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Research Note

Effects of Storage Temperature on Tyramine Production by *Enterococcus faecalis* R612Z1 in Water-Boiled Salted Ducks

FANG LIU,[1] LIHUI DU,[2] HAIHONG WU,[1]* DAOYING WANG,[1] YONGZHI ZHU,[1] ZHIMING GENG,[1] MUHAN ZHANG,[1] AND WEIMIN XU[1]

[1]*Institute of Agricultural Products Processing, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, People's Republic of China; and* [2]*College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing 210046, People's Republic of China*

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ABSTRACT

Tyramine production by *Enterococcus faecalis* R612Z1 in water-boiled salted ducks was evaluated during storage at different temperatures. The results showed that *E. faecalis* R612Z1 could produce tyramine in meat samples when the storage temperature was no less than 4°C. The *E. faecalis* R612Z1 counts of the meat samples reached 10^8 CFU/g on day 7 at 4°C and on day 4 at 10°C. However, the tyramine content of the meat samples stored at 10°C increased to 23.73 μg/g (on day 10), which was greater than the level in the samples stored at 4°C (7.56 μg/g). Reverse transcription quantitative PCR detection of the expression level of the *tyrDC* gene in *E. faecalis* R612Z1 in the meat samples revealed no significant changes at different storage temperatures. Thus, the changes in tyramine production of *E. faecalis* R612Z1 may be due to the different enzymatic activities at different storage temperatures.

Biogenic amines (BAs) are low-molecular-weight organic bases formed mainly through the decarboxylation of amino acids *(19)*. The most common BAs found in foods are histamine, tyramine, cadaverine, 2-phenylethylamine, spermine, spermidine, putrescine, tryptamine, and agmatine *(16)*. Tyramine is one of the most toxicologically significant BAs and is found in some food products, including low-temperature and low-fermentation meat products *(11, 14)*. Tyramine is produced by the decarboxylation of tyrosine through tyrosine decarboxylase-specific enzymes derived from bacteria present in food *(13)*. Numerous articles have focused on the tyramine content of foods since the identification of the ''cheese reaction'' hypertensive crisis induced by dietary tyramine in individuals taking monoamine oxidase inhibitor drugs *(1, 3)*. Studies show that the dietary intake of 200 mg to 800 mg of tyramine could moderately increase the blood pressure of normal adults *(2)*. However, 6 mg of tyramine in one or two servings is considered sufficient to exert a mild adverse effect on those taking monoamine oxidase inhibitor drugs *(15)*. Therefore, studies are needed to determine how to prevent tyramine formation in foods and how to reduce their levels once formed.

Water-boiled salted duck is a famous traditional pasteurized meat product enjoyed by many Chinese people *(4)*. In this meat product, our group has previously detected tyramine and found it to be primarily produced by *Enterococcus faecalis (11)*. Accordingly, this study aimed to evaluate the tyramine production ability of *E. faecalis* strain R612Z1 in water-boiled salted ducks during storage at different temperatures.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Tyramine- producing strain *E. faecalis* R612Z1 was isolated from water-boiled salted duck *(11)*, and it was routinely grown in de Man Rogosa Sharpe (MRS) medium (LuQiao Co., Beijing, People's Republic of China, China) at 30°C.

Sample preparation. *E. faecalis* R612Z1 was grown in 500 ml of MRS broth at 37°C for 16 h and harvested by centrifugation at 3,700 × *g* for 5 min at 4°C. The bacterial cells obtained were resuspended in sterile normal saline (0.90% NaCl, wt/vol). The suspension density was adjusted to about 10^6 to 10^7 CFU/ml, which was measured by serial dilution of the suspension with sterile normal saline. The dilution was inoculated onto MRS agar and incubated for 48 h at 30°C.

Water-boiled salted ducks were obtained from a local meat factory in July 2012. The processing of this product was reported by us previously *(12)*. The meat of about 30 water-boiled salted ducks was removed from the bones and mixed together. After the chopped meat was sterilized at 121°C for 15 min, it was divided into two groups with 80 portions each. Every portion in one group was sprayed uniformly with 10 ml of *E. faecalis* R612Z1 suspension to obtain initial bacterial counts of 10^5 CFU/g in meat samples, while the other control group was sprayed uniformly with 10 ml of sterile normal saline. Each portion in the two groups was packaged in a separate tray. The storage temperatures were 0, 4, and 10°C. After 1, 4, 7, and 10 days of storage, samples (four samples for each treatment) were randomly chosen for the next experiments.

Bacterial counts. Twenty grams of each sample was homogenized in 180 ml of sterile peptone saline (1 g of peptone

* Author for correspondence. Tel: +86 25 84390065; Fax: +86 25 84390065; E-mail: wuhaihong169@163.com.

