Iranian Journal of Microbiology
Volume 14 Number 4 (August 2022) 529-534

ORIGINAL ARTICLE

Effect of eliminating *hdcA* gene of *Staphylococcus epidermidis* TYH1 on Histamine production

Safoora Pashangeh[1*], Enayat Berizi[2], Majid Majlesi[3], Sajad Ghaderi[3], Victor Nizet[4], Samira Dahesh[4]

[1]Department of Food Science and Technology, School of Agriculture, Jahrom University, Jahrom, Iran
[2]Department of Food Hygiene and Quality Control, School of Nutrition and Food Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Department of Nutrition, School of Health & Nutrition Sciences, Yasuj University of Medical Sciences, Yasuj, Iran
[4]Department of Pediatrics, School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, USA

Received: January 2022**, Accepted:** July 2022

ABSTRACT

Background and Objectives: The possible adverse effect of histamine on human health has made it a detrimental aspect to the quality and safety of many fermented food products especially fish sauce.
Materials and Methods: In the present study, *hdcA* gene in *Staphylococcus epidermidis* TYH1 was knocked out and its effect on histamine production was evaluated. *HdcA* encodes histidine decarboxylase, an enzyme that produces histamine from histidine. Both strains of TYH1, the wild type (WT) and mutant (ΔhdcA) were then incubated in tryptic soy broth (TSB) supplemented with histidine (0.5 mM). The histamine content determined by capillary zone electrophoretic (CZE) analysis. Safety assessment of this mutant of food origin was conferred by virulence genes.
Results: It was found that *S. epidermidis* TYH1 exhibited production of histamine (50.09 ± 0.06 µg/mL), while ΔhdcA strain of TYH1 exhibited no histamine forming activity. Safety assessment of ΔhdcA revealed the presence of *nuc* gene, while superantigenic toxins and *coa* genes were not observed. Therefore, it has the ability to be used as a starter culture to decrease the histamine content in any fermented food products.
Conclusion: Our study findings may contribute to provide a novel approach of promoting the food safety of fish sauce and other fermented food products regarding the regulation of histamine content.

Keywords: *Staphylococcus epidermidis*; Histamine; Histidine decarboxylase; Capillary electrophoresis; Enterotoxin

INTRODUCTION

Histamine is a heterocyclic biogenic amine (BA) found in a variety of organisms and is formed by the bacterial decarboxylation of free histidine (histidine decarboxylase, *hdc*). It is known as a foodborne chemical hazard and causes scombroid poisoning (1). Adverse effects of histamine on the health of consumers are including tachycardia, headache, flush, itch and decreasing in blood pressure (2). The toxic and defect action levels of histamine in fish products are 50 and 500 ppm respectively which established by FDA (3).

Foods containing high levels of histamine are fish,

*Corresponding author: Safoora Pashangeh, Ph.D, Department of Food Science and Technology, School of Agriculture, Jahrom University, Jahrom, Iran. Tel: +98-9173018808 Fax: +7154372254 Email: s.pashangeh@yahoo.com

seafood and fermented products (4). A popular and commonly used fermented fish product in South-east Asian countries is fish sauce, now consuming worldwide. It contains 20 g/L nitrogen, composed of 80% amino acids that introduced it as a source of dietary protein. The highest ever reported histamine concentration in fish sauce was 1220 ppm (5). High levels of free amino acids in fish and its fermented products resulted in bacterial decarboxylase activity during the insufficient refrigeration process. Therefore, possible methods for controlling histamine content of these products must be applied (6). Potential approaches of reducing histamine accumulation in foods have been proposed, including the prevention of histamine-producing bacterial activity, limiting the BAs constitutive amino acids by preventing the proteolytic activity, inhibition of amino acid decarboxylase activities and controlling the manufacturing process. The oxidative deamination of histamine by histamine oxidase has the potential to degrade it. This method could be applied by the addition of microorganisms or enzymes that decompose histamine (6-8). Since controlling the bacterial decarboxylase activity is more promising than the inhibition of histamine-producing bacteria and the oxidative activities of microbial enzymes and chemical compounds are insufficient for decreasing histamine to acceptable levels, we explored eliminating the histidine decarboxylase gene that governs the formation of histamine from histidine in preliminary trials. In this preliminary study, a mutant strain of *S. epidermidis* TYH1, a halotolerant histamine-producing bacterium isolated from Japanese fermented fish paste (fish-miso) (9), lacking *hdcA* gene was generated. Since staphylococci were frequently isolated from fermented fish products, it can be introduced as a fermenting organism able to decrease histamine content. Therefore safety assessment of this mutant of food origin was conferred by investigating the existence of virulence genes for possibility of using this mutant as a starter culture in fermented food products.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A new histamine forming strain of *S. epidermidis* TYH1 was originally isolated from fish-miso, kindly provided to our laboratory (9). Bacterial strains were cultured in TSB at 37°C in shaking incubator for 24 h. Chloramphenicol (20 µg/mL) was used for plasmid expression of *hdcA* or empty vector control. All the experiments were conducted with *S. epidermidis* TYH1, WT and ΔhdcA mutant strains containing the empty vector control as a proper comparison for experiments using the complemented strain. The diluted bacterial cultures (1:30 in fresh TSB) were grown to logarithmic phase A_{600} = 0.4 (2×10^8 CFU/mL). The experiment conditions resulted in similar growth kinetic for all strains (9, 10).

Targeted mutagenesis and complementation vector construction. Exact allelic replacement of *S. epidermidis* TYH1 was conducted using PCR-based methods as described (10). Primer sequences were designed using the *hdc* gene of the *S. epidermidis* TYH1 characterized by Yokoi et al. (9). Briefly, 300 bp of 1/3 middle sequence of *hdcA* was PCR-amplified from chromosomal DNA of *S. epidermidis* TYH1 with the primers *hdc*-F+attB1, 5´-ggggacaagtttgtacaaaaaagcaggctcctgaagtcgatgaatc-3´ and *hdc*-R+attB1, 5´-ggggaccactttgtacaagaaagctgggtcctgcatcttcaacgaa-3´. The resulting PCR products were fused in a second round of PCR using oligonucleotide primers, *hdc*-F+attB1 and *hdc*-R+attB1, and were then cloned into the temperature-sensitive vector pKOR1 using BP clonase enzyme mix reaction (Invitrogen). The resulting plasmid pKOR1 was first electroporated into *S. aureus* RN4220 and then into *S. epidermidis* TYH1. PCR and sequence analysis were acknowledged the exact in-frame allelic displacement of the *hdc* gene which was settled by a two-step procedure of temperature change and antisense counterselection (10).

Histamine production by bacteria. *S. epidermidis* TYH1 was cultured overnight in 10 mL of TSB medium at 30ºC. Aliquots (5 mL) were then transmitted into 100 mL of fresh TSB and incubated for an additional 24 h at 30°C. Centrifuged bacterial pellet (10000 × g for 10 min) was washed with phosphate buffer (0.1 M, pH 6.0), adjusted to the final concentration of 2×10^8 cells/mL and then inoculated into histidine-supplemented medium (TSB containing 0.5 mM histidine (TSBH)). Recent culture was incubated for 24 h at 30°C. Following, after sedimentation (10000 × g for 5 min at 4°C) the collected supernatant was filtered through a 0.45 µm sterile filter membrane (8, 10).

Histamine analysis. Capillary zone electrophoretic analysis (CZE) was performed for determination

of histamine content using the method of Vitali et al. (11) with some modification. One mL of the prepared supernatant was mixed with 2 mL of 0.1 M HCl in a centrifuge tube. The mixture was homogenized using LabINCO vortex for 2 min (LabINCO, England). Subsequently, the supernatant was obtained by centrifugation at 10000×g for 5 min (Eppendorf, Hamburg) at 25ºC and filtered using a Whatman No. 1 filter paper. This procedure was duplicated for each residue. Both of the supernatants were mixed into a 5 mL microtube and filled up to 4 mL with 0.1 M HCl, filtered with a 0.45 µm filter and injected into the CZE-DAx system. Analysis was carried out by a CZE apparatus (Prince Autosample, Model 1-Lift, 450 Series, Netherland) equipped with a UV-visible detector set at 210 nm, a temperature control device (set at 25ºC) and data treatment software (Data Acquisition and Analysis Software, DAx). Calculation of peak area was used for the quantification of histamine in samples.

Safety assessment of knockout strain: DNA extraction. *S. epidermidis* TYH1 WT and ΔhdcA were inoculated in 2 mL of TSB at 30ºC for 24 h. After centrifugation (5000 × g for 10 min) the harvested cells were washed with distilled water, suspended in 0.5 mL of TE-buffer containing 10 mM Tris-HCl; 1 mM EDTA (pH 8.0), and then 20% sodium dodecyl sulfate was used for cell lysis. The solution was boiled for 20 minutes, and then the cellular debris was removed by centrifugation (10000 × g for 5 minutes). The total DNA which obtained by centrifugation was precipitated with ethanol (70%) and used as template DNA for PCR (10).

