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Histamine and tyramine production by bacteria isolated from spoiled sardine (*Sardina pilchardus*)

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Bacterial strains (32) from spoiled sardine were isolated and investigated for their ability to produce histamine and tyramine in histidine and tyrosine decarboxylase broth, respectively by a rapid high-performance liquid chromatography (HPLC) method. The predominant microflora of sardine consisted of the genera *Enterobacteriaceae*, *Pseudomonas*, *Chryseobacterium*, *Vibrio, Photobacterium*, and *Stenotrophomonas*. In histidine-enriched broth, the highest levels of histamine were observed in *Proteus mirabilis* (5201.95 mg/L), followed by *Enterobacter cloacae* (2333.99 mg/L), whilst the lowest histamine accumulation was found for *Kluyvera* spp. and *Listeria monocytogenes* at the level of 0.38 and 0.45 mg/L, respectively. However, *Pseudomonas oryzihabitans, Chryseobacterium indologenus* and *Vibrio vulnificus* showed the highest accumulation of tyramine in tyrosine decarboxylase broth with values of 1648.85, 774.20 and 187.96 mg/L, respectively. *Serratia liquefaciens* produced more than 1000 mg/L putrescine in both enrichment broths, although *Serratia rulnidace* did not have the ability to produce amines (except for dopamine, serotonin and agmatine) in tyrosine decarboxylase broth. The study results show that *P. mirabilis* and *Enterobacter cloacae*, which were dominantly found in spoiled sardine, were strong amine producers.

Key words: Biogenic amines, histamine, tyramine, spoilage, sardine.

INTRODUCTION

Fatty fish, such as sardine (*Sardina pilchardus*) are extremely perishable and generally spoil faster than other muscle foods. A number of chemical agents that are products of microbial metabolism have been found to be associated with fish spoilage. Included most notably among these are biogenic amines derived from the bacterial decarboxylation of the amino acids (Levin, 2010). In fact, the consumption of food containing high concentrations of biogenic amines has been associated with toxic effects and constitutes a potential health hazard. The most important food-borne intoxication caused by biogenic amines results from histamine action (Lehane and Olley, 2000; Taylor, 1986). However, histamine does not appear to be the only causative agent of scombroid poisoning; other amines such as cadaverine, putrescine and tyramine are also implicated in this illness as they enhance the toxicity of histamine (Halász et al., 1994; Rice et al., 1976).

Furthermore, biogenic amines are indicators of fish spoilage because their precursor amino acids are decarboxylated by bacterial enzymes (Dainty, 1996). Several Gram positive and Gram negative bacteria are implicated in the formation of biogenic amines. These biogenic amine-forming microorganisms may constitute part of the

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endogenous microbiota associated with the microflora of the fish or may be introduced by contamination during processing and storage of these fish (Ruiz-Capillas and Jiménez-Colmenero, 2010). In freshly caught fish, bacterial contamination is located initially on the skin and gills; from there, these microorganisms invade the fish muscle and grow rapidly in response to a number of factors relating to processing and the storage conditions such as temperature, time, etc. In this case, it is important to identify which bacteria possess amino acid decarboxylase activity in order to estimate the risk of biogenic amine production in seafood and to prevent its build up in seafood products (Ruiz-Capillas and Jiménez-Colmenero, 2010).

However, the variation observed in the ability to produce biogenic amines of different species is extremely wide, and this variation has even also been observed between strains of the same species. Therefore, more research is needed to distinguish the ability of fish spoilage bacteria to produce biogenic amines. The objective of this study was to investigate the capabilities of bacteria isolated from spoiled sardine to produce ammonia and biogenic amines in histidine and tyrosine decarboxylase broth.

MATERIALS AND METHODS

Sample preparations and bacterial isolations

Sardines (*S. pilchardus*) were purchased from local market in Adana, Turkey. The fish were held in room temperature (25°C) until it was spoiled. The fish samples were taken from three parts of the fish which were muscle, skin and gill surface. The surface of the fish was sampled using sterile swap and transferred onto agar plates. Muscles of fish were aseptically weighed (10 g) and mixed with 90 mL of Ringer solution and then stomached for 3 min. Further decimal dilutions were made and then 0.1 mL of each dilution was pipetted onto the surface of agar plates in triplicate. Agars used for each groups consist of Plate Count Agar (Fluka 70152 Steinheim, Switzerland). All groups were incubated for 2 days at 30°C. Gram staining was tested by the KOH-method (Powers, 1995). Motility of isolated strains was determined using motility test medium (Fluka, M1053). Oxidase production was tested using oxidase disk (Fluka, 70439 Oxidase Test) and catalase production by suspending cell material in 3% hydrogen peroxide. Glucose metabolism was investigated by the O/F-test. All these tests were performed for initial classification of isolated strains. After that, each of the chosen individual bacterial colonies was spread out several times on the agar plates using sterile loops in order to produce pure colonies. Isolates were identified according to the manufacturer's instructions of API 20E, API 20NE and API Listeria strip system (BioMerieux, France). The inoculated strip was incubated for 16 to 24 h and the colour reactions were noted either as positive or negative. The results obtained were analysed using the APILAB PLUS software (Biomerieux, France).

Biogenic amine analysis

Chemical reagents

L-histidine monohydrochloride (H8125), tyrosine and all BA standards were purchased from Sigma-Aldrich (Munich, Germany). The mobile phase consisted of acetonitrile and HPLC grade water for amine analyses.

Preparation of standard amine solution

Histamine dihydrochloride (165.7 mg), tyramine hydrochloride (126.7 mg), tryptamine hydrochloride (122.8 mg), putrescine dihydrochloride (182.9 mg), 2-phenylethylamine hydrochloride (130.1 mg), cadaverine dihydrochloride (171.4 mg), spermidine trihydrochloride (175.3 mg), spermine tetrahydrochloride (172.0 mg), 5-hydroxytryptamine (serotonin) (133.9 mg), 3-hydroxytyramine hydrochloride (dopamine) (123.8 mg), agmatine sulphate (175.4 mg), trimethylamine hydrochloride (161.7 mg) and ammonium chloride (296.9 mg) were dissolved in 10 mL HPLC grade water. The final concentration of free base for each amine was 10 mg/mL solution.

Bacterial extraction

The production of biogenic amines by all strains used in this work was monitored using histidine decarboxylase broth (HDB) and tyrosine decarboxylase broth (TDB) proposed by Klausen and Huss (1987). 1 g peptone, 0.5 g Lab-Lemco powder, 2.5 NaCl, 4.01 g L-histidine HCl or L-tyrosine and 2.5 mg pyridoxal HCl (Sigma) were added in 500 mL distilled water and the pH was adjusted according to their optimum growth pH (6.5) with 1 M NaOH or 0.1 N HCl. The HDB or TDB was pipetted in 10 mL bottles and then autoclaved at 121°C for 15 min prior to use. Nutrient broth was used for propagation of bacterial cultures. Bacterial strains were incubated at 37°C for 24 h, which after 0.5 mL of these bacterial cultures was removed and put into the HDB or TDB to allow them decarboxylate histidine or tyrosine. For extraction of amines from bacterial cultures, 5 mL of the HDB or TDB containing bacterial strains were removed to separate bottles and then 2 mL of 6% trichloroacetic acid was added. They were centrifuged at 3000 × *g* for 10 min and then filtered through a filter paper (Millipore). After that, 4 mL of bacterial supernatant were taken for derivatisation from each of bacterial strains.

Derivatisation of extraction from bacterial broth culture

A stock solution was prepared by dissolving 2% benzoyl chloride in acetonitrile to enhance the reaction with amines. For derivatisation of standard amine solutions, 100 mL were taken (4 mL for extracted bacterial cultures) from each free base standard solution (10 mg/mL). Sodium hydroxide (1 mL of 2 M) was added, followed by 1 mL of 2% benzoyl chloride (dissolved in acetonitrile) and the solution was mixed on a vortex mixer for 1 min. The reaction mixture was left at room temperature for 5 min and then centrifuged for 10 min. After that, the benzoylation was stopped by adding 2 mL of saturated sodium chloride solution and the solution was extracted twice with 2 mL of diethyl ether. The upper organic layer was transferred into a clean tube after mixing. Afterwards, the organic layer was evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1 mL of acetonitrile and 10 µL aliquots were injected into the HPLC (Özogul, 2004).

Apparatus and columns

A Shimadzu Prominence HPLC apparatus (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A diode array detector and two binary gradient pumps (Shimadzu LC-10AT), auto sampler (SIL 20AC), column oven (CTO-20AC), and a communication bus

Table 1. Bacterial flora isolated from spoiled sardine muscle, skin and gills.

Microorganism	Number of bacterial species (%)
Enterobacter cloacae	6 (18.7)
Chryseobacterium indologenus	3 (9.4)
Pseudomonas oryzihabitans	2 (6.3)
Proteus mirabilis	2 (6.3)
Listeria grayi	2 (6.3)
Vibrio vulnificus	2 (6.3)
Shigella spp.	2 (6.3)
Listeria monocytogenes	1 (3.1)
Listeria ivanovii	1 (3.1)
Enterobacter intermedius	1 (3.1)
Serratia liquefaciens	1 (3.1)
Photobacterium damselae	1 (3.1)
Pseudomonas luteola	1 (3.1)
Pseudomonas putida	1 (3.1)
Providencia stuantrii	1 (3.1)
Serratia rulnidace	1 (3.1)
Stenotrophomonas maltophilia	1 (3.1)
Kluyvera spp.	1 (3.1)
Unidentified	2 (6.3)
Total	32

module (CBM-20A) with valve unit FCV-11AL was used. For data analysis, the LC solution version 1.11 SP1 program (Shimadzu, Kyoto, Japan) was used. The column used was a reverse-phase; Spherisorb 5 Si C18 pH-St, 250 × 4.6 mm column (Phenomenex, Macclesfield, Cheshire, UK).

Statistical analysis

The mean value and standard deviation of each amine were calculated from the data obtained from triplicate samples for each bacterial strain. Data were subjected to analysis of variance and Duncan's multiple range tests using the SPSS Version 13.0 statistical package (SPSS Inc., Chicago, USA).

RESULTS

Microbial flora of spoiled sardine is shown in Table 1. The predominant microflora of the sardine was found to be Gram-negative, belonging to the genera *Enterobacteriaceae*, *Pseudomonas*, *Chryseobacterium*, *Vibrio, Photobacterium*, and *Stenotrophomonas*. Gram-positive microorganisms were also found and were dominated by the genera of *Listeria*. The mean concentration of histamine and other biogenic amines produced by bacterial strains in histidine decarboxylase broth was estimated using triplicate samples (Table 2). Significant differences in ammonia and biogenic amine production were found among the bacterial isolates ($P < 0.05$). *P. mirabilis* and *E. cloacae* showed the highest histidine decarboxylase activity and were able to produce 5201.95 and 2333.99 mg histamine per litre, respectively. The most accumulated amines by bacteria in histidine enrichment medium (HDB) were putrescine, cadaverine and spermidine. Ammonia production by bacterial isolates ranged from 112.24 mg/L for *Listeria monocytogenes* to 549.63 mg/L for *C. indologenus* (Table 2).

The production of tyramine and other biogenic amines by bacteria isolated from sardine in tyrosine-enriched broth was estimated using triplicate samples (Table 3). Significant differences in ammonia and biogenic amine production apart from TMA were also observed among the bacteria ($P < 0.05$). Tyrosine is converted to tyramine by tyrosine decarboxylase. In this experiment, the highest accumulation of tyramine was in *Pseudomonas oryzihabitans, C. indologenus* and *V. vulnificus* at 1648.85, 774.20 and 187.96 mg/L, respectively. The most accumulated amine by bacterial isolates in tyrosine enrichment medium (TDB) was putrescine followed by tyramine and dopamine, whilst histamine, TMA and tryptamine were the lowest accumulated amines. Histamine production by bacteria was below 4.3 mg/L, while some *Enterobacteriaceae* and *Pseudomonas* spp. strains did not produce this amine in TDB (Table 3).

DISCUSSION

At ambient temperature (25°C), the microflora is dominated by mesophilic *Vibrionaceae* (Gorczyca and Len, 1985; Gram et al., 1990) and, particularly if the fish are

Table 2. Ammonia and biogenic amine production by bacteria isolated from spoiled sardine in histidine decarboxylase broth (mg/L).

Biogenic amine (mg/L)	AMN	PUT	CAD	SPD	TRP	PHEN	SPN	HIS	SER	TYR	TMA	DOP	AGM
Enterobacter cloacae	447.56^{xb}	792.45^{b}	56.49^{b}	25.95^{d}	0.31^{e}	49.05^{b}	23.89^{b}	2333.99^{b}	3.61^{b}	6.30^{c}	1.36^{c}	14.50^{fg}	5.07^{fg}
	39.76^{y}	77.62	5.54	2.53	0.03	4.01	1.73	164.96	0.29	4.28	0.85	1.15	0.27
Chryseobacterium indologenus	549.63^{a}	395.23^{c}	23.88^{d}	24.40^{de}	0.00^{e}	7.48^{ghi}	2.65^{ef}	11.95^{c}	0.00^{b}	0.62^{e}	0.07^{fg}	19.53^{de}	5.75^{ef}
	48.87	21.71	1.77	1.62	0.00	0.70	0.07	0.95	0.00	0.08	0.06	0.79	0.34
Pseudomonas oryzihabitans	301.76^{cd}	384.63^{c}	19.60^{de}	31.01^{c}	0.00^{e}	12.96^{de}	4.02^{def}	0.94^{c}	0.45^{b}	0.69^{e}	0.09^{efg}	12.53^{gh}	4.99^{g}
	16.19	11.75	1.50	2.57	0.00	0.21	0.42	0.08	0.07	0.02	0.01	1.06	0.49
Proteus mirabilis	303.38^{cd}	148.17^{g}	14.61^{ef}	21.65^{e}	3.93^{e}	91.25^{a}	74.17^{a}	5201.95^{a}	688.12^{a}	125.10^{a}	1.55^{b}	12.77^{gh}	15.57^{a}
	9.79	6.87	1.72	1.46	0.33	2.13	4.48	258.83	60.98	5.78	0.07	0.96	0.62
Listeria grayi	203.49^{ef}	3.49^{f}	2.88^{gh}	11.77^{fgh}	0.40^{e}	5.78^{ghik}	2.80^{ef}	2.31^{c}	0.23^{b}	4.08^{cd}	0.24^{de}	24.59^{d}	10.32^{b}
	6.11	0.22	0.02	1.14	0.02	0.30	0.18	0.17	0.01	0.40	0.02	2.25	0.63
Vibrio vulnificus	132.09^{gf}	392.33^{c}	281.37^{a}	16.33^{fg}	0.15^{e}	15.52^{d}	3.35^{ef}	18.76^{c}	3.74^{b}	0.69^{e}	0.07^{fg}	19.14^{def}	7.18^{c}
	6.18	11.67	9.09	0.74	0.00	0.46	0.58	0.95	0.17	0.52	0.01	1.72	0.23
Shigella spp.	128.59^{gf}	28.83^{f}	10.02^{fg}	8.40^{hi}	3.93^{e}	11.65^{ef}	5.12^{de}	37.76^{c}	2.22^{b}	2.03^{de}	0.23^{def}	0.78^{k}	5.85^{e}
	7.91	2.46	1.09	0.31	0.33	0.06	0.34	1.07	0.18	0.16	0.02	0.03	0.22
Listeria monocytogenes	112.24^{f}	22.24^{f}	1.43^{h}	12.50^{fgh}	3.10^{d}	2.16^{k}	2.57^{ef}	0.45^{c}	1.22^{b}	1.44^{de}	0.04^{g}	38.05^{c}	0.62^{k}
	8.49	1.13	0.09	1.10	0.17	0.08	0.00	0.03	0.04	0.04	0.00	2.72	0.02
Listeria ivanovii	274.19^{d}	124.84^{d}	3.75^{gh}	14.73^{fg}	0.00^{e}	7.98^{gh}	18.00^{c}	8.45^{c}	27.21^{b}	15.54^{b}	1.81^{a}	77.16^{a}	10.12^{b}
	18.38	9.19	0.11	0.37	0.00	0.76	1.70	0.00	1.39	0.00	0.20	7.50	0.82
Enterobacter intermedius	289.49^{cd}	107.08^{de}	0.00^{h}	12.68^{fgh}	6.23^{a}	42.11^{c}	2.54^{ef}	1.05^{c}	0.62^{b}	1.73^{de}	0.04^{g}	16.98^{efg}	3.92^{hi}
	18.38	9.90	0.00	0.85	0.28	2.83	0.01	0.01	0.00	0.04	0.00	1.41	0.03
Serratia liquefaciens	284.10^{cd}	1055.73^{a}	286.28^{a}	54.90^{a}	4.48^{b}	0.00^{l}	3.21^{ef}	7.04^{c}	0.00^{b}	0.89^{de}	0.06^{g}	57.65^{b}	3.83^{hi}
	14.14	28.28	7.07	4.95	0.42	0.00	0.07	0.14	0.00	0.01	0.01	5.66	0.14
Photobacterium damselae	133.39^{gf}	789.25^{b}	15.68^{ef}	40.33^{b}	0.00^{e}	3.79^{ik}	2.42^{ef}	1.07^{c}	0.00^{b}	0.17^{e}	0.12^{defg}	0.00^{l}	6.43^{e}
	11.31	14.14	1.41	2.83	0.00	0.28	0.07	0.01	0.00	0.00	0.01	0.00	0.07
Pseudomonas luteola	307.06^{c}	112.56^{de}	0.54^{h}	9.23^{hi}	0.00^{e}	6.25^{ghi}	2.27^{ef}	0.61^{c}	0.88^{b}	2.97^{cde}	0.27^{d}	3.43^{jk}	4.95^{g}
	7.07	9.90	0.02	0.71	0.00	0.28	0.04	0.01	0.02	0.03	0.00	0.02	0.20
Pseudomonas putida	226.98^{e}	5.05^{f}	0.43^{h}	15.06^{fg}	0.00^{e}	6.84^{ghi}	2.53^{ef}	25.65^{c}	0.89^{b}	0.38^{e}	0.15^{defg}	13.81^{fg}	3.24^{i}
	11.31	0.42	0.04	1.47	0.00	0.25	0.14	2.55	0.02	0.00	0.00	0.57	0.20
Providencia stuantrii	131.92^{gf}	50.64^{ef}	6.99^{gh}	16.46^{f}	0.00^{e}	14.54^{de}	3.64^{ef}	0.48^{c}	0.00^{b}	0.34^{e}	0.08^{efg}	0.00^{k}	0.30^{k}
	7.07	4.95	0.25	1.56	0.00	1.44	0.00	0.03	0.00	0.00	0.00	0.00	0.03
Serratia rulnidace	179.08^{f}	1001.74^{a}	35.76^{c}	51.48^{a}	0.00^{e}	15.56^{d}	6.63^{d}	1.82^{c}	5.91^{b}	0.52^{e}	0.14^{defg}	8.16^{hi}	3.68^{i}
	16.97	87.68	2.91	3.96	0.00	1.06	0.00	0.08	0.49	0.00	0.02	0.64	0.24
Stenotrophomonas maltophilia	224.52^{e}	4.40^{f}	0.93^{h}	7.89^{hi}	0.00^{e}	9.25^{fg}	1.87^{f}	0.54^{c}	0.00^{b}	0.93^{de}	0.16^{defg}	0.00^{l}	7.91^{c}
	16.97	0.28	0.02	0.07	0.00	0.02	0.00	0.03	0.00	0.01	0.03	0.00	0.06
Kluyvera spp.	146.23^{g}	411.89^{c}	1.88^{h}	22.75^{de}	0.00^{e}	4.95^{ghik}	1.91^{f}	0.38^{c}	0.00^{b}	1.34^{de}	0.14^{defg}	0.00^{l}	4.48^{gh}
	12.73	7.07	0.07	1.41	0.00	0.71	0.03	0.00	0.00	0.00	0.00	0.00	0.07