TABLE 1. E. faecalis *counts in the treated samples during storage at different temperatures*

Storage temp (°C)	*E. faecalis* count (log CFU/g) on day[a]: 1	4	7	10
0	5.81 ± 0.10 A b	5.78 ± 0.10 A c	5.82 ± 0.11 A c	5.42 ± 0.09 B b
4	6.27 ± 0.12 C a	7.48 ± 0.02 B b	8.18 ± 0.08 A b	8.24 ± 0.03 A a
10	6.55 ± 0.09 C a	8.04 ± 0.08 B a	8.54 ± 0.08 A a	8.36 ± 0.13 A a

[a] Values are averages ± standard deviations. Different uppercase letters in a row and different lowercase letters in a column indicate a significant difference ($P < 0.05$).

and 9 g of NaCl per liter) under aseptic conditions. After shaking at low speed for 15 min in a temperature-controlled incubator shaker at 4°C, this suspension was serially diluted (1:10) in sterile normal saline. A total of 1 ml of the dilution was inoculated onto an MRS agar plate (9 cm in diameter) to determine the *E. faecalis* R612Z1 counts. Plates were then incubated for 48 h at 30°C.

Detection of the BA contents. The BAs were extracted from the meat samples according to the method used in our previous study *(11)*. The fluorescent dansyl derivatives of BA were determined by gradient elution using an HP 1200 liquid chromatography system (Agilent Technologies, Wilmington, DE) with a Zorbax Eclipse XDB C_{18} column and were detected with a UV/visible light detector at 254 nm. Gradient elution was performed with a mobile phase consisting of solvents A (H_2O) and B (acetonitrile) (0 to 5 min, 65 to 70% B; 5 to 20 min, 70 to 100% B; 20 to 24 min, 100% B; 24 to 25 min, 100 to 65% B; and 25 to 30 min, 65% B) at 30°C at a flow rate of 1 ml/min. A standard solution of mixed BAs (tryptamine, β-phenylethylamine, putrescine, tyramine, spermidine, spermine, and histamine) was prepared similarly and used as a control.

RNA preparation. For direct extraction of total bacterial RNA from duck meat samples, the initial bacterial suspension of each sample which had been used for bacterial count analysis was centrifuged at 4°C for 10 min at 4,000 × *g*. Of the supernatant, 20 ml was transferred to a 50-ml sterile centrifuge tube, and a second centrifugation was performed at 10,000 × *g* for 20 min at 4°C. The pellet was transferred to a sterile 1.5-ml tube, and RNA was extracted using the TIANamp RNAprep pure cell/bacteria kit (Tiangen, Beijing, People's Republic of China). RNA integrity, concentration, and purity were checked by electrophoresis on a 2% (wt/vol) agarose gel and by measurement of the ratio of optical density at 260 nm to optical density at 280 nm.

Quantification of gene expression by RT-qPCR. Removal of contaminating DNA and synthesis of cDNA were carried out by using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, People's Republic of China) according to the manufacturer's recommendations.

Gene expression was analyzed by reverse transcription quantitative PCR (RT-qPCR) according to the method used in previous articles *(8, 10, 17)*. Primers tdc-F (5′-TAT GGA CGT GCC ATC TTC TTA GAC G-3′) and tdc-R (5′-CGC ATA CGG ATA TCT TGG ATA ACG A-3′) were used to detect the expression level of the tyrosine decarboxylase gene (*tyrDC*). The 16S rDNA gene was used as the reference to normalize data. Quantification of cDNA was performed using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in a 7500 Fast Real-Time PCR system (Applied Biosystems). The reaction mixtures were comprised of the following components: 12.5 μl of SYBR premix Ex Taq II (TaKaRa), 8.5 μl of water, 2 μl of primer mix (containing 5 pmol of each primer), and 2 μl of each cDNA sample. The amplification program was 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. All the samples, including no-RT and no-template controls, were analyzed in triplicate. Data analysis was performed using the $2^{-\Delta\Delta CT}$ cycle threshold (C_T) method, where $\Delta\Delta C_T = \Delta C_T$ (treated sample) − ΔC_T (untreated sample), $\Delta C_T = C_T$ (*tyrDC*) − C_T (16S rDNA), and C_T is the threshold cycle value for the amplified gene. A fold change of ≤2 or >2 was considered a real difference in transcript level.

RESULTS

Bacterial enumeration. Changes in the *E. faecalis* R612Z1 counts of the treated samples throughout storage at different temperatures are shown in Table 1. The *E. faecalis* R612Z1 counts of the treated samples stored at 0°C changed gradually, whereas those of the samples stored at 4 and 10°C increased dramatically from day 1 to day 7 ($P < 0.05$) and remained stable from day 7 to day 10. The *E. faecalis* R612Z1 counts reached 10^8 CFU/g on day 7 at 4°C and on day 4 at 10°C.

Tyramine production of *E. faecalis* R612Z1. The results in Table 2 give an overview of the individual BA content of control and *E. faecalis* R612Z1-treated samples. Four BAs, putrescine, cadaverine, spermidine, and spermine, were detected simultaneously in all of these samples. The contents of the four BAs did not change during storage, indicating that they already existed in the meat samples. Tyramine was detected only in the treated samples, suggesting that *E. faecalis* R612Z1 can produce tyramine in water-boiled salted duck. The tyramine contents increased significantly ($P < 0.05$) during storage at 4 and 10°C, whereas no change was noted during storage at 0°C. The results also revealed that storage temperature significantly affected tyramine production ($P < 0.05$). The tyramine content increased to 23.73 μg/g (day 10) at 10°C, which was greater than the level at 4°C.

***tyrDC* transcriptional analysis.** The results in Figure 1A show that a 250-bp fragment characteristic of the *tyrDC* gene and 150 bp of 16S rDNA were amplified from cDNA, indicating that the primers could be used for subsequent RT-qPCR experiments. The *tyrDC* transcription levels during storage under different conditions, normalized to 16S rDNA levels, are shown in Figure 1B. The expression of the *tyrDC* gene gradually increased during storage (less than threefold), but the storage temperature did not significantly affect the expression of the *tyrDC* gene on the same day (less than twofold).

TABLE 2. *Biogenic amine contents of the control and* E. faecalis *R612Z- treated samples during storage*

Biogenic amine[a]	Storage temp (°C)	Amt of amine (μg/g) in group on indicated day[b]							
		Control group				*E. faecalis* R612Z1-treated group			
		1	4	7	10	1	4	7	10
PUT	0	2.40 ± 0.21 A	2.53 ± 0.20 A	2.18 ± 0.16 A	2.31 ± 0.01 A	2.40 ± 0.15 A	2.26 ± 0.21 A	2.58 ± 0.21 A	2.46 ± 0.02 A
	4	2.39 ± 0.18 A	2.32 ± 0.23 A	2.54 ± 0.19 A	2.42 ± 0.15 A	2.29 ± 0.29 A	2.45 ± 0.28 A	2.38 ± 0.22 A	2.37 ± 0.19 A
	10	2.58 ± 0.32 A	2.32 ± 0.22 A	2.51 ± 0.41 A	2.17 ± 0.34 A	2.28 ± 0.16 A	2.14 ± 0.31 A	2.66 ± 0.55 A	2.42 ± 0.18 A
CAD	0	2.48 ± 0.23 A	2.45 ± 0.16 A	2.44 ± 0.31 A	2.61 ± 0.43 A	1.92 ± 0.41 B	2.66 ± 0.30 A	2.45 ± 0.20 A	2.78 ± 0.22 A
	4	2.78 ± 0.23 A	2.80 ± 0.20 A	2.69 ± 0.19 A	2.84 ± 0.65 A	2.85 ± 0.42 A	2.76 ± 0.35 A	2.70 ± 0.44 A	2.65 ± 0.35 A
	10	2.89 ± 0.08 A	2.87 ± 0.11 A	2.75 ± 0.32 A	2.76 ± 0.21 A	2.73 ± 0.19 A	2.92 ± 0.28 A	2.78 ± 0.43 A	2.65 ± 0.45 A
SPD	0	8.34 ± 0.05 A	8.63 ± 0.43 A	8.37 ± 0.32 A	8.73 ± 0.56 A	8.26 ± 0.40 A	8.75 ± 45 A	8.72 ± 0.44 A	8.89 ± 0.20 A
	4	8.07 ± 0.65 A	8.12 ± 0.46 A	8.00 ± 0.42 A	7.94 ± 0.28 A	7.96 ± 0.45 A	7.96 ± 0.54 A	7.96 ± 0.25 A	7.96 ± 0.0.45 A
	10	7.26 ± 0.44 A	7.96 ± 0.20 A	7.80 ± 0.17 A	7.99 ± 0.31 A	7.91 ± 0.45 A	7.72 ± 0.23 A	7.43 ± 0.09 A	7.31 ± 0.12 A
SPM	0	22.55 ± 1.09 A	21.59 ± 1.12 A	22.09 ± 2.89 A	21.21 ± 0.88 A	21.83 ± 0.56 A	21.65 ± 0.64 A	22.45 ± 1.24 A	19.78 ± 1.36 B
	4	23.56 ± 0.35 A	22.34 ± 0.42 A	22.52 ± 0.22 A	21.92 ± 0.68 A	23.51 ± 0.25 A	21.87 ± 0.56 A	22.74 ± 0.45 A	22.99 ± 0.86 A
	10	19.8 ± 1.02 A	19.67 ± 0.98 A	19.82 ± 0.35 A	19.69 ± 0.04 A	19.79 ± 0.55 A	19.54 ± 0.29 A	19.31 ± 1.20 A	19.45 ± 0.89 A
TYR	0	—[c]	—	—	—	—	—	—	—
	4	—	—	—	—	—	0.61 ± 0.35 A d	2.24 ± 0.42 B d	7.56 ± 0.51 C d
	10	—	—	—	—	—	18.11 ± 1.21 B a	24.45 ± 0.16 A a	23.73 ± 1.38 A a