Pathogenicity factors. *S. epidermidis* TYH1 WT and ΔhdcA mutant strains were examined for the presence of genes coding pathogenicity factors, including classical enterotoxin genes *sea, seb, sec, sed, see,* enterotoxin-like toxin Q gene (*selq*), and TSST-1 gene (*tst1*) (12), coagulase (13), and nuclease (14). Table 1 shows the PCR primer pairs. PCR amplifications were performed as described by Rahmdel et al. (15). For *selq, tst1, coa* and *nuc* detection, the uniplex PCR assay using each primer pair was applied. The reaction mixture (25 µL) contained 12.5 µL of Taq DNA Polymerase 2.0× Master Mix RED (1.5mM $MgCl_2$; Ampliqon, Copenhagen, Denmark), 0.4 µM of each primer, and 50-100 ng DNA template. Superantigenic toxin genes, (SEs), were co-amplified by multiplex PCR according to Omoe et al. (16). The positive control strains used in this study were *S. aureus* DSM 19040 (*sec, see*), and *S. aureus* DSM 19041 (*sea, seb,* and *sed*).

RESULTS

Construction of *hdcA* deletion mutant. The homologous recombination method was applied for the construction of ΔhdcA mutant. As shown in Fig. 1, the targeted 300 bp bands, designed to validate the elimination of *hdcA* gene, were obtained from the genome of TYH1 WT, but they could not be obtained from the *HdcA* deletion mutant, ΔhdcA. Consequently the recombinant cassette fragment was successfully introduced into the locus of prosequence of TYH1 and the prosequence was replaced and the mutant strain TYH1 ΔhdcA was acquired and recognized by PCR.

Histamine production by bacteria. The histamine forming activity of TYH1 strains, WT and ΔhdcA, was tested by inoculating of resting cells in TSB containing 0.5 mM histidine. The histamine content was determined by the Capillary zone electrophoresis (CZE) method. Fig. 2 showed the histamine contents in WT and ΔhdcA strains of TYH1. *S. epidermidis* TYH1 exhibited production of histamine, forming histamine to about 50.09 ± 0.06 µg/mL, possessed histidine decarboxylase activity. ΔhdcA strain of TYH1 exhibited no histamine forming activity which is in agreement with the lack of *hdcA* gene.

Pathogenicity factors. To the virulence of *HdcA* deletion mutant strain of TYH1, ΔhdcA, a series of PCR assays were conducted. PCR analysis of genes coding for pathogenicity factors in the genome of *S. epidermidis* TYH1 have been summarized in Fig. 3. We investigated the staphylococcal pathogenicity factors in 2 categories: exotoxins and exoenzymes. Exotoxin genes including staphylococcal enterotoxins (SEs) and the SE-related toxin, toxic shock syndrome toxin-1 (TSST-1), are members of the superantigenic toxin. ΔhdcA did not harbor any of the superantigenic toxin genes. Exoenzymes, such as nuclease and coagulase were explored by the uniplex PCR assay using each primer pair. Amplification with *nuc* primers revealed that ΔhdcA was positive for amplification of a 270-300 bp specific band, corresponding to *nuc* gene. However, more virulent exoenzymes genes, such as coagulase was absent.

Table 1. The oligonucleotide primers used in PCR assay for virulence genes detection in TYH1 and TYH1 ΔhdcA.

PCR set	Gene	Nucleotide sequence (5´-3´)	Application size (bp)
Multiplex a	*Sea*	CCTTTGGAAACGGTTAAAACG	127
		TCTGAACCTTCCCATCAAAAAC	
Multiplex	*Seb*	TCGCATCAAACTGACAAACG	477
		GCAGGTACTCTATAAGTGCCTGC	
Multiplex	*Sec*	CTCAAGAACTAGACATAAAAGCTAGG	271
		TCAAAATCGGATTAACATTATCC	
Multiplex	*Sed*	CTAGTTTGGTAATATCTCCTTTAAACG	318
		TTAATGCTATATCTTATAGGGTAAACATC	
Multiplex	*see*	CAGTACCTATAGATAAAGTTAAAACAAGC	178
		TAACTTACCGTGGACCCTTC	
Uniplex b	*selq*	AATCTCTGGGTCAATGGTAAGC	122
		TTGTATTCGTTTTGTAGGTATTTTCG	
Uniplex b	*tst1*	AAGCCCTTTGTTGCTTGCG	447
		ATCGAACTTTGGCCCATACTTT	
Uniplex c	*Coa*	CGAGACCAAGATTCAACAAG	Variable
		AAAGAAAACCACTCACATCA	
Uniplex d	*Nuc*	GCGATTGATGGTGATACGGTT	270-300
		AGCCAAGCCTTGACGAACTAAAGC	

