doi: 10.17221/545/2015-CJFS

Application of Nisin – the Well-known Lactic Acid Bacteria Bacteriocin – against Spoilage Bacteria in Tangerine Wine

Jinjin PEI[1], Lei JIANG[1], Huiping DAI[2] and Pei CHEN[3]

[1]Shaanxi Key Laboratory of Shaanxi Bioresources and Biology, Shaanxi University of Technology, Hanzhong, Shaanxi, P.R. China; [2]College of Northwest Plateau Botany, Chinese Academy, Xining, Qing Hai, P.R. China; [3]Shaanxi Business College, Shaanxi University of Technology, P.R. China

Abstract

Pei J.J., Jiang L., Dai H.P., Chen P. (2016): **Application of nisin – the well-known lactic acid bacteria bacteriocin – against spoilage bacteria in tangerine wine**. Czech J. Food Sci., 34: 488–494.

The application of nisin to tangerine wