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Relation of Biogenic Amines' Formation with Microbiological and Sensory Attributes in *Lactobacillus*-Inoculated Vacuum-Packed Rainbow Trout (*Oncorhynchus mykiss*) Fillets

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The biogenic amine (BA) content of vacuum-packed filleted rainbow trout (*Oncorhynchus mykiss*) inoculated or not with two different *Lactobacillus* strains, individually or in combination, was monitored during refrigerated storage for 20 days and related to respective bacteriological and sensory changes occurring during the same period. Eight amines, namely putrescine, cadaverine, tyramine, tryptamine, β-phenylethylamine, histamine, spermine, and spermidine, were determined, whereas agmatine was not detected in any of the samples. In all cases, BA concentration was higher ($P \leq 0.05$) in the controls compared to all inoculated treatments, whereas the trend with regard to the bacterial populations (Enterobacteriaceae, pseudomonads, and H_2S-producing bacteria) and the off-odor scores was similar. Inoculation with *Lactobacillus sakei* CECT 4808 showed the best preservative effect among inoculated treatments. Concentrations of putrescine and cadaverine, the main BAs formed, correlated well with both spoilage bacterial counts and off-odor scores and can be useful indicators of shelf life. Spermine and spermidine contents decreased during storage, while levels of the other determined BAs remained below 10 mg/kg even after sensory rejection.

KEYWORDS: Biogenic amines; rainbow trout fillets; *Lactobacillus sakei*; *Lactobacillus curvatus*; vacuum-packaging; *Oncorhynchus mykiss*

INTRODUCTION

Fresh fish are known to be extremely perishable food commodities, and consequently their quality and safety are parameters of major concern to both industry and consumers. Initial quality deterioration of fresh fish is primarily caused by autolytic changes, whereas subsequent quality loss and spoilage occur as a result of bacteriological activity. A wide variety of bacterial species are involved in the spoilage of refrigerated fish under aerobic and vacuum storage conditions, including *Pseudomonas* spp., Enterobacteriaceae, *Shewanella putrefaciens*, *Alteromonas* spp., *Photobacterium phosphoreum*, *Brochothrix thermosphacta*, and lactic acid bacteria (LAB) (*1*). Although abundant data exist regarding spoilage of marine fish stored under vacuum, little is known about the spoilage bacteriology of vacuum-packed freshwater fish. It is unlikely, though, that *P. phosphoreum* plays a major role as it is a sodium-requiring species. Moreover, TMAO-reducing organisms such as *S. putrefaciens* are commonly found at lower levels in vacuum-packed freshwater fish than in marine fish and usually at later stages of storage (*1*).

Evaluation of fish quality and safety is based on the measurement of various microbiological, chemical, and/or sensory parameters, which are associated with both freshness and changes leading to fish spoilage. Sensory evaluation is believed to be the simplest and most reliable method for establishing freshness as well as the shelf life of fish and, consequently, for the assessment of fish spoilage; however, in order to be objective it requires both highly trained and specialized assessors and statistical treatment of the data, which can be a problem under industrial conditions. For this reason alternative methods have been proposed, among which is the determination of nonvolatile amines (*2*). Biogenic amines (BAs) are nonvolatile compounds, most of which are normally present in fresh fish at very low levels. Gradual accumulation of BAs is associated with the growth of bacteria, whereas their presence at high concentrations is indicative of bacterial spoilage (*3*). BAs are formed by decarboxylation of amino acids as a result of bacterial metabolism; amino acid decarboxylases are found in, among others, certain Enterobacteriaceae, *Pseudomonas*, *Clostridium*, *Lactobacillus*, *Streptococcus*, and *Micrococcus* species. Enterobacteriaceae are usually implicated in the forma-

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tion of cadaverine and histamine. On the other hand, pseudomonads are usually responsible for the formation of putrescine, whereas certain LAB strains can be tyramine or histamine producers (*4*). It is therefore evident that the type of BAs formed in fish under specific storage conditions will depend on the bacterial species present.

Histamine is considered to be the most important indicator of fish freshness because of its toxic effects (histamine intoxication) and is the only amine with a European Union-established regulatory limit of 100 mg/kg (*5*). Other BAs have also been proposed as freshness indicators either individually or in combination by numerous researchers. Křížek et al. (*6*), for example, have suggested that putrescine, cadaverine, and the sum of both amines are useful quality indicators for carp meat, whereas Dawood et al. (*7*) also stated that these two amines can be used to assess freshness of chill-stored rainbow trout. In the same context, Mietz and Karmas (*8*) have proposed the complex biogenic amines index (BAI) for tuna based on five BAs, given by the formula BAI = (putrescine + cadaverine + histamine)/(1 + spermine + spermidine).

The use of LAB as biopreservative agents in fish, in contrast to meat and meat products, is a relatively unexploited method. LAB can contribute in a number of ways toward improving the quality of fish and fish products. Microbiological safety and stability can be improved through control of growth of other microorganisms, including inhibition of pathogenic bacteria such as *Listeria monocytogenes* and *Clostridium* spp. (*9*) and antagonism toward bacterial species such as Enterobacteriaceae, *Pseudomonas* spp., and H_2S-producing bacteria, which are commonly associated with fish and fish product spoilage (*1*). Although numerous studies have been reported regarding the formation of BAs during storage of marine and freshwater fish (*6*−*8*, *10*−*15*), few of them have related bacterial and sensory changes to BA formation (*12*−*14*). In addition, available data related to the formation of BAs during storage of LAB-inoculated freshwater fish are very limited and mainly involve cold-smoked fish (*16*, *17*). The objectives, therefore, of the present study were (a) to determine biogenic amines formed during refrigerated storage of filleted rainbow trout inoculated with two different *Lactobacillus* strains as compared to non-inoculated controls and (b) to correlate BA formation to microbiological and sensory changes occurring in these samples.

MATERIALS AND METHODS

Materials and Reagents. Rainbow trout (*O. mykiss*) with an average weight of ≈370 g were obtained fresh from an aquaculture unit in northern Greece. *Lactobacillus sakei* CECT 4808 and *Lactobacillus curvatus* CECT 904^T, the strains used for the inoculation experiments, were obtained lyophilized from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT). Previous work (*18*) has shown that both *Lb. sakei* CECT 4808 and *Lb. curvatus* CECT 904^T were able to produce bacteriocin-like inhibitory substances (BLIS), with the one produced by *Lb. sakei* 4808 having a wider antimicrobial spectrum. Trichloroacetic acid (p.a.) and benzoyl chloride (for synthesis) were obtained from Merck (Darmstadt, Germany). Water and acetonitrile (Merck) used for chromatographic separations were of HPLC grade. Analytical grade tryptamine hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and agmatine sulfate were obtained from Sigma-Aldrich (St. Louis, MO). These were used in suitable amounts to prepare 1 mg/mL stock solutions for each amine and were then diluted appropriately to construct calibration curves.

Preparation of Trout Fillet Samples. Rainbow trout were transferred to the laboratory in an insulated ice box containing ice packs within 2 h of killing. They were then gutted, filleted, and skinned. The fillets were then placed in individual polyacrylamide−polyethylene bags and stored at 4 °C until inoculation with the microbial culture suspensions. During these operations utensils and working surfaces were frequently washed and disinfected with absolute ethanol to minimize cross-contamination of the fillets.

Inoculation Experiments. The lactobacilli were rehydrated in de Man−Rogosa−Sharpe Broth (MRS, BBL, Sigma-Aldrich) and incubated at 30 °C for 18−20 h to obtain cells in the early stationary phase of growth when maximum bacteriocin production is reported to occur (*19*). The cultures were centrifuged at 4 °C and 10000*g* for 10 min (centrifuge Hermle Z 383 K, Hermle, Gosheim, Germany), washed three times, and resuspended in 0.85% NaCl to obtain a cell concentration of at least 10^8 colony-forming units (cfu)/mL. The suspensions were freshly prepared when needed.

Pairs of fillets from 40 fish were randomly assigned to four treatments: (1) uninoculated controls (control), (2) inoculated with 1 mL of *Lb. sakei* CECT 4808 suspension on each side of each fillet (Ls4808), (3) inoculated with 1 mL of *Lb. curvatus* CECT 904^T suspension on each side of each fillet (Lc904), or (4) inoculated with 0.5 mL of both suspensions on each side of each fillet (S+C). After inocula were spread by manually massaging the exterior of each bag, the fillets were individually vacuum-packaged in polyacrylamide−polyethylene bags (O_2 transmission rate = 42 cm^3/m^2/24 h at 23 °C, 1 atm, and 0% relative humidity) and stored in a refrigerator at 4 ± 0.5 °C. The permeability of the packaging material was chosen to favor the growth of LAB by creating a microaerophilic environment and at the same time to prevent formation of sulfides by the *Lactobacillus* strains, which can occur in the complete absence of oxygen (*20*). The experiments were repeated three times, to remove any effects deriving from the initial quality of the raw material.

Analyses were performed on days 0, 5, 10, 15, and 20 of refrigerated storage on two samples in duplicate. Analyses on day 0 were conducted only for the control samples, except for the determination of LAB counts, which was conducted for all treatments and also for the lactic culture suspensions used for the inoculation. Sample allocation for each examined storage day and treatment was the following: the two fillets from each fish were transversely divided in three parts, equal in width. Samples for BA determinations were from the middle third of each fillet, whereas samples for microbiological analysis were from the bottom third.

Biogenic Amine Determination. The extraction, separation, and quantification of BAs were carried out according to the procedure described by Paleologos et al. (*21*). According to this procedure a solution of BAs was obtained after sample extraction with trichloroacetic acid and centrifugation. BAs were separated after derivatization with benzoyl chloride according to the method of Yen and Hsieh (*22*). Separation and quantification of each amine were performed by HPLC with UV detection. The liquid chromatographer consisted of a Shimadzu 10AD series for HPLC equipped with a UV visible variable wavelength detector (Shimadzu) set at 254 nm. A LiChrospher 100 RP-18 [244 × 4.4 mm i.d., 5 μm linked to a LiChrospher guard precolumn (10 × 4.6 mm i.d.)] and thermostated at 30 °C in a CTO-10A Shimadzu column oven was used for all separations. The mobile phase consisted of water and acetonitrile (ACN). The gradient elution program adopted was set at a flow rate of 1.1 mL min^{-1}, starting with an ACN/water mixture (30:70, v/v) for 4 min. The program proceeded linearly to ACN/water (50:50, v/v) over 2 min and remained for another 3 min. This was followed by a linear decrease to ACN/water (30:70, v/v) over 2 min, and the same composition was maintained for another 4 min.

