this effect has not been documented. The spoilage microbiota of sliced and vacuum-packed cold-smoked salmon is dominated by lactic acid bacteria (LAB), which can occur with *Photobacterium phosphoreum* or members of the *Enterobacteriaceae (20, 21, 24, 36).* Low concentrations of biogenic amines can be formed in sliced and vacuum-packed cold-smoked salmon, and at 5°C these compounds are primarily produced by *P. phosphoreum (24, 25).* Fortunately, Atlantic salmon (*Salmo salar*) has a low concentration of free histidine of about 100 to 200 mg/kg in muscle tissue *(17),* and sliced and vacuum-packed cold-smoked salmon has not been associated with HFP, although this product is produced and consumed in large quantities.

In contrast, fillets of tuna contain high concentrations of free histidine, typically more than 10,000 mg/kg *(2, 16)*. Therefore, growth and activity of histamine-producing bacteria must be controlled in CST. *P. phosphoreum* and psychrotolerant bacteria similar to *Morganella morganii* are of particular concern because they form histamine at low temperatures and are known to be present in fresh tuna *(16)*. Nevertheless, we have found no studies on interventions used with CST for the control of histamine formation during chilled distribution of this product.

The objective of the present study was to evaluate formation of histamine and other biogenic amines in CST. CST involved in a single case and two outbreaks of HFP in Denmark during 2004 were evaluated with respect to product characteristics, profiles of biogenic amines, and dominating microbiota. The microorganisms most likely responsible for histamine formation were identified. Challenge tests were then conducted at 5°C to evaluate the ability of psychrotolerant variants of *M. morganii* and *P. phosphoreum* to form histamine in vacuum-packed (VP) CST. Storage trials with naturally contaminated VP CST also were conducted to evaluate the effects of storage temperature (5 or 10°C) and a previous period of frozen storage (1 month at −24°C) on the formation of histamine and biogenic amines in this product.

MATERIALS AND METHODS

HFP cases associated with consumption of CST. HFP outbreak 1 took place in January 2004. Two persons became ill after eating CST in their home. Clinical signs, including nausea, rash, and a tingling sensation in the mouth, were observed 45 min after consumption and lasted for about 12 h. In late March 2004, one person at a private party became ill with HFP after eating CST. Clinical signs, including rash, increased pulse, and hyperventilation, became evident shortly after consumption and lasted for about 2 h. Outbreak 2 occurred in September 2004 and involved 10 persons who experienced diarrhea, general malaise, and vomiting after ingestion of CST from a buffet at a cantina *(31)*.

CST samples from the single case and the two outbreaks were analyzed in duplicate by the Danish Veterinary and Food Administration. Analyses were conducted within 24 h after the illnesses were reported. Prior to analysis, samples were stored on ice to reduce further formation of biogenic amines and growth of bacteria. Biogenic amines were evaluated by extraction with perchloric acid, derivatization with dansyl-chloride, and separation by high-pressure liquid chromatography (HPLC) *(15)*. The pH values and dry matter, NaCl, lactic acid, lipid, smoke component, and free amino acid concentrations were determined as previously described *(3, 9, 10, 16, 19)*. Microbiological analyses included counts of aerobic bacteria (APCs; on Long and Hammer agar at 15°C for 5 days), *Enterobacteriaceae* (on tryptic soy agar–violet red bile glucose agar at 25°C for 48 h), and LAB (on nitrite-actidion-polymyxin agar at 25°C for 3 days) as previously described *(16)*.

For samples from outbreak 1 and the single case, 21 and 13 colonies were isolated from the Long and Hammer plates, respectively. The number of each type of isolated colony was proportional to the percentage of the total number of colonies. For samples from outbreak 2, no bacteria were isolated because of very low concentrations of microorganisms in the samples, which had been frozen before analysis. The bacteria were isolated in pure culture and characterized based on cell morphology, motility, Gram reaction (3% KOH), catalase and cytochrome oxidase production, and glucose fermentation *(4)*. Gram-negative isolates were further tested for sensitivity to vibriostaticum (O/129, 150 μg), arginine, lysine, and ornithine metabolism and reduction of trimethylamine-*N*-oxide. Gram-negative, fermentative, oxidase-negative (or late positive), O/129-sensitive isolates (similar to *P. phosphoreum*) were further tested for bioluminescence, reduction of nitrate, production of gas from glucose, hydrolysis of gelatin, assimilation of D-mannitol, growth without NaCl, and growth at 0 and 37°C *(11)*. Gram-negative, catalase- and oxidase-positive, nonfermentative, O/129-resistant motile rods unable to reduce trimethylamine-*N*-oxide (similar to *Pseudomonas*) were further tested for liquefaction of gelatin, production of acid from maltose, production of indole from tryptophane, and fluorescence on King B agar *(47, 49, 52)*. *Enterobacteriaceae*-like gram-negative, oxidase-negative, catalase-positive, fermentative, O/129-resistant isolates were characterized with API 20E (bioMérieux, Marcy l'Etoile, France) and tested for production of phenylalanine deaminase, formation of gas from glucose, and fermentation of trehalose. Growth at 2 and 37°C also was determined for isolates similar to *M. morganii* in a nutrient-rich broth with 1.0% NaCl (growth medium broth [GMB]) *(12)*. Gram-negative, oxidase-positive, fermentative, O/129-resistant isolates assumed to be *Aeromonas* spp. were further tested for production of indole and hydrolysis of gelatin. Gram-positive, fermentative isolates were tested depending on their catalase reaction. Catalase-positive isolates expected to be *Brochothrix* spp. were tested for growth on streptomycin sulfate–thallium acetate–actidion agar (STAA; CM881, Oxoid, Basingstoke, UK) supplemented with glycerol and inhibiting agents (SR151, Oxoid), growth with 8 and 10% NaCl, and for fermentation of rhamnose *(50)*. Catalase-negative isolates expected to be LAB were tested for growth on acetate agar, final pH after growth in La broth (deMan, Rogosa, Sharpe [MRS] broth without phosphate buffer with 0.3% [wt/vol] sodium citrate replacing ammonium citrate and adjusted to an initial pH of 6.8), and production of gas from glucose and gluconate. From this group, *Carnobacterium*-like isolates were tested for production of acetoin from glucose and NH_3 from arginine, fermentation of inulin, D-mannitol, methyl-α-D-glycoside, methyl-α-D-mannoside, and D-xylose *(7)*. All biochemical identification tests were conducted as previously described *(11, 13)*.

Isolates were identified with general keys *(4)*, keys for specific species, or the API LAB plus software (bioMérieux) for *Enterobacteriaceae*-like isolates. To confirm the identifications, the following type and reference strains were included in the study: *Brochothrix thermosphacta* (ATCC 11509^T), *Carnobacterium piscicola* (DSM 20730^T), *M. morganii* subsp. *morganii* (LMG 7874^T), *M. morganii* subsp. *sibonii* (DSM 14850^T), and *P. phosphoreum* (NCIMB 13481).

Selected isolates from each group of bacteria were individually tested for production of histamine and biogenic amines using GMB at pH 5.8 with added arginine (200 mg/liter), lysine (500 mg/liter), histidine (15,000 mg/liter), phenylalanine (200 mg/liter), and tyrosine (200 mg/liter) (GMB-AA) to match the concentration of these compounds in tuna (Table 1). Tubes with GMB-AA were inoculated with the isolate in question at approximately 10^5 CFU/ml and incubated at 10°C. During incubation, growth was measured as the increase in absorbance at 540 nm until 24 h after the time when the absorbance no longer increased. After filter sterilization and appropriate dilution of the growth medium, the concentrations of biogenic amines (agmatine, β-phenylethylamine, cadaverine, histamine, putrescine, spermidine, spermine, trypt-

TABLE 1. *Characteristics, including microbial counts and concentrations of biogenic amines of cold-smoked tuna involved in one case and two outbreaks of histamine fish poisoning in Denmark, 2004*[a]

Characteristics	Outbreak 1 (2 persons)[b]	Single case (1 person)[c]	Outbreak 2 (10 persons)[d]
Dry matter (%)	28.5 ± 0.4	27.6 ± 0.6	NT[e]
NaCl (%)	0.9 ± 0.3	1.6 ± 0.3	3.6
Water phase salt (%)	1.3 ± 0.1	2.2 ± 0.4	NT
Smoke components (mg phenol/kg)	11.8 ± 6.3	NT	NT
Lipid (%)	0.4 ± 0.1	NT	NT
pH	6.1 ± 0.01	5.9 ± 0.01	NT
Free amino acids (mg/kg)			
Alanine	397 ± 28	647 ± 37	NT
Aspartic acid	87 ± 6	409 ± 29	NT
Cysteine	17 ± 24	44 ± 0	NT
Glutamic acid	27 ± 9	444 ± 42	NT
Glycine	158 ± 11	267 ± 17	NT
Histidine	7,156 ± 317	16,363 ± 867	NT
Isoleucine	169 ± 14	238 ± 9	NT
Leucine	318 ± 17	344 ± 6	NT
Lysine	15 ± 2	401 ± 13	NT
Methionine	167 ± 10	155 ± 10	NT
Ornithine	128 ± 31	379 ± 11	NT
Phenylalanine	123 ± 2	311 ± 9	NT
Proline	129 ± 8	264 ± 7	NT
Serine	98 ± 7	126 ± 10	NT
Theronine	204 ± 16	305 ± 13	NT
Tryptophan	<5[f]	129 ± 2	NT
Tyrosine	5 ± 2	51 ± 6	NT
Valine	277 ± 18	451 ± 17	NT
Lactic acid (mg/kg)	12,612 ± 972	NT	NT
Microbial counts (log CFU/g)			
Aerobic plate count	7.3 ± 0.6	8.2	3.7
Enterobacteriaceae	2.5 ± 2.1	5.8	<1
Lactic acid bacteria	6.8 ± 0.9	8.0	NT
Biogenic amines (mg/kg)			
Agmatine	99 ± 7	NT	NT
β-Phenylethylamine	54 ± 4	<5	<5
Cadaverine	212 ± 5	132 ± 5	68 ± 1
Histamine	4,548 ± 123	1,972 ± 4	914 ± 8
Putrescine	20 ± 0.3	6 ± 0.2	<5
Serotonin	<5	<5	<5
Spermidine	<5	<5	<5
Spermine	14 ± 0.0	<5	<5
Tryptamine	<5	<5	<5
Tyramine	150 ± 0	88 ± 3	23 ± 0.3

[a] Values are the mean ± standard deviation.
[b] One piece of CST was divided into two samples and analyzed in duplicate.
[c] One sample was analyzed in duplicate.
[d] A very small sample of frozen tuna was delivered for analysis.
[e] NT, not tested.
[f] Tested but not detected.

amine, and tyramine) were determined by HPLC as previously described *(25)*.

Formation of biogenic amines in a challenge test with psychrotolerant bacteria in chilled CST. CST used in the challenge test was obtained from a local processor as four whole frozen loins. The product was produced from frozen tuna loins from Indonesia. The loins were thawed, salted with a combination of brine injection and addition of dry salt, and then stored at 0 to 2°C for 2.5 days. The frozen loins were steeped in water (30% loins and 70% water) at 2°C for 1 h, dried, and then smoked at 27°C for 3 h. The smoked loins were chilled to 0°C, vacuum packed, and kept frozen at −24°C until used in the challenge tests.

Prior to the challenge test, VP CST loins were thawed for 24 h at 5°C and sliced. The characteristics of the CST were de-

termined with respect to concentrations of NaCl, free amino acids, lipids, lactic acid, and smoke components. The analyses were conducted in triplicate as described previously. The CST slices were divided randomly into three treatment groups. One group was inoculated with a mixture of strains of *M. morganii*–like bacteria (mix M) at approximately 10^4 to 10^5 CFU/g. This mix included three isolates from CST implicated in the single 2004 HFP case and three isolates from the spoilage microbiota in fresh tuna *(16)*. Another group of slices was inoculated with a mixture of *P. phosphoreum* strains (mix P) at approximately 10^4 to 10^5 CFU/g. This mix included three isolates from outbreak 1 and three isolates from the spoilage microbiota in fresh tuna *(16)*. All isolates were precultured at 10°C in GMB at pH 5.8. These 10°C cultures in late exponential growth phase were mixed and diluted to 10^6 to 10^7 CFU/g in chilled 0.85% NaCl prior to inoculation. The CST slices were placed in sterile plastic bags, inoculated with 1% diluted mixtures, and mixed for about 2 min. The third group of slices served as an uninoculated control. CST slices from all three groups were vacuum packed in 50-g portions with a packaging film of low gas permeability (NEN 40 HOB/LLPDE 75, Amcore Flexibles, Horsens, Denmark) and stored at 5°C. At regular intervals during storage, three packages from each group were removed for determination of APCs, *Enterobacteriaceae* and *P. phosphoreum* counts, biogenic amine concentrations, and pH as previously described *(16)*. Growth of *Brochothrix* and *Pseudomonas* was evaluated on STAA and cetrimide-fucidin-cephaloridine medium (CN559, SR103, Oxoid) at 25°C for 48 h in the noninoculated control group. At each sampling time, off-odor development for each sample group was evaluated by two trained persons with experience in sensory evaluation of seafood. Off-odors were graded as acceptable or unacceptable. The shelf life of a group of CST samples was defined as the time when off-odors were considered unacceptable in one of the three analyzed samples. This method was chosen because of uneven distribution of NaCl in the CST samples, which resulted in substantial variation in concentrations of bacteria and in sensory characteristics among samples.

Storage trial with naturally contaminated CST. CST was obtained from the same local processor that provided the product used in the challenge tests. After production, one third of the loins were sliced, vacuum packed, and stored at 5.5°C. The remaining two thirds of the loins were sliced, vacuum packed, and stored at −24°C for 4 weeks, after which they were thawed and used in storage trials at 5.5 and 10°C. On day 0, the CST slices were evaluated for pH and concentrations of NaCl, dry matter, organic acids, smoke components, and lipids. Total volatile nitrogen (TVN) concentration also was determined, as described previously *(9)*. At regular intervals during storage, samples were withdrawn for microbial (APCs, LAB, and *Enterobacteriaceae*) and sensory analyses, which were performed in triplicate. When APCs rose above 6 log CFU/g, TVN, pH, and the concentration of organic acids and biogenic amines were also determined. On the day that off-odors were deemed unacceptable, isolates from the dominating microbiota were obtained. Twenty-nine isolates were obtained from the CST samples stored at 5.5°C, and 12 and 13 isolates were obtained from the CST samples that had been frozen, thawed, and then stored at 5.5 or 10°C, respectively. Bacteria were isolated in pure culture and tentatively identified.