^{x}Mean; ^{y}standard deviation (n = 3). AMN, ammonia; PUT, putrescine; CAD, cadaverine; HIS, histamine; SPD, spermidine; TRP, tryptamine; PHEN, 2-Phenyl-ethylamine; SPN, spermine; SER, serotonin; TYR, tyramine; TMA, trimethylamine; DOP, dopamine; AGM, agmatine. Different lowercase letters ($^{a-l}$) in a column indicate significant differences ($P < 0.05$) among bacterial species.

Table 3. Ammonia and biogenic amine production by bacteria isolated from spoiled sardine in tyrosine decarboxylase broth (mg/L)

Biogenic amine (mg/L)	AMN	PUT	CAD	SPD	TRP	PHEN	SPN	HIS	SER	TYR	TMA	DOP	AGM
Enterobacter cloacae	334.97^{xb}	1051.75^{b}	52.83^{d}	50.97^{b}	0.93^{fg}	50.63^{b}	92.75^{c}	3.75^{c}	140.33^{c}	64.04^{de}	10.75^{a}	403.84^{c}	147.10^{e}
	28.21^{y}	35.85	4.24	4.14	0.12	3.61	6.70	0.27	13.53	3.91	0.81	30.17	5.63
Chryseobacterium indologenus	202.53^{d}	497.12^{e}	32.21^{e}	19.11^{f}	0.96^{fg}	8.17^{de}	94.06^{c}	1.46^{e}	9.74^{hi}	774.20^{b}	1.30^{b}	40.82^{hik}	227.26^{c}
	16.61	37.89	1.98	0.81	0.08	0.33	1.48	0.13	0.44	60.43	0.13	3.63	10.33
Pseudomonas oryzihabitans	136.77^{ef}	14.38^{k}	6.63^{g}	25.87^{e}	0.00^{g}	8.66^{de}	58.32^{d}	0.56^{g}	316.80^{a}	1648.85^{a}	0.75^{b}	85.42^{g}	192.22^{d}
	9.76	0.61	0.76	1.39	0.00	0.12	1.62	0.04	6.58	158.70	0.00	4.47	7.67
Proteus mirabilis	518.99^{a}	371.83^{f}	132.64^{c}	32.39^{d}	6.42^{c}	123.14^{a}	54.42^{d}	4.07^{b}	98.22^{d}	57.70^{de}	2.75^{b}	439.92^{b}	137.33^{ef}
	42.88	35.74	7.06	3.93	0.59	13.68	2.67	0.17	2.44	0.56	0.27	27.33	3.88
Listeria grayi	278.52^{c}	185.21^{h}	30.98^{e}	20.38^{f}	2.72^{ef}	4.86^{def}	15.45^{fg}	0.36^{gh}	19.20^{gh}	117.45^{cd}	0.26^{b}	58.33^{gh}	157.03^{e}
	3.18	11.86	0.47	1.64	3.85	0.00	0.95	0.05	0.74	3.17	0.02	2.94	3.04
Vibrio vulnificus	518.58^{a}	299.84^{g}	467.29^{a}	49.70^{b}	16.42^{a}	11.95^{d}	31.53^{e}	4.30^{a}	140.97^{c}	187.96^{e}	4.99^{b}	369.54^{d}	192.90^{d}
	47.24	16.42	20.47	3.86	1.08	1.06	0.24	0.26	1.67	4.40	0.17	24.07	6.76
Shigella spp.	100.57^{fg}	15.72^{k}	3.95^{g}	1.56^{i}	0.34^{g}	4.48^{def}	13.05^{fgh}	0.82^{f}	171.61^{b}	38.61^{de}	0.38^{b}	297.67^{e}	276.65^{b}
	10.45^{y}	0.21	0.07	0.05	0.00	0.46	1.03	0.05	16.62	1.03	0.15	29.36	24.93
Listeria monocytogenes	97.47^{g}	20.23^{k}	5.53^{g}	13.86^{g}	0.00^{g}	3.31^{ef}	9.66^{i}	0.23^{h}	24.27^{g}	114.00^{cd}	0.72^{b}	22.22^{ikl}	101.62^{g}
	4.53	1.41	0.28	1.32	0.00	0.21	0.16	0.00	0.00	11.31	0.02	0.99	7.35
Listeria ivanovii	184.63^{d}	135.43^{hi}	9.89^{fg}	14.70^{g}	0.00^{g}	6.24^{def}	25.84^{de}	1.70^{d}	12.69^{h}	6.86^{e}	0.22^{b}	50.63^{hi}	391.79^{a}
	5.52	6.93	0.42	1.26	0.00	0.42	1.41	0.07	0.99	0.16	0.01	3.11	36.77
Enterobacter intermedius	197.83^{xd}	135.50^{hi}	0.41^{g}	12.51^{g}	5.59^{cd}	34.99^{c}	12.69^{fgh}	0.00^{i}	137.39^{c}	87.84^{de}	0.04^{b}	61.56^{gh}	111.17^{fg}
	11.31^{y}	7.50	0.03	1.15	0.31	1.46	1.10	0.00	4.53	5.23	0.00	5.66	2.97
Serratia liquefaciens	138.75^{e}	1126.38^{a}	427.03^{b}	71.26^{a}	9.97^{b}	0.00^{f}	48.64^{d}	0.27^{h}	49.65^{ef}	76.18^{de}	0.05^{b}	16.38^{kl}	70.63^{h}
	7.07	70.71	19.80	4.24	0.57	0.00	2.83	0.02	2.83	4.95	0.01	1.56	2.97
Photobacterium damselae	186.63^{d}	932.74^{c}	33.08^{e}	43.66^{c}	4.07^{de}	11.74^{d}	86.57^{c}	1.44^{e}	10.82^{hi}	65.63^{de}	0.07^{b}	13.11^{kl}	262.72^{b}
	3.54	12.73	3.25	1.84	0.10	1.03	7.07	0.14	1.13	2.26	0.00	0.74	25.46
Pseudomonas luteola	195.60^{d}	113.95^{i}	0.98^{g}	6.68^{h}	0.38^{g}	6.53^{def}	19.73^{def}	0.00^{i}	0.00^{i}	46.83^{de}	0.26^{b}	121.06^{f}	110.14^{fg}
	4.24	7.07	0.03	0.00	0.01	0.08	1.41	0.00	0.00	1.70	0.00	11.31	7.07
Pseudomonas putida	106.35^{efg}	25.17^{k}	4.60^{g}	25.38^{e}	0.35^{g}	4.21^{def}	22.32^{def}	0.00i	14.43^{gh}	126.50^{cd}	1.83^{b}	60.17^{gh}	98.30^{g}
	6.36	1.70	0.17	1.27	0.08	0.05	1.94	0.00	1.34	8.49	0.03	4.24	2.83
Providencia stuantrii	34.13^{e}	9.50^{k}	0.00^{g}	0.00^{i}	0.00^{g}	0.00^{f}	9.97^{gh}	0.00^{i}	1.91^{i}	3.24^{e}	0.44^{b}	123.24^{f}	13.07^{i}
	1.70	0.85	0.00	0.00	0.00	0.00	0.99	0.00	0.07	0.00	0.04	7.07	1.27
Serratia rulnidace	0.00^{e}	0.00^{k}	0.00^{g}	0.00^{i}	0.00^{g}	0.00^{f}	0.00^{h}	0.00^{i}	42.66^{f}	0.00^{e}	0.99^{b}	1039.38^{a}	46.84^{h}
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.26	0.00	0.07	45.25	4.24
Stenotrophomonas maltophilia	122.22^{efg}	115.18^{i}	9.32^{fg}	0.00^{i}	0.00^{g}	8.20^{de}	132.08^{b}	0.00^{i}	53.75^{e}	50.76^{de}	0.36^{b}	461.23^{b}	147.62^{e}
	5.66	4.24	0.71	0.00	0.00	0.42	6.36	0.00	5.37	3.39	0.02	24.04	2.83
Kluyvera spp.	266.21^{c}	714.07^{d}	24.80^{ef}	40.39^{c}	0.00^{g}	8.02^{de}	224.56^{a}	0.00^{i}	1.55^{i}	8.88^{e}	0.38^{b}	9.18^{l}	153.31^{e}
	7.07	26.87	2.83	3.39	0.00	0.28	22.63	0.00	0.07	0.04	0.00	0.57	3.68

^{x}Mean; ^{y}standard deviation (n = 3). AMN, ammonia; PUT, putrescine; CAD, cadaverine; HIS, histamine; SPD, spermidine; TRP, tryptamine; PHEN, 2-Phenyl-ethylamine; SPN, spermine; SER, serotonin; TYR, tyramine; TMA, trimethylamine; DOP, dopamine; AGM, agmatine. Different lowercase letters ($^{a-l}$) in a column indicate significant differences ($P < 0.05$) among bacterial species.

caught in polluted waters, mesophilic *Enterobacteriaceae* (Gram, 1992). In this experiment, the microflora of sardine was predominantly *Enterobacteriaceae* (46.8%), *Pseudomonas* (12.5%), *Chryseobacterium* (9.4%) and *Vibrio* (6.3%). Storage of fish at ambient temperature leads to rapid growth of mesophilic Gram negative bacteria but a large number of specific spoilage bacteria of iced tropical fresh water fish such as *Pseudomonas, Alteromonas* and *Shewanella* were also found at this temperature. The microflora of temperate water fish is dominated by psychotropic Gram-negative and rod-shaped bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella, Flavobacterium, Vibrionaceae* and *Aeromonadaceae*, but Gram-positive bacteria such as *Bacillus, Micrococcus, Clostridium, Lactobacillus* and *Corynebacterium* can also be found in varying proportions (Gram and Huss, 1996).