Determination and quantification of the amines was performed by measuring the absorbance of the benzene ring at 254 nm and integrating the resulting peaks with the CLASS-VP Shimadzu for chromatography software. Results are reported as milligrams per kilogram.

Microbiological Analyses. A 20 g portion from each treatment was homogenized in a Stomacher bag with 180 mL of sterilized chilled 0.1% peptone (BBL) water for 2 min using a Stomacher blender (Stomacher 400, Lab. Blender, Seward, London, U.K.). Serial decimal dilutions were prepared in 9 mL volumes of peptone water. For determination of LAB counts in culture suspensions, serial decimal dilutions were made in 9 mL volumes of peptone water using 1 mL

Table 1. Concentrations of Biogenic Amines (Milligrams per Kilogram) during Storage of Control or Inoculated with *Lb. sakei* (Ls4808), *Lb. curvatus* (Lc904), or Both Organisms (S+C) Trout Fillets[a]

		storage day				
amine	treatment	0	5	10	15	20
putrescine	control	6.45 ± 0.132	6.50 ± 0.110 b	20.83 ± 0.517 c	30.44 ± 1.442 d	55.82 ± 0.953 d
	Ls4808		3.48 ± 0.399 a	5.63 ± 0.567 a	7.10 ± 0.762 a	16.91 ± 2.633 a
	Lc904		4.07 ± 0.264 a	13.29 ± 0.166 b	26.05 ± 1.432 c	52.38 ± 1.155 c
	S+C		4.27 ± 0.356 a	6.68 ± 0.889 a	9.60 ± 0.956 b	29.33 ± 1.198 b
cadaverine	control	1.88 ± 0.078	1.46 ± 0.171 a	23.70 ± 2.230 c	45.50 ± 0.826 d	62.23 ± 0.414 d
	Ls4808		0.80 ± 0.042 a	3.38 ± 0.422 a	15.53 ± 1.803 a	24.05 ± 0.944 a
	Lc904		1.87 ± 0.252 a	17.28 ± 0.857 b	31.57 ± 0.734 c	55.74 ± 0.915 c
	S+C		1.67 ± 0.195 a	4.87 ± 0.590 a	18.35 ± 1.063 b	29.04 ± 1.651 b
tyramine	control	1.91 ± 0.305	3.08 ± 0.126 c	3.41 ± 0.151 c	4.49 ± 0.061 b	6.76 ± 0.199 c
	Ls4808		1.88 ± 0.412 a	2.47 ± 0.203 a	3.57 ± 0.181 a	4.21 ± 0.232 a
	Lc904		2.78 ± 0.233 bc	2.92 ± 0.148 b	3.54 ± 0.071 a	5.59 ± 0.318 b
	S+C		2.53 ± 0.177 b	2.29 ± 0.111 a	3.52 ± 0.153 a	4.46 ± 0.181 a
histamine	control	2.92 ± 0.111	3.65 ± 0.057 d	4.39 ± 0.067 d	4.78 ± 0.058 d	6.11 ± 0.197 d
	Ls4808		1.64 ± 0.025 a	2.02 ± 0.084 a	3.06 ± 0.116 a	3.58 ± 0.201 a
	Lc904		3.18 ± 0.042 c	3.83 ± 0.032 c	4.18 ± 0.050 c	5.40 ± 0.284 c
	S+C		2.53 ± 0.030 b	3.06 ± 0.042 b	3.60 ± 0.089 b	5.37 ± 0.222 b
tryptamine	control	3.93 ± 0.039	7.54 ± 0.141 d	8.11 ± 0.067 d	9.08 ± 0.180 d	11.60 ± 0.159 c
	Ls4808		3.60 ± 0.152 a	2.56 ± 0.433 a	3.78 ± 0.428 b	4.31 ± 0.219 a
	Lc904		6.79 ± 0.319 c	6.90 ± 0.368 c	6.35 ± 0.620 c	9.03 ± 0.444 b
	S+C		4.41 ± 0.183 b	3.56 ± 0.290 b	2.94 ± 0.628 a	4.75 ± 0.514 a
β-phenylethylamine	control	3.14 ± 0.285	2.51 ± 0.144 c	2.38 ± 0.204 c	2.14 ± 0.186 c	2.48 ± 0.076 d
	Ls4808		1.21 ± 0.077 a	0.63 ± 0.021 a	0.96 ± 0.113 ab	1.04 ± 0.167 b
	Lc904		1.92 ± 0.256 b	1.63 ± 0.233 b	1.15 ± 0.084 b	1.82 ± 0.031 c
	S+C		1.66 ± 0.060 b	0.79 ± 0.076 a	0.77 ± 0.088 a	0.60 ± 0.058 a
spermine	control	24.71 ± 0.951	18.85 ± 0.212 c	15.14 ± 0.226 c	13.57 ± 0.178 c	11.17 ± 0.113 d
	Ls4808		14.37 ± 0.496 a	11.71 ± 0.630 a	11.39 ± 0.358 a	8.25 ± 0.127 a
	Lc904		16.34 ± 0.596 b	14.01 ± 0.330 b	12.61 ± 0.462 b	10.46 ± 0.107 c
	S+C		14.56 ± 0.907 a	11.32 ± 0.872 a	10.86 ± 0.664 a	9.20 ± 0.313 b
spermidine	control	7.06 ± 0.122	4.69 ± 0.131 b	3.39 ± 0.184 c	2.59 ± 0.082 c	2.37 ± 0.092 d
	Ls4808		4.06 ± 0.263 a	2.38 ± 0.031 a	1.73 ± 0.087 a	0.89 ± 0.120 a
	Lc904		4.07 ± 0.054 a	2.60 ± 0.061 b	2.03 ± 0.018 b	1.91 ± 0.086 c
	S+C		3.87 ± 0.050 a	2.36 ± 0.048 a	1.95 ± 0.039 b	1.56 ± 0.033 b