Statistical analysis. All microbial counts were converted to log CFU per gram or log CFU per milliliter before analysis. Significant differences ($P < 0.05$) among means were determined with a one-way analysis of variance.

RESULTS

Cases of HFP associated with CST. The mean (±standard deviation) concentrations of histamine in the CST associated with HFP outbreak 1, the single case, and outbreak 2 were 4,548 ± 123, 1,972 ± 4, and 914 ± 8 mg/kg, respectively (Table 1). These relatively high concentrations of histamine most likely caused the observed HFP clinical signs. However, the implicated CST samples also contained high concentrations of free histidine, indicating that substantially more histamine could potentially be formed by microbial activity (Table 1). The concentrations of other biogenic amines were markedly lower than those of histamine (Table 1). For the CST implicated in outbreak 1, the microbiota of about 18×10^6 CFU/g was dominated by LAB, *P. phosphoreum*, *B. thermosphacta*, and *Pseudomonas fluorescens* (Table 2). Only *P. phosphoreum* formed histamine, and the types and concentrations of biogenic amines produced by *P. phosphoreum* was very similar to those produced by the total microbiota in the CST implicated in outbreak 1 (Table 2). Thus, *P. phosphoreum* most likely formed the histamine responsible for the clinical signs of HFP in outbreak 1.

In the sample of CST associated with the single case of HFP, the microbiota of about 160×10^6 CFU/g was dominated by psychrotolerant *M. morganii*–like bacteria (39%), *Carnobacterium* spp. (31%), *Pseudomonas* (15%), and *Aeromonas*- and *Providencia*-like isolates (15%). Histamine was formed by the psychrotolerant *M. morganii*–like isolates (8,234 ± 368 mg/liter) and *Aeromonas* (52 ± 18 mg/liter), whereas other bacteria did not form detectable concentrations. Thus, histamine formation by psychrotolerant *M. morganii*–like bacteria most likely explained the high concentration of histamine detected in the sample of CST associated with the single case of HFP (Table 1). The *M. morganii*–like bacteria grew at 2°C but not at 37°C. The *Aeromonas*-like isolate formed cadaverine (462 ± 15 mg/kg), and the *Carnobacterium* spp. isolates formed tyramine (252 ± 17 mg/kg). The detected concentrations of cadaverine and tyramine in CST (Table 1) therefore may result from activity of these bacteria. Very little of the CST associated with HFP outbreak 2 was available for analyses, and only a few selected tests could be conducted (Table 1).

The CST samples associated with outbreak 1 contained 1.3% ± 0.1% water phase salt (WPS), the sample associated with the single HFP case contained 2.2% ± 0.4% WPS, and that associated with outbreak 2 contained approximately 5% WPS (extrapolated from the measured 3.6% NaCl in the product). Similar cold-smoked products from the Danish retail market contained significantly higher WPS concentrations ($P < 0.05$) than the products associated with these cases of HFP (Table 3). No temperature profiles were available for the CST samples associated with these HFP cases, and it is not known whether the products had been exposed to temperatures above 5°C. At 5°C, psychrotolerant *M. morganii*–like bacteria and *P. phosphoreum* are expected to form histamine in products with 1.3% ± 0.1% and 2.2% ± 0.4% WPS, respectively, but it is not

TABLE 2. *Production of biogenic amines by microbiota isolated from cold-smoked tuna involved in outbreak 1*

Microorganisms	No. of isolates	No. of isolates tested	Biogenic amine concentration[a]					
			Put	Tyr	Cad	His	β-phe	Tryp
Broth culture at 10°C (mg/ml)								
Brochothrix thermosphacta	4	1	9 ± 0.4	<5[b]	<5	<5	<5	<5
Lactic acid bacteria	10	5	9–9	23–275	<5	<5	<5	<5–154
Photobacterium phosphoreum	5	1	6 ± 1	290 ± 17	107 ± 12	3,777 ± 334	60 ± 5	6 ± 1
Pseudomonas fluorescens	2	1	78 ± 0	<5	<5	<5	<5	<5
Total from product (mg/kg)			20 ± 0.3	150 ± 0.0	212 ± 5	4,548 ± 123	54 ± 4	<5

[a] Put, putrescine; Tyr, tyramine; Cad, cadaverine; His, histamine; β-phe, β-phenylethylamine; Tryp, tryptamine. Values for all except lactic acid bacteria are reported as the mean ± standard deviation. Values for LAB are reported as a range because of taxonomic variability within the group, which included *Lactobacillus* and *Weisella*–like bacteria, *Carnobacterium divergens*, and two nonaciduric but unidentified LAB isolates.