The *Enterobacteriaceae* strains isolated were *E. cloacae, Enterobacter intermedius, P. mirabilis, Serratia liquefaciens, Serratia rulnidace* and *Providencia stuantrii.* Ababouch et al. (1991) reported that *P. mirabilis* was also isolated from sardine, along with *Morganella morganii*, *Proteus vulgaris, Providencia stuartii,* and unidentified species of *Proteus*. *E. cloacae, E. intermedius, Hafnia alvei, Klebsiella oxytoca, Plesiomonas shigelloides, P. vulgaris, Pseudomonas fluorescens, Serratia liquefaciens,* and *Serratia plymuthica* have all been isolated from samples of tuna collected from markets in Spain (Lopez-Sabater et al., 1996).

Bernardet et al. (2006) reported that the genus *Chryseobacterium* and the new genus *Flavobacterium* encompass species essentially confined to soil and water environments and includes some major fish pathogens. In addition to these, *Stenotrophomonas maltophia* was re-cently isolated from albacore tuna (*Thunnus alalconga*) (Ben-Gigirey et al., 1998) and *Photobacterium* spp., a psychrophilic organism, has been proposed as a significant histamine-producing organism (ten Brink et al., 1990). In the current study, *Chryseobacterium* spp. and *Stenotrophomonas* were also isolated in spoiled sardine. Bremer et al. (2003) reported that *Listeria* species can attach to and grow on a wide range of materials used in seafood processing plants. *L. monocytogenes* has also been isolated from fresh, frozen, smoked and dried salted seafood products (Weagent et al., 1988; Farber, 1991). Similarly, the results obtained from this experiment showed the presence of the genera *Listeria* (12.5%) in spoiled fish.

Histamine production in histidine decarboxylase broth

The production of histamine by *E. cloacae, P. mirabilis, E. intermedius, S. liquefaciens* and *P. fluorescens* isolated from tunafish, bonito and mackerel was 86.4, 65.5, 51, 94 and 17 ppm, respectively in culture broth assay (Lopez-Sabater et al., 1996). Accumulation of histamine by *Shigella* spp. was 37.76 mg/L in a histidine-enriched broth. In addition, *V. vulnificus* and *C. indologenus* produced 18.76 and 11.95 mg/L of histamine in histidine-enriched broth, respectively. Ababouch et al. (1991) found that maximum histamine accumulation by bacteria isolated from sardine was observed for *P. stuantrii* (5.77 µmol/mL) and *Vibrio* spp. (0.46 µmol/mL) in sardine fish infusion broth supplemented with histidine. Ben-Gigirey et al. (2000) reported that three strains of *S. maltophilia* produced less than 25 ppm of histamine when grown in trypticase soy broth supplemented with 2% histidine, which was considerably higher than that of our results. In the present study, *S. maltophilia* showed the lowest production of histamine (0.54 mg/L). *P. damselae* and *P. stuantrii* was found to be a weak histamine former, producing less than 2 mg/L of histamine. Kanki et al. (2007) reported that *P. damselae* was able to produce 649 mg/kg histamine in tuna samples kept for 2 h at 30°C. Bjornsdottir et al. (2009) also found that *P. damsela* produced >1,000 ppm (3372 ppm) of histamine in culture broth and *L. innocua* was non-histamine producer (<125 ppm). In the current study, gram-positive microorganisms such as *L. ivanovii, L grayi* and *L monocytogenes* produced less than 9 mg/L in histidine-enriched broth.

Tyramine production in tyrosine decarboxylase broth

P. putida, P. phosphoreum and *Vibrio* spp. were able to produce tyramine less than 5.58 mg/L in tyrosine decarboxylase broth (Özogul and Özogul, 2007). Emborg and Dalgaard (2006) reported that tyramine accumulation by *P. phosphoreum* and *P. fluorescens* isolated from seafood was 290 and < 5 mg/L, respectively.

Tyrosine decarboxylase activity is more common among Gram positive bacteria (Bover-Cid and Holzapfel, 1999). The production of tyramine by *L. grayi*, *L. monocytogenes* and *P. putida* was higher than 100 mg/L in tyrosine-enriched broth. Pircher et al. (2007) reported that 95.3% of *Enterobacteriaceae* species isolated from meat, fermented sausages and cheeses were able to produce less than 10 mg/l of tyramine, and the remaining 4.7% produced 10 mg/L to a maximum of 35.3 mg/L. In the current study, tyramine production by *E. cloacae, P. damselae, E. intermedius, S. liquefaciens*, *P. mirabilis* and *S. maltophilia* was found higher than 50 mg/L in tyrosine-enriched broth. All the other strains tested produced little amounts of tyramine except *S.rulnidace*, thus they can be classified in the poor amine former group. Marino et al. (2000) reported that *S. liquefaciens* and *E. cloacae* formed significant concentrations of tyramine at 24 and 28 ppm, respectively in a laboratory medium. This is in agreement with the result obtained from this study since high amount of tyramine production was observed with these bacteria at 76.18 and 64.04 mg/L,

respectively in tyrosine decarboxylase broth.Tyrosine and histidine decarboxylase activities have been detected in *E. coli* (Blackwell and Mabbitt, 1965) and *Pseudomonas spp*. (Rice et al., 1976; Diaz-Cinco et al., 1992). In a laboratory medium, tyramine was produced by *Enterococcus*, *Lactococcus*, *Proteus* and *Pseudomonas* but not by *Acinetobacter*, *Bacillus, Escherichia, Hafnia, S. arizonae, S. marcescens, Shigella* and *Y. enterocolitica* (Beutling, 1993).