[a] Concentrations on day 0 refer to the raw material. Data are presented as mean value ± standard error ($n = 6$). Means in the same column for the same determined amine bearing a different letter differ significantly ($P \leq 0.05$).

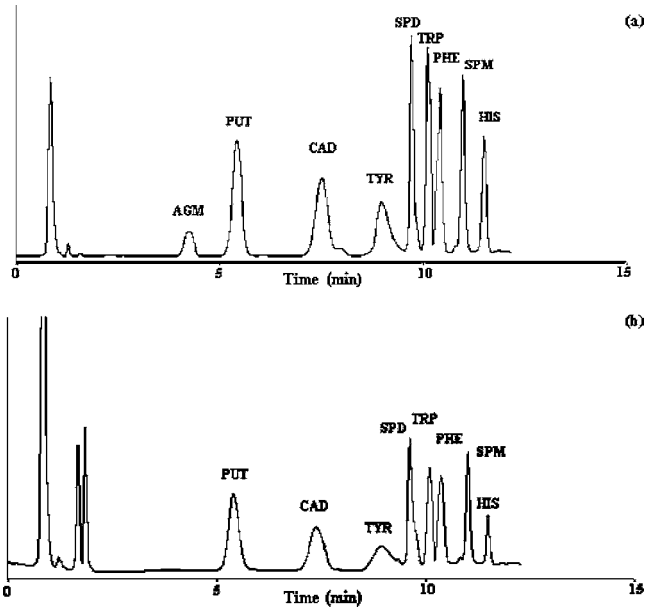

Figure 1. HPLC chromatograms at 254 nm of (**a**) the mixture of the nine standard amine derivatives and (**b**) the eight separated amine derivatives detected in the samples of the present study. AGM, agmatine; PUT, putrescine; CAD, cadaverine; TRY, tryptamine; PHE, β-phenylethylamine; SPD, spermidine; SPM, spermine; HIS, histamine; TYR, tyramine.

from each suspension. LAB were enumerated using duplicate 1 mL volumes of suitable dilutions in overlaid pour-plates of MRS agar (Fluka, Sigma-Aldrich), incubated at 30 °C for 3 days (*23*, *24*). Enterobacteriaceae counts were determined in overlaid pour plates of violet red bile glucose agar (VRBG; Oxoid, Basingstoke, U.K.), incubated at 37 °C for 24 h (*25*). *Pseudomonas* counts were determined using *Pseudomonas* agar base (Oxoid) supplemented with cetrimide−fucidin−cephaloridine (C-F-C) selective supplement (Oxoid), incubated at 25 °C for 48 h. Finally, H_2S-producing bacteria were determined using 1 mL of suitable dilutions on overlaid pour-plates of iron agar medium (*26*) incubated at 25 °C; black colonies formed by the production of H_2S were enumerated after 3 days. All results are reported as $\log_{10}$ colony-forming units per gram except for LAB counts in culture suspensions, which are reported as $\log_{10}$ colony-forming units per millliliter.

Off-Odor Evaluation. Odor was evaluated 5 min after each vacuum bag had been opened by a three-member trained panel, assigning off-odor scores from 1 = none to 5 = extreme, with a score of 3.5 being set as the limit of acceptability.

Statistical Analysis. All data were analyzed using the General Linear Model of ANOVA with treatment and time as factors, after normality and homogeneity of variances were confirmed. The statistical significance of the differences was checked using the Student−Newman−Keuls (SNK) test at the 0.05 level, whereas correlations between different parameters were evaluated with the Spearman correlation coefficient. All statistical analyses were conducted by use of the SPSS statistical package (SPSS 10.05, SPSS Inc., Woking, Surrey, U.K.).

RESULTS AND DISCUSSION

Formation of Biogenic Amines. In the present study eight BAs were detected (**Figure 1**), namely putrescine, cadaverine, tyramine, spermidine, tryptamine, spermine, β-phenylethylamine, and histamine (**Table 1**), whereas agmatine was not detected in any of the trout samples analyzed. Concentrations of all detected BAs were significantly higher ($P \leq 0.05$) in control samples than in those of the inoculated treatments throughout the storage period. Among the three inoculated