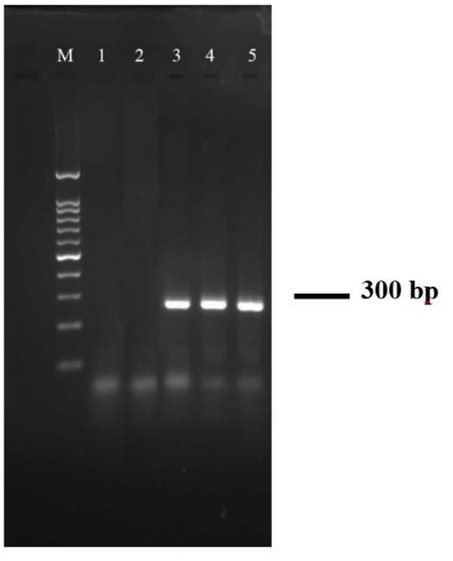

Fig. 1. PCR validation of TYH1 recombinant strains. M: 100 bp ladder; 1, 2: TYH1 ΔhdcA; 3: TYH1 WT; 4: PKOR1 containing 300 bp *hdcA* gene; 5: *S. aureus* RN4220 containing PKOR1.

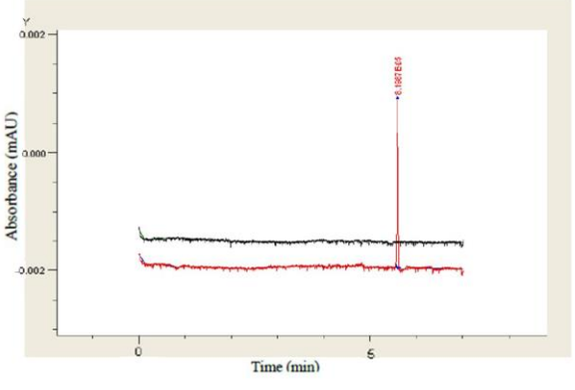

Fig. 2. Capillary zone electropherograms of the histamine contents in WT (red) and ΔhdcA (black) strains of *S. epidermidis* TYH1, while separation by CZE-DAx.

DISCUSSION

Histamine is detected in various fermented food especially in fish and seafoods, which has adverse effect on health of its consumers. Therefore, an importance arises to inhibit or decrease the histamine accumulation in these food sources. Among the potential approaches, controlling the bacterial decarboxylase activity is more reliable than others.

In the present study, *HdcA* deletion mutant was more successful than the inhibition of histamine-producing bacteria and degradation activities of microorganisms for controlling the production of histamine. Therefore, controlling histamine-related enzymes activity, inhibition the enzyme activity or elimination of the gene can be introduced as more practical approaches than others (8, 17). However,