Production of other amines in HDB and TDB

The production of the other biogenic amines was in varying quantities since the broth contained small amounts of the other amino acids such as lysine, arginine and glutamic acid (calculated as 132, 170 and 304 mg/L) originating from peptone and beef extract (Özogul and Özogul, 2005).

Putrescine was the main amine produced by bacterial isolates in HDB, which are formed from ornithine via either glutamic acid or arginine. The highest putrescine accumulation was found for *S. liquefaciens* (1055.73 mg/L). Glutamic acid or arginine was present in the broth at high concentrations enough to produce that level of putrescine. Significant cadaverine accumulation was observed for *S. liquefaciens* and *V. vulnificus*, corresponding to 286.28 and 281.37 mg/L, respectively. The cadaverine production might be due to lysine (132 mg/L) present in peptone and beef extract. Moreover, production of tyramine by *P. mirabilis* (125.10 mg/L) was significantly higher than for other strains ($P < 0.05$), although the histidine-enriched broth did not contain any tyrosine. This might be explained by the presence of tyro-sine in peptone and beef extract in the broth. All strains isolated formed high levels of spermidine (>7.89 mg/L) in HDB. The highest spermine (74.17 mg/L) and agmatine (15.57 mg/L) levels were observed by *P. mirabilis* in a histidine-enriched broth. The other biogenic amines, not mentioned here, were also produced in low levels (Table 2).

In the present study, putrescine was one of the most accumulated amines by bacteria in TDB. Significant putrescine accumulation (>1000 mg/L) was observed for *S. liquefaciens* and *E. cloacae,* though, *S. rulnidace* did not produce putrescine. Marino et al. (2000) found that *Enterobacteriaceae* species produced only histamine, cadaverine, putrescine and tyramine but did not accumulate spermine, spermidine, 2-phenylethylamine and tryptamine production in a laboratory medium. Apart from *P. putida*, *P. oryzihabitans, L. monocytogenes*, *P. stuantrii* and *Shigella*, most of bacterial strains produced more than 100 mg/L putrescine in TDB (Table 3). Özogul and Özogul (2005) reported that metabolic pathways to putrescine occur from either ornithine or arginine via agmatine. The production of agmatine by all strains was higher than 13.07 mg/L in tyrosine-enriched broth and this amount can be converted from arginine (170 mg/L) present in peptone and beef extract. *S. rulnidace* and *P. stuantrii* did not accumulate cadaverine, whereas *S. liquefaciens*, *V. vulnificus* and *P. mirabilis* showed the highest accumulation of cadaverine with corresponding value of 427.03, 467.29 and 132.64 mg/L, respectively.

The amino acids glutamine, ornithine and arginine are involved in the biological pathways leading to the formation of putrescine, agmatine, spermidine and spermine. In the present study, the highest spermidine (71.26 mg/L) and spermine (224.56 mg/L) levels were observed for *S. liquefaciens* and *Kluyvera* spp., respectively. *P. mirabilis* and *E. cloacae* showed highest accumulation of 2-phenylethylamine with levels of 123.14 and 50.63 mg/L, respectively. The availability of free 5-hydroxytryptophan and tyrosine in the broth may lead to the production of serotonin and dopamine, respectively. Dopamine production by bacteria ranged from 9.18 mg/L for *Kluyvera* spp. to 1039.38 mg/L for *S. rulnidace*. The serotonin formation was at significant levels (316.80 mg/L) for *P. oryzihabitans* ($P < 0.05$), though *P. luteola* did not produce serotonin. Tryptamine is formed from amino acid tryptophan by action of tryptophan-decarboxylating bacteria. The tryptamine accumulation by all strains isolated was less than 16.42 mg/L in tyrosine-enriched broth.

Conclusion

The results obtained from this study show that the highest histamine production was for *P. mirabilis*, followed by *E. cloacea*, whereas, *P. oryzihabitans, C. indologenus* and *V. vulnificus* resulted in the highest tyramine accumulation in tyrosine-enriched broth. The other strains isolated produced the other biogenic amines in varying quantities in histidine and tyrosine decarboxylase broth. These results confirm that all strains tested are capable of decarboxylating one or more amino acids, but this production of amines differed among bacterial species of the same family. However, the ability of microorganisms to decarboxylate amino acids is highly variable. It depends not only on the species, but also on the strain and the environmental conditions.

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