Table 2. Microbiological Counts (Log_{10} Colony-Forming Units per Gram) during Storage of Control or Inoculated with *Lb. sakei* (Ls4808), *Lb. curvatus* (Lc904), or Both Organisms (S+C) Trout Fillets[a]

		storage day				
microbial group	treatment	0	5	10	15	20
Enterobacteriaceae	control	<1.00 ± 0.000	2.83 ± 0.124 d	5.52 ± 0.343 d	7.10 ± 0.204 d	7.73± 0.050 c
	Ls4808		1.33 ± 0.030 a	3.03 ± 0.225 a	4.91 ± 0.030 a	6.05 ± 0.067 a
	Lc904		2.56 ± 0.171 c	5.12 ± 0.179 c	6.50 ± 0.139 c	7.25 ± 0.017 b
	S+C		1.84 ± 0.183 b	3.60 ± 0.096 b	5.36 ± 0.020 b	6.22 ± 0.051 a
Pseudomonas spp.	control	<1.70 ± 0.000	4.01 ± 0.044 c	6.43 ± 0.445 d	7.78 ± 0.187 d	8.27± 0.034 d
	Ls4808		2.72 ± 0.102 a	4.51 ± 0.087 a	6.04 ± 0.111 a	6.80 ± 0.086 a
	Lc904		3.80 ± 0.021 c	6.00 ± 0.243 c	7.17 ± 0.066 c	7.85 ± 0.092 c
	S+C		3.16 ± 0.197 b	4.82 ± 0.143 b	6.41 ± 0.055 b	7.31 ± 0.136 b
H_2S-producing bacteria	control	1.13 ± 0.085	1.69 ± 0.225 b	3.76 ± 0.189 c	4.76 ± 0.042 c	5.01± 0.013 c
	Ls4808		<1.00 ± 0.000 a	2.09 ± 0.354 a	2.29 ± 0.411 a	2.86 ± 0.221 a
	Lc904		1.28 ± 0.100 a	3.06 ± 0.239 b	3.94 ± 0.169 b	4.27 ± 0.168 b
	S+C		<1.00 ± 0.000 a	2.34 ± 0.429 a	2.52 ± 0.481 a	3.11 ± 0.356 a
LAB	control	2.04 ± 0.220 a	3.47 ± 0.309 a	5.71 ± 0.042 a	7.08 ± 0.140 a	8.10± 0.138 a
	Ls4808	7.02 ± 0.019 b	7.56 ± 0.033 b	8.02 ± 0.102 b	8.40 ± 0.027 b	8.43 ± 0.021 b
	Lc904	7.09 ± 0.011 b	7.84 ± 0.057 c	8.02 ± 0.155 b	8.46 ± 0.053 b	8.26 ± 0.120 ab
	S+C	7.18 ± 0.015 b	7.79 ± 0.035 c	8.22 ± 0.078 b	8.44 ± 0.017 b	8.19 ± 0.058 a

[a] Counts on day 0 refer to the raw material, except for LAB counts. Data are presented as mean value ± standard error ($n = 6$). Means in the same column for the same microbial group bearing a different letter differ significantly ($P \leq 0.05$).

treatments, Lc904 samples were found to have significantly higher ($P \leq 0.05$) contents for all of the detected amines, whereas differences between S+C and Ls4808 samples were significant ($P \leq 0.05$) only for putrescine, cadaverine, histamine, and spermidine. The observed differences in BA contents among treatments are probably related to the significantly higher ($P \leq 0.05$) counts of spoilage organisms (Enterobacteriaceae, *Pseudomonas*, and H_2S-producing bacteria) in the control and Lc904 samples compared to samples of the other two treatments (**Table 2**).

For all treatments, putrescine and cadaverine were the main BAs formed during storage of the samples. Levels of putrescine and cadaverine increased from initial values of 6.45 and 1.88 mg/kg in the raw material, respectively, to 55.82 and 62.23 mg/kg, respectively, in the control samples on day 20 of storage. Much lower levels of these two amines have been detected by Rodriguez et al. (*15*) in refrigerated vacuum-packed trout after 12 days of storage and by Chytiri et al. (*12*) and Paleologos et al. (*21*) in filleted rainbow trout after 18 days in ice. Similar or higher contents than ours are reported by Özogul and Özogul (*27*) in fresh ice-stored vacuum-packed trout after 18 days for both amines and by Emborg et al. (*11*) in fresh modified-atmosphere-packed salmon stored at 2 °C for cadaverine. Putrescine and cadaverine contents on day 20 were 16.91 and 24.05 mg/kg for Ls4808, 52.38 and 55.74 mg/kg for Lc904, and 29.33 and 29.04 mg/kg for S+C, respectively. In agreement with our results, Baixas-Nogueras et al. (*28*), studying hake, found that cadaverine usually starts to increase later than putrescine, but its levels at the end of storage are generally higher. Putrescine values lower than 10 mg/kg have been proposed by Křížek et al. (*6*) as indicative of good carp meat quality, values between 10 and 20 mg/kg as indicative of acceptable quality, and values >20 mg/kg as indicative of poor quality carp meat based on sensory scores. In the same context, they have also suggested that the sum of putrescine and cadaverine concentrations could also be used as a promising criterion for carp meat, although slightly preceding the sensory signals, with maximum total values of 20, 20−45, and >45 mg/kg for good, acceptable, and poor quality, respectively.

Low concentrations of tyramine, tryptamine, β-phenylethylamine, and histamine have been detected in the raw material. The levels of these amines either increased slightly or fluctuated around their initial values throughout the storage period, without exceeding the concentration of 10 mg/kg, with the exception of tryptamine in the control samples on day 20. With regard to histamine, the values obtained for all treatments, even after 20 days of storage, are well below the EU regulatory limit of 100 mg/kg (*5*). Such low levels of histamine are expected in substrates such as trout, where there is little or no free histidine present, which is the precursor of histamine. Not detectable or similarly low (<10 mg/kg) levels of these amines have also been reported during low-temperature storage of rainbow trout (*12*, *15*), freshwater vacuum-packed carp (*10*), and Mediterranean sea bass (*13*). On the other hand, higher concentrations of histamine and tyramine have been found in fresh and thawed modified-atmosphere-packed salmon after 18 days of storage at 2 °C (*11*).

Spermine and spermidine were initially present at rather high concentrations in the raw material, but both steadily declined during the 20-day storage period for all sample treatments. This decrease has also been reported in aerobically stored gilthead sea bream (*14*), sea bass (*13*), and various sea fish species including salmon, rockfish, lobster, and shrimp (*8*). The literature on spermine and spermidine behavior during fish storage is contradictory, as other authors have reported either increasing trends (*12*, *15*) or no change (*28*) in these amines. The initial presence of spermine and spermidine at increased concentrations is common in metabolically highly active tissues, such as those of animals and plants, as these amines are considered to be involved in cell growth and proliferation (*29*). In animal origin foods, concentrations of spermine are normally greater than those of spermidine, as confirmed by the results of the present work and others (*8*, *13*, *15*, *28*), whereas in plant products the relationship of their contents is reversed (*29*). There are, however, reports on spermine and spermidine contents of meat and fish in the literature that contradict this trend (*12*, *21*, *30*).

Changes in Microbiological Counts and Off-Odor Scores during Storage. The numbers of *Lb. sakei* and *Lb. curvatus* in the inocula were 8.41 ± 0.019 and 8.59 ± 0.033 $\log_{10}$ cfu/mL, respectively (mean ± standard error). Microbiological counts of the trout fillets during the 20-day storage period are presented in **Table 2**. In all cases counts of spoilage bacteria, namely Enterobacteriaceae, pseudomonads, and H_2S-producing bacteria, were higher for the control samples, followed by Lc904, S+C, and Ls4808. Enterobacteriaceae were not initially detectable in the raw material, indicating low cross-contamination during the filleting process. During the 20-day storage period, Enterobacteriaceae counts differed significantly among all treatments, with

samples inoculated with *Lb. sakei* showing the lowest values, reaching the level of 6 $\log_{10}$ cfu/g on day 20. A similar number was reached approximately on days 11, 13, and 19 for the control, Lc904, and S+C samples, respectively. Enterobacteriaceae counts were lower at the end of the storage period in aerobically ice-stored trout fillets (*12*) and sea bass fillets (*13*) compared to the control samples of our study. This is attributed to factors favoring the growth of Enterobacteriaceae in our experiments such as the higher storage temperature (4 °C) and vacuum-packaging. Enterobacteriaceae counts similar to our control sample have been reported by Arashisar et al. (*31*) in vacuum-packaged rainbow trout fillets stored at 4 °C.

Pseudomonads were also not detectable in the raw material. As in the case of Enterobacteriaceae, *Pseudomonas* spp. counts also differed significantly ($P \leq 0.05$) among all sample treatments during the storage period. The level of 7 $\log_{10}$ cfu/g was reached approximately after 12, 14, 18, and 20 days of storage for the control, Lc904, S+C, and Ls4808 samples, respectively. Pseudomonads are known to be the dominant spoilage organisms of freshwater fish (*1*). *Pseudomonas* spp. counts in the present study gradually increased, although fillets were vacuum-packaged; this can be attributed to the oxygen transmission rate of the packaging material, as oxygen levels as low as 1% are enough to support the growth of these organisms under vacuum-packaging conditions (*32*). As expected, the reported counts of pseudomonads during aerobic storage of fresh filleted fish are higher than ours, despite ice storage (*12*, *13*).

H_2S-producing bacteria (including *S. putrefaciens*) counts did not differ significantly ($P > 0.05$) between Ls4808 and S+C samples, whereas both treatments showed significantly different ($P \leq 0.05$) levels from Lc904 and control samples, especially after the 10th day of storage. Initial counts were low (**Table 2**) in the raw material; maximum counts of 5 $\log_{10}$ cfu/g were attained at the end of the storage period in the control samples, whereas the respective counts for the Ls4808 samples were lower by $\approx$2 $\log_{10}$ cfu/g. Similar to our results, H_2S-producing bacteria counts were at the level of 5 $\log_{10}$ cfu/g in fresh modified-atmosphere-packed salmon after 28 days of storage at 2 °C (*11*), whereas higher counts were observed in aerobically ice-stored filleted fish (*12*, *13*). Finally, LAB counts for the control sample rose from 2 to >8 $\log_{10}$ cfu/g, whereas the counts for the inoculated samples were constantly >8 $\log_{10}$ cfu/g after storage day 10. It is apparent that for all spoilage bacteria examined, populations were consistently higher in the control samples compared to the inoculated treatments throughout the storage period. These results indicate that inoculation with LAB prevented, to different extents, the growth of these organisms. *Lb. sakei* 4808 showed the best protective effect, which is probably a result of its broader antimicrobial spectrum and more efficient acid production (*18*).

Off-odor scores differed significantly ($P \leq 0.05$) among all sample treatments at all times (**Figure 2**). The limit of acceptability was reached approximately on days 13, 15, 17, and 18 for the control, Lc904, S+C, and Ls4808 samples, respectively, whereas the off-odor detected by the panelists at the time of spoilage was described as putrid. For all treatments the limit of off-odor rejection coincided with the time when the Enterobacteriaceae and pseudomonads counts were around 6 and 7 $\log_{10}$ cfu/g, respectively; no such connection was observed with regard to H_2S-producing bacteria counts. This is to be expected in vacuum-packed freshwater fish, where the latter organisms grow more slowly and are usually present during the later stages of fish spoilage (*1*).

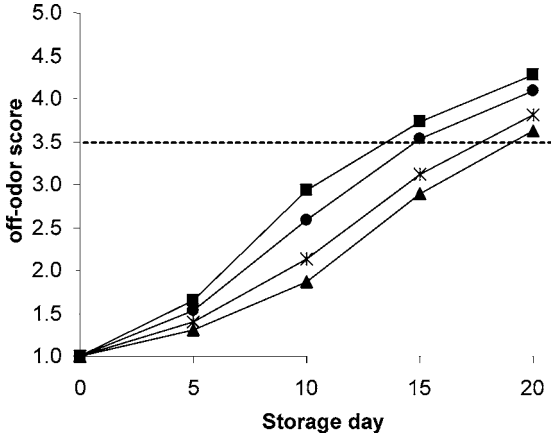

Figure 2. Off-odor scores of the experimental trout samples during the 20-day storage period: (■) control; (▲) Ls4808; (●) Lc904; (∗) S+C.

Relationship of BA Formation to Microbial and Sensory Changes. As mentioned previously, BAs are usually formed through bacterial enzyme activity, whereas the types and levels of BAs will depend on the specific microflora and count (*4*). Of all BAs examined in the present study, concentrations of putrescine and cadaverine were highest at the time of sensory rejection. The limit of acceptability for putrescine (20 mg/kg), as proposed by Křížek et al. (*6*), was reached approximately on days 10, 13, 17, and 20 for control, Lc904, S+C, and Ls4808 samples, respectively, whereas the levels of 25 mg/kg of cadaverine and 45 mg/kg for the sum of putrescine and cadaverine were reached at about the same time. As mentioned before, putrescine is mainly formed by pseudomonads, whereas Enterobacteriaceae are usually responsible for the formation of cadaverine (*4*). Interestingly, both cadaverine and Enterobacteriaceae showed a steep increase in control and Lc904 samples after the fifth day of storage, whereas for the other two treatments both parameters increased in a smoother trend. Similar are the trends with regard to putrescine contents and *Pseudomonas* spp. counts. It should be noted that an increase of the packaging material's oxygen permeability would probably result in higher *Pseudomonas* spp. and lower Enterobacteriaceae counts, with a subsequent increase in putrescine and decrease in cadaverine formation, respectively. This could be supported by the results of Křížek et al. (*10*), who compared BA contents in vacuum-packed and non-vacuum-packed carp stored at 3 °C and found higher putrescine and lower cadaverine contents in the latter samples. This is also the case with other fish stored under aerobic conditions, where higher *Pseudomonas* spp. and lower Enterobacteriaceae counts than those of the present study have been reported (*12*, *13*).

For all treatments, off-odor scores correlated strongly ($P \leq 0.01$) with counts of spoilage microorganisms and putrescine and cadaverine concentrations (**Table 3**). In agreement with the results of the present study, Křížek et al. (*6*) pointed out that the sum of putrescine and cadaverine contents could be a promising criterion for carp meat quality, as both amines showed similar kinetics, slightly preceding the sensory signals. Our results therefore indicate that the acceptability limits proposed by Křížek et al. (*6*) for putrescine and cadaverine contents also apply in the case of rainbow trout, at least with regard to the experimental conditions used in the present study.

Levels of histamine, β-phenylethylamine, tyramine, and tryptamine remained below 10 mg/kg in samples of all treatments at least until the respective time of sensory rejection. At the same time counts of Enterobacteriaceae, usually responsible for histamine formation, were around the level of 6 $\log_{10}$ cfu/g,

Table 3. Spearman Correlations between the Tested Parameters ($n = 120$)[a]

	ENT	PSE	LAB	H_2S-pb	ODOR	PUT	CAD	TRY	PHE	SPD	SPM	HIS	TYR
ENT	1.000												
PSE	0.992**	1.000											
LAB	0.537**	0.544**	1.000										
H_2S-pb	0.852**	0.822**	0.264**	1.000									
ODOR	0.983**	0.985**	0.585**	0.810**	1.000								
PUT	0.779**	0.760**	0.220*	0.878**	0.763**	1.000							
CAD	0.897**	0.895**	0.481**	0.830**	0.900**	0.821**	1.000						
TRY	0.545**	0.514**	–0.119	0.717**	0.466**	0.617**	0.468**	1.000					
PHE	–0.309**	–0.319**	–0.598**	–0.133	–0.362**	0.048	–0.204**	0.329**	1.000				
SPD	–0.781**	–0.792**	–0.767**	–0.553**	–0.832**	–0.475**	–0.706**	–0.066	0.714**	1.000			
SPM	–0.720**	–0.746**	–0.795**	–0.393**	–0.797**	–0.382**	–0.629**	0.028	0.642**	0.876**	1.000		
HIS	0.801**	0.794**	0.196*	0.787**	0.778**	0.870**	0.757**	0.687**	0.112	–0.431**	–0.380**	1.000	
TYR	0.811**	0.829**	0.428**	0.650**	0.847**	0.745**	0.745**	0.514**	–0.036	–0.595**	–0.658**	0.806**	1.000