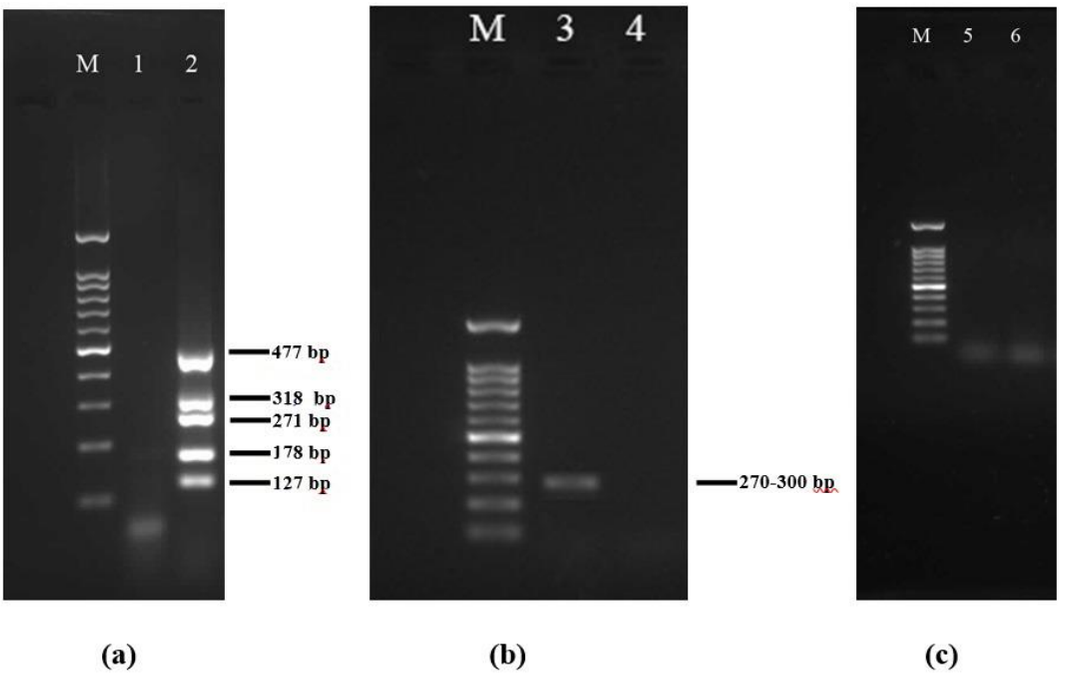

Fig. 3. (a) Representative PCR amplification of TYH1 superantigenic toxin genes. Lanes: M, 100-bp DNA ladder; 1, TYH1-ΔhdcA; 2, Positive control. (b) Representative PCR amplification of TYH1 nuclease gene. Lanes: M, 100-bp DNA ladder; 3, TYH1-ΔhdcA; 4, negative control. (c) Representative PCR amplification of TYH1 coagulase gene. Lanes: M, 100-bp DNA ladder; 5, TYH1-ΔhdcA, 6; negative control.

the lack of information about histamine producing genes in staphylococci is considerable. Interestingly, our results are similar to the study performed by Guo et al. (17), in which *PEP4* gene in *Saccharomyces cerevisiae* was knocked out. The *PEP4* gene encodes an enzyme capable of producing free amino acids. They observed that, this knockout strain exhibited less ability in production of biogenic amines. The PrA activity and the amino acid concentration of this mutant strain reduced remarkably when compared to the wild type strain. The main reason for these reductions was the low concentration of free amino acids. In the study conducted by Pashangeh et al. (8) histamine degrading activity of staphylococcal isolates was investigated. The highest histamine degrading activity was 58.33% related to isolate No. 605 identified as *S. epidermidis.* Also Zaman et al. (18) observed that *S. carnosus* FS19, isolated from fish sauce, was able to degrade 15.1% and 13.8% of histamine content. Due to the bacteria inability to degrade all the added histamine, Diamine oxidase (DAO) was examined as more promising method to degrade histamine (8). No studies had been conducted on eliminating histidine decarboxylase gene, which is the first and most effective agent in preventing histamine accumulation.

ΔhdcA did not harbor any of the superantigenic toxin genes. Similar result of virulence genes is also found in *S. epidermidis* strain RP62A (19). These findings can explain why *S. epidermidis* strains are common inhabitants of skin or mucous membrane, but usually do not invade deeper tissues. To the best of our knowledge, there are a few existent reports on the prevalence of virulence genes in *S. epidermidis* to make a comparison. Similar results of safety assessment of staphylococcal strains which is in accordance with our study reported by Zhang et al. (19), showing that compared to the pathogenicity factors in *S. aureus*, virulence genes were not found in *S. epidermidis* strain (ATCC 12228). Interestingly, Rahmdel et al. (15) observed that *S. epidermidis* 4S09 did not show any of the five classical enterotoxin genes. In this study ΔhdcA did not show a prevalence of superantigenic toxin and *coa* genes. Despite the presence of *nuc* gene, existence of the genes does not indicate their expression and toxin production. From the opposed point of view, the absence of virulence genes is a main criterion for selection of strains as potential starter cultures supplied by the strain TYH1.

CONCLUSION

In conclusion, eliminating of *hdcA* reduced the histamine formation activity in TYH1, and decreased the release of histamine. The finding of this research indicated that among the potential method of decreasing histamine content, generating the ΔhdcA mutant may be a reliable method for increasing the food safety of fish sauce and other fermented food products. Regarding the lack of information about histamine producing and degrading gene in staphylococcal strains, this information can be of great worth to the field of decreasing or inhibition of biogenic amines content in fermented foods.