[b] Tested but not detected.

clear whether histamine production occurs at this temperature in more appropriately salted CST.

Formation of biogenic amines in challenge tests with psychrotolerant bacteria in chilled CST. CST used for the challenge test contained 4.4% ± 0.8% WPS, and substantial WPS variation was detected among samples (3.2 to 5.1%). Concentrations of smoke components at 3.0 ± 1.3 mg/kg phenol, lipids at 1.1% ± 0.5%, pH at 5.8 ± 0.1 were measured. Concentrations of lactic acid and free histidine were 14,864 ± 779 and 12,470 ± 72 mg/kg, respectively, and thus the substrate for production of histamine was available. None of the other amino acids were detected in such high concentrations: alanine, 225 ± 3 mg/kg; aspartic acid, 19 ± 1 mg/kg; cysteine, 0 ± 0 mg/kg; glutamic acid, 189 ± 1 mg/kg; glycine, 78 ± 1 mg/kg; isoleucine, 91 ± 2 mg/kg; leucine, 169 ± 0 mg/kg; lysine, 180 ± 3 mg/kg; methionine, 73 ± 2 mg/kg; phenylalanine, 93 ± 0 mg/kg; proline, 33 ± 6 mg/kg; serine, 67 ± 3 mg/kg; threonine, 108 ± 1 mg/kg; tyrosine, 80 ± 2 mg/kg; and valine, 125 ± 18 mg/kg.

The psychrotolerant *M. morganii*–like bacteria grew from 4.1 ± 0.2 to 6.9 ± 0.3 log CFU/g and produced >3,500 mg/kg histamine in CST samples during 55 days of storage at 4.9 ± 0.5°C (Fig. 1). However, CST inoculated with *M. morganii* developed unpleasant off-odors after 26 to 36 days, and at this stage maximum histamine concentrations in individual packs were 642 to 1,139 mg/kg (Fig. 1). The CST samples were inoculated with *P. phosphoreum* at 3.7 ± 0.4 log CFU/g; however, these bacteria did not grow and did not produce histamine (Fig. 1 and Table 4). In the noninoculated CST samples stored at 5.1 ± 0.5°C, no histamine was detected during the 55 days of storage (Fig. 1 and Table 4) and no indication of spoilage was observed. At day 55, the microbiota was dominated by *Brochothrix* (Table 4). The tyramine concentration of 170 mg/kg (Table 4) was probably formed by LAB, which reached a maximum concentration of 6.4 ± 0.3 log CFU/g after 35 days of storage.

Storage trial with naturally contaminated CST. The concentration of free histidine in the CST samples was 14,488 ± 1,928 mg/kg, but regardless of whether the product had been previously frozen, little histamine was formed in the product within the shelf life of approximately 40 days at 5.5 ± 0.8°C (Table 5). None of the other amino acids were detected in high concentrations: alanine, 311 ± 96 mg/kg; aspartic acid, 123 ± 68 mg/kg; cysteine, 33 ± 13 mg/kg; glutamic acid, 214 ± 100 mg/kg; glycine, 125 ± 32 mg/kg; isoleucine, 142 ± 86 mg/kg; leucine, 276 ± 135 mg/kg; lysine, 412 ± 286 mg/kg; methionine, 163 ± 62 mg/kg; phenylalanine, 159 ± 42 mg/kg; proline, 109 ± 121 mg/kg; serine, 123 ± 47 mg/kg; threonine, 171 ± 80 mg/kg; tyrosine, 163 ± 46 mg/kg; and valine, 206 ± 108 mg/kg. The concentration of lactic acid was 11,810 ± 351 mg/kg.

For the fresh CST used for the storage trial, the aerobic bacteria, LAB, and *Enterobacteriaceae* counts were 3.8 ± 0.4, 4.5 ± 1.6, and 2.52 ± 0.2 log CFU/g, respectively. In the thawed CST samples, the respective concentrations

TABLE 3. *Concentrations of dry matter and NaCl in samples of various commercial products*[a]

Product	Salt (%)	Dry matter (%)	Water phase salt (%)
Cold-smoked tuna			
Product a	6.5 ± 0.01	37.3 ± 0.06	9.4 ± 0.02
Product b	7.8 ± 0.1	46.4 ± 0.04	12.7 ± 0.1
Product c	5.2 ± 0.1	41.2 ± 0.03	8.1 ± 0.07
Product d	5.0 ± 0.1	43.2 ± 0.5	8.03 ± 0.2
Product e	3.8 ± 0.1	38.6 ± 0.2	5.8 ± 0.1
Cold-smoked blue marlin	2.7 ± 0.5	36.3 ± 0.0	4.1 ± 0.5

[a] Values are the mean ± standard deviation.

were 3.6 ± 1.2, 2.4 ± 0.5, and 1.2 ± 0.8 log CFU/g. The product shelf life of approximately 40 days was limited by development of unacceptable flavors, and concentrations of LAB higher than 7 log CFU/g were detected in the individual package with off-odors. During storage, the APC rose slowly, and LAB dominated the microbiota. No growth of *Enterobacteriaceae* was observed (Table 5). Frozen storage of CST did not prolong the shelf life of the thawed product (Table 5). As expected, shelf life at 10.6 ± 0.4°C was much shorter than that at 5.5 ± 0.8°C, but no histamine was formed and the microbiota was dominated by LAB (Table 5).

Regardless of treatment, variability in bacterial concentrations was observed among the three groups of CST samples during the storage period (Table 5). This variation may be explained by an uneven distribution of NaCl in samples; the WPS range for 12 samples was 3.7 to 11.4%, with a mean of 7.9% ± 2.6%.

The microbiota in CST samples stored at 5.5°C was dominated by aciduric and facultatively heterofermentative LAB (48%), *Brochothrix*-like bacteria (28%), nonaciduric and homofermentative LAB (10%), and nonaciduric and obligately heterofermentative LAB (7%). The remaining 7% of the bacteria were unidentified but were gram negative and nonfermenting. The microbiota of the thawed CST samples that were stored at 5.5°C consisted of unidentified aciduric facultatively heterofermentative LAB (75%) and *Brochothrix* spp. (25%). For CST stored at 10.6°C, the spoilage microbiota was dominated by aciduric facultatively heterofermentative LAB (46%), *Brochothrix*-like bacteria (30%), yeast (8%), nonaciduric and homofermentative LAB (8%), and nonaciduric and obligately heterofermentative bacteria (8%).

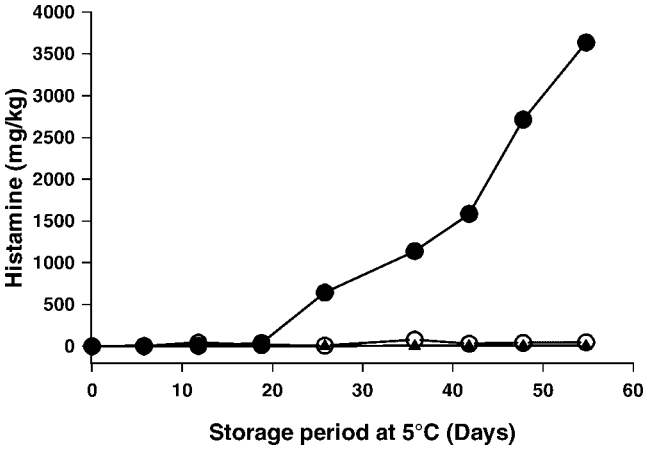

FIGURE 1. *Maximum concentration of histamine when three samples of vacuum-packed uninoculated cold-smoked tuna (▲), cold-smoked tuna inoculated with psychrotolerant* Morganella morganii*–like bacteria (●), and cold-smoked tuna inoculated with* Photobacterium phosphoreum *(○) were withdrawn at regular intervals during the storage period.*

CST samples used in the storage trial contained 0.6% ± 0.1% lipid, had a pH of 5.7 ± 0.04, and had a phenol concentration of 4.7 ± 1.2 mg/kg. The initial TVN concentration for the CST samples was 24.2 ± 2.3 mg/kg, and at the time that off-odors were detected only a small increase in TVN was measured (Table 5), indicating that this parameter is not suitable as an index for sensory spoilage of CST.

DISCUSSION

Biogenic amines including cadaverine and putrescine may increase the toxicity of histamine if their concentration is, for example, five times the concentration of histamine *(33)*. Profiles of biogenic amines are seldom included in outbreak reports. However, in CST samples associated with cases of HFP, we found histamine in concentrations much higher than those of other biogenic amines, in agreement with the concentrations of free amino acids in CST (Table 1). Thus, HFP associated with CST seems primarily due to high concentrations of histamine in the product.

Previously, *M. morganii*, *Raoultella planticola*, *P. phosphoreum*, and *Hafnia* have been identified as responsible for histamine formation in tuna and sardine products implicated in HFP cases, but only a few products from a limited number of outbreaks and cases have been studied *(16, 22, 26, 27, 34, 48)*. The present study identified for the first time the bacteria most likely responsible for histamine formation in CST associated with HFP in a single case and in outbreak 1, i.e., *M. morganii*–like bacteria and *P. phosphoreum*. Both of these histamine-producing bacteria are psychrotolerant, indicating that the products implicated in the HFP incidences may not necessarily have been exposed to elevated storage temperatures.

P. phosphoreum formed histamine in high concentrations (4,548 ± 123 mg/kg) in CST. The organism is widespread in the marine environment *(8)* and may have contaminated the tuna raw material or product during processing. *P. phosphoreum* grows well even at 0°C, and high

TABLE 4. *Shelf life and spoilage characteristics of vacuum-packed cold-smoked tuna in challenge tests at 5°C with psychrotolerant* Morganella morganii *and* Photobacterium phosphoreum[a]

Spoilage characteristics	Vacuum-packed CST inoculated with:		
	No bacteria (control)	*M. morganii*	*P. phopshoreum*
Bacterial counts (log CFU/g)			
Total viable bacteria	5.9 ± 0.5	6.5 ± 0.9	6.1 ± 0.1
Enterobacteriaceae	1.5 ± 0.8	6.3 ± 0.8	2.1 ± 0.3
P. phosphoreum	<0.6	NT	1.3 ± 0.4
Lactic acid bacteria	4.8 ± 0.8	4.5 ± 0.7	NT
Pseudomonas-like bacteria	4.0 ± 0.2	NT	NT
Brochothrix-like bacteria	5.5 ± 0.6	NT	NT
pH	5.8 ± 0.0	5.8 ± 0.0	5.8 ± 0.0
Biogenic amines (mg/kg)			
Histamine	<5[b]	256 ± 339	12 ± 16
Tyramine	170 ± 37	140 ± 64	115 ± 5
Cadaverine	<5	<5	<5
Putrescine	<5	<5	<5
β-Phenylethylamine	<5	<5	<5
Tryptamine	<5	13 ± 4	13 ± 1
Spermine	20 ± 1.9	52 ± 6	41 ± 4
Spermidine	5 ± 1.1	11 ± 4	6 ± 0.5
Agmatine	<5	<5	<5
Shelf life (days)	>55	26	42

[a] Values are the mean ± standard deviation. NT, not tested.
[b] Tested but not detected.

concentrations have been observed previously in VP fresh tuna and VP cold-smoked salmon *(16, 25, 43)*. For *P. phosphoreum*, histamine formation is optimal at 1 to 3% NaCl, and at 2 to 10°C formation of 4,000 mg/kg or more has been detected in broth and in VP fresh tuna *(16, 45)*. Thus, histamine formation by this bacterium in chilled CST with only 1.3% ± 0.1% WPS is possible. However, about 4% NaCl seems sufficient to prevent growth and histamine formation by *P. phosphoreum* when stored at 20°C *(44)*. In the present study, both growth and histamine formation

TABLE 5. *Shelf life and spoilage characteristics of naturally contaminated cold-smoked tuna stored at 5.5 and 10.6°C*[a]

Spoilage characteristics	Vacuum-packed CST stored at:		
	5.5°C, fresh	5.5°C, thawed	10.6°C, fresh
Bacterial counts (log CFU/g)			
Total viable bacteria	4.7–7.1	3.9–5.7	3.6–5.5
Enterobacteriaceae	1.9–3.8	0.7–1.8	1.3–3.6
Lactic acid bacteria	3.7–7.5	2.7–5.9	3.5–7.2
pH	5.74 ± 0.07	5.81 ± 0.09	5.73 ± 0.08
Biogenic amines (mg/kg)			
Histamine	0–4	0–15	<5[b]
Tyramine	0–12	7–18	0–43
Cadaverine	0–1	<5	0–6
Putrescine	<5	<5	<5
β-Phenylethylamine	<5	<5	0–11
Tryptamine	0–6	4–12	0–4
Spermine	15–21	15–20	12–18
Spermidine	4–7	5–7	4–7
Agmatine	<5	<5	<5
TVN	28.5 ± 4.9 (day 43)	31 ± 4.7 (day 49)	26.2 ± 1.7 (day 20)
Shelf life (days)	40	41	16

[a] Values are ranges or mean ± standard deviation.
[b] Tested but not detected.

(Fig. 1) were prevented in CST with 4.4% ± 0.8% WPS that was stored at 5°C.

Mesophilic *M. morganii* can produce toxic concentrations of histamine in seafood at or above 7 to 10°C *(28, 29, 39, 54)*. However, the psychrotolerant *M. morganii*–like bacteria most likely responsible for histamine formation in CST grew well at 2°C but not at 37°C (Table 3) and thus differ from the mesophilic *M. morganii*, which was previously studied extensively in relation to histamine formation in seafood. In contrast, the *M. morganii*–like bacteria from CST seem similar to the psychrotolerant histamine producers recently found in fresh tuna *(16)*. Kimata and Kawai *(30)* also described *M. morganii*–like bacteria (*Achromobacter histaminum* and *Proteus morganii*) that were unable to grow at 37°C, but evaluation of their growth at 2°C was not reported and we cannot determine whether these bacteria are similar to the psychrotolerant *M. morganii*–like bacteria isolated in the present study from cold-smoked and previously from fresh tuna *(16)*. Mesophilic *M. morganii* in tuna has been reported frequently as a naturally inhabitant of the gills and intestines that can be spread to other sites during handling *(14, 38–40)*. However, it is uncertain whether the behavior of psychrotolerant variants of *M. morganii* is similar. Nevertheless, to control histamine formation in seafood it is important to distinguish between mesophilic and psychrotolerant variants of *M. morganii*. Growth and histamine formation of the well-known mesophilic variant is controlled by storage of seafood below 5°C. In contrast, the psychrotolerant variant can grow and form toxic concentrations of histamine at 2°C in VP fresh tuna *(16)* and at 5°C in VP CST with 4.4% ± 0.8% WPS, 3.0 ± 1.3 mg/kg phenol, and pH 5.8 ± 0.1 (Fig. 1 and Table 4). We isolated psychrotolerant *M. morganii* from CST with 2.2% ± 0.4% WPS (Table 1), and this very low NaCl concentration most likely explains why the product caused HFP.

Little information was available for outbreak 2, but the NaCl concentration in the implicated product was relatively high (~5% WPS; Table 1). No bacteria were isolated from this product, and it cannot be determined whether the histamine detected (914 ± 8 mg/kg) was formed prior to the cold-smoking process or whether the product had been stored at elevated temperatures.

VP CST products randomly collected from retail stores contained 5.8 to 12.7% WPS (Table 3). These salt concentrations seem appropriate to prevent histamine formation by psychrotolerant *M. morganii* in chilled CST with a shelf life of 3 to 4 weeks. For mesophilic *M. morganii*, NaCl concentrations above 7.5 to 8% have been required to prevent growth and histamine formation in seafoods *(1, 55)*. Further studies are needed to determine the concentrations of NaCl that alone or in combination with other preserving techniques, including low temperatures and smoking, are required to prevent critical histamine formation by psychrotolerant *M. morganii*–like bacteria in CST or other chilled and lightly preserved seafoods.

To prevent toxin formation by *Clostridium botulinum*, ready-to-eat smoked fish in reduced-oxygen packaging (vacuum and/or modified atmosphere) should contain at least 3 to 3.5% WPS if stored between 3 and 5°C and >5% WPS if stored at 5 to 10°C *(6, 53)*. A WPS of 3 to 3.5% did not prevent critical histamine formation by psychrotolerant *M. morganii*–like bacteria in CST at 5°C (Fig. 1). Consequently, to control histamine formation in CST more NaCl must be added or the declared shelf life must be limited. In CST with 4.4% ± 0.8% WPS, psychrotolerant *M. morganii* formed toxic histamine concentrations in some but not all packs (Fig. 1). Although not specifically studied, histamine may have been formed in packs of CST with 3.2% WPS, whereas in packs of CST with 5.1% WPS histamine formation was insignificant. Thus, the WPS concentration required to control histamine formation by psychrotolerant *M. morganii*–like bacteria in CST probably is 5% or higher.

Gravad salmon, products with 4.5 to 5.0% WPS, have an optimal salty taste *(23)*. Similar information is not available for CST, although it would be interesting to determine whether WPS concentrations required to control histamine formation in chilled CST coincide with optimal sensory properties. If lower WPS concentrations improve sensory properties, a reduced declared shelf life may become important for controlling histamine formation in CST. In Denmark, salty products seem to be accepted and consumed (Table 4). Clearly, >5% WPS would reduce the risk of HFP in CST, and the results of the present study indicate a need for improved technology to obtain CST with a more even distribution of NaCl.

Using mathematical models developed to predict shelf life for cold-smoked salmon *(32, 35)*, we found that a shelf life of 40 to 41 days determined in the present study for naturally contaminated CST at 5°C (Table 5) corresponds to the 40-day shelf life predicted for cold-smoked salmon at the same temperature and with the same concentrations of NaCl and smoke components. However, at 10.6°C the predicted shelf life of 25 days was higher than the 16 days observed for CST (Table 5). Lakshmanan and Dalgaard *(32)* also found a difference between observed and predicted shelf life for cold-smoked salmon at 9.6°C, indicating potential limitations of the available models for shelf-life prediction of cold-smoked fish as a function of temperature and concentrations of NaCl and smoke components.

Growth of *Listeria monocytogenes* to critical concentrations is a human health risk that should be considered when safety of CST is evaluated. As a function of storage conditions (5°C and vacuum packed) and product characteristics (5% WPS, pH 5.9, 2% water phase lactate, and 3 to 10 mg/kg phenol, i.e., smoke components), the predicted time for *L. monocytogenes* to multiply 100-fold is more than 4 weeks *(19)*, which suggests that the environmental factors that control histamine formation in CST also limit growth of *L. monocytogenes* in the product. More information concerning histamine formation in CST is needed. Nevertheless, to control this hazard we suggest that CST have >5% WPS and a declared shelf life equivalent to no more than 3 to 4 weeks at 5°C. These controls targeting histamine formation would also be beneficial for suppression of microbial pathogens.

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