[a] Abbreviations: ENT, Enterobacteriaceae; PSE, *Pseudomonas* spp.; LAB, lactic acid bacteria; H_2S-pb = H_2S-producing bacteria; ODOR, off-odor; PUT, putrescine; CAD, cadaverine; TRY, tryptamine; PHE, β-phenylethylamine; SPD, spermidine; SPM, spermine; HIS, histamine; TYR, tyramine. *, $P < 0.05$. **, $P < 0.01$.

which is considered to be low for histamine production (*12*−*14*). Histamine formation is also attributed to H_2S-producing bacteria, and especially *P. phosphoreum* (*33*), which is not considered to be important in the spoilage of freshwater fish (*1*). H_2S-producing bacteria counts in the present study remained low (<4 $\log_{10}$ cfu/g) until the time of sensory rejection and slightly exceeded 5 $\log_{10}$ cfu/g at an advanced decomposition stage (day 20, control sample). In conjunction with the generally low levels of free histidine reported in salmonids (*11*), these factors could account for the low histamine contents observed in rainbow trout fillets in the present study. Indeed, the maximum histamine concentration determined in the present study (6.11 mg/kg in day 20 control sample) is lower by a factor of $\approx$16 than the toxicological limit of 100 mg/kg (*5*), although this value was measured much later than the sensory rejection point, at an advanced stage of decomposition. This is probably the reason that salmonids are not considered to be causative agents of histamine poisoning and are not included in the species for which a regulatory limit is applied (*5*, *11*).

It is also noteworthy that spermine and spermidine contents decreased to significantly lower contents in the samples of the inoculated treatments compared to the control. Guirard and Snell (*34*) have reported that the growth of LAB, and especially lactobacilli, is stimulated by the presence of spermine and spermidine in the substrate. The same authors have reported that LAB naturally contain very low spermine and spermidine quantities and that they tend to accumulate these amines intracellularly when found in abundance in their substrate. This trait is not equal, quantity-wise, among different LAB strains and could account for the observed overall significant differences in spermine and spermidine contents between samples of treatments Ls4808 and Lc904.

In agreement with Křížek et al. (*6*), when calculating the BAI criterion of Mietz and Karmas (*8*) we found an obvious delay in its onset, probably because of the very low histamine contents of our samples. The proposed acceptability value of 10 for this index (*8*) is considered to be high also for rainbow trout flesh, as in the case of carp meat (*6*). On the other hand, the critical value of 3.2 for the BAI criterion, calculated according to the putrescine and cadaverine acceptability limits proposed by Křížek et al. (*6*), corresponds in our study to storage days 12, 14, 16, and 18 for the control, Lc904, S+C, and Ls4808 samples, respectively (data not shown). It is apparent that the BAI value of 3.2 in the present study correlates almost perfectly with the sensory rejection scores and is proposed as a critical value for assessment of shelf life during refrigerated storage of filleted rainbow trout.

In the present study all inoculated samples had generally significantly lower ($P \leq 0.05$) contents for all determined BAs than the noninoculated controls. The observed differences between treatments followed the same pattern for all determined BAs (Ls4808 < S+C < Lc904 < control) and were obviously related to the strain used for inoculation. It is therefore evident that inoculation with the strain *Lb. sakei* 4808 resulted in the lowest BA contents, which is also in agreement with the lowest bacteriological counts and the longest time until sensory rejection observed in the Ls4808 samples. Reduction of cadaverine, tyramine, and histamine contents in cold-smoked fish inoculated with LAB strains has also been observed by Petäjä et al. (*16*), but no such effect was noted for putrescine or the other BAs. Similarly, inoculation with two non-amine-producing *Lb. sakei* strains significantly inhibited the formation of putrescine, cadaverine, and tyramine in minced meat stored for 7 days at 20 °C (*30*). Histamine contents were also significantly lower in tuna mince samples inoculated with different LAB strains after 51 days of refrigerated storage (*35*). Brillet et al. (*17*), however, found no significant differences in the putrescine, cadaverine, and histamine contents of vacuum-packed cold-smoked salmon inoculated with *Carnobacterium divergens* after 28 days of storage; on the contrary, tyramine concentrations were found to be higher in the inoculated samples, indicating the production of this amine by the inoculated strain.

In conclusion, although BAs are not considered to be responsible for spoilage off-odors in fresh fish (*33*), their concentrations, especially those of putrescine and cadaverine, correlated rather well with the development of spoilage off-odors in the present study. In this context, BAs could be useful indicators for the assessment of filleted trout shelf life. Moreover, inoculation with both *Lactobacillus* strains caused a significant ($P \leq 0.05$) reduction in the BA contents of the respective samples. However, it seems that the strain *Lb. sakei* 4808 showed a significantly better performance with regard to control of both bacteriological and sensory parameters as well as in reducing the BA contents in the present study. Further studies involving the use of various LAB strains are required (a) to improve understanding of the relationship between BA formation and bacterial counts/sensory attributes and (b) to investigate the potential of commercial use of biopreservation by LAB for extending the shelf life of fresh fish.

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