[a] PUT, putrescine; CAD, cadaverine; SPD, spermidine; SPM, spermine; TYR, tyramine.

[b] Values are averages ± standard deviations. Different letters in a row for the four BAs PUT, CAD, SPD, and SPM and different letters in a row (uppercase letters) or column (lowercase letters) for TYR indicate a significant difference ($P < 0.05$).

[c] —, not detected (the limit of detection was 0.5 μg/g).

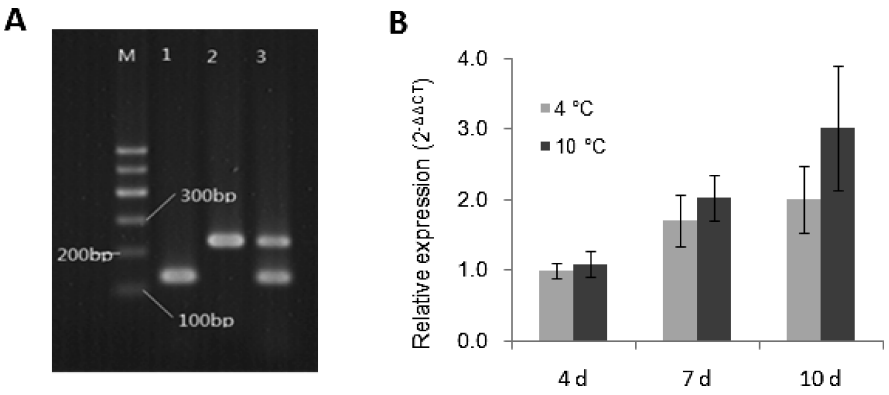

FIGURE 1. *Determination of the specificity of primers and RT-qPCR analysis of the relative* tyrDC *gene expression. (A) Detection of the specificity of primers. M, DNA molecular marker; 1, PCR product of 16S rDNA; 2, PCR product of* tyrDC*; 3, multiple PCR products of 16S rDNA and* tyrDC. *(B) RT-qPCR analysis of the relative* tyrDC *gene expression of* E. faecalis *R612Z1.*

DISCUSSION

BAs in food that are produced by amino acid decarboxylation result mainly from bacterial activity. Their formation can be controlled by inhibiting bacterial growth or the decarboxylase activity of microbes *(20)*. In this study, we found that *E. faecalis* R612Z1 could produce tyramine in water-boiled salted duck when the storage temperature was at least 4°C. The storage temperature (from 4 to 10°C) did not affect the final bacterial counts and *tyrDC* expression levels, but it significantly affected tyramine production. This phenomenon may be explained by the difference in enzymatic activities, as shown in our previous study *(13)*. Although purified recombinant tyrosine decarboxylase from *E. faecalis* R612Z1 could catalyze L-tyrosine within the temperature range of 4 to 60°C, the enzymatic activities were drastically reduced when the reaction temperature was below 10°C *(13)*. Thus, temperature was the most effective parameter in the control of BAs. Considering that bacteria can still produce BAs at 4°C, which was also shown in this study, freezing is more effective than cooling in preventing BA production *(5, 6, 16)*.

In recent years, many studies have been conducted to estimate the influence of the gene expression level of amino acid decarboxylase on the production of BAs during food processing and storage *(7, 9, 18)*. The transcription levels of amino acid decarboxylase did not correspond to the various concentrations of the histamine produced because the enzymatic activities were also affected by the food processing variables *(18)*. In this study, no significant change was observed in the expression level of *tyrDC* at different storage temperatures. Therefore, the differences in tyramine contents may be due to differences in enzymatic activities. It was also found previously *(9)* that the *tyrDC* transcript was undetectable in the cultures inoculated with heat-treated bacteria, which indicated that the expression of *tyrDC* may be regulated by the global changes of the bacteria occurring after heat shock. Currently, there are few articles in the literature focusing on the regulatory mechanisms acting on the *tyrDC* gene. In future work, we plan to study the regulation mechanisms of the *E. faecalis tyrDC* gene locus under different processing and storage conditions in order to prevent tyramine production in water-boiled salted ducks.

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