REFERENCES

1. Tabanelli G. Biogenic Amines and Food Quality: Emerging challenges and public health concerns. *Foods* 2020; 9: 859.
2. Gonzaga VE, Lescano AG, Huamán AA, Salmón-Mulanovich G, Blazes DL. Histamine levels in fish from markets in Lima, Peru. *J Food Prot* 2009; 72: 1112-1115.
3. Lehane L, Olley J. Histamine fish poisoning revisited. *Int J Food Microbiol* 2000; 58: 1-37.
4. Linares DM, Martín MC, Ladero V, Alvarez MA, Fernández M. Biogenic amines in dairy products. *Crit Rev Food Sci Nutr* 2011; 51: 691-703.
5. Zaman MZ, Abu Bakar F, Jinap S, Bakar J. Novel starter cultures to inhibit biogenic amines accumulation during fish sauce fermentation. *Int J Food Microbiol* 2011; 145: 84-91.
6. Naila A, Flint S, Fletcher G, Bremer P, Meerdink G. Control of biogenic amines in food-existing and emerging approaches. *J Food Sci* 2010; 75: R139-150.
7. Mah JH, Hwang HJ. Inhibition of biogenic amine formation in a salted and fermented anchovy by *Staphylococcus xylosus* as a protective culture. *Food Control* 2009; 20: 796-801.
8. Pashangeh S, Shekarforoush SS, Aminlari M, Hosseinzadeh S, Nizet V, Dahesh S, et al. Inhibition of histamine accumulation by novel histamine degrading species of *Staphylococcus* sp. isolated from goats and sheep milk. *Food Sci Nutr* 2022; 10: 354-362.
9. Yokoi KJ, Harada Y, Shozen KI, Satomi M, Taketo A, Kodaira KI. Characterization of the histidine decarboxylase gene of *Staphylococcus epidermidis* TYH1 coded on the staphylococcal cassette chromosome. *Gene* 2011; 477: 32-41.
10. Bae T, Schneewind O. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* 2006; 55: 58-63.
11. Vitali L, Valese AC, Azevedo MS, Gonzaga LV, Costa ACO, Piovezan M, et al. Development of a fast and selective separation method to determine histamine in tuna fish samples using capillary zone electrophoresis. *Talanta* 2013; 106: 181-185.
12. Morandi S, Brasca M, Lodi R, Cremonesi P, Castiglioni B. Detection of classical enterotoxins and identification of enterotoxin genes in *Staphylococcus aureus* from milk and dairy products. *Vet Microbiol* 2007; 124: 66-72.
13. Javid F, Taku A, Bhat MA, Badroo GA, Mudasir M, Sofi TA. Molecular typing of *Staphylococcus aureus* based on coagulase gene. *Vet World* 2018; 11: 423-430.
14. González-Domínguez MS, Carvajal HD, Calle-Echeverri DA, Chinchilla-Cárdenas D. Molecular detection and characterization of the *mecA* and *nuc* genes from *Staphylococcus* species (*S. aureus, S. pseudintermedius,* and *S. schleiferi*) isolated from dogs suffering superficial pyoderma and their antimicrobial resistance profiles. *Front Vet Sci* 2020; 7: 376.
15. Rahmdel S, Hosseinzade S, Shekarforoush SS, Torriani S, Gatto V, Pashangeh S. Safety hazards in bacteriocinogenic *Staphylococcus* strains isolated from goat and sheep milk. *Microb Pathog* 2018; 116: 100-108.
16. Omoe K, Hu DL, Takahashi-Omoe H, Nakane A, Shinagawa K. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. *FEMS Microbiol Lett* 2005; 246: 191-198.
17. Guo X, Guan X, Wang Y, Li L, Wu D, Chen Y, et al. Reduction of biogenic amines production by eliminating the PEP4 gene in *Saccharomyces cerevisiae* during fermentation of Chinese rice wine. *Food Chem* 2015; 178: 208-211.
18. Zaman MZ, Abu Bakar F, Selamat J, Bakar J, See Ang S, Chong CY. Degradation of histamine by the halotolerant *Staphylococcus carnosus* FS19 isolate obtained from fish sauce. *Food Control* 2014; 40: 58-63.
19. Zhang YQ, Ren SX, Li HL, Wang YX, Fu G, Yang J, et al. Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol Microbiol* 2003; 49: 1577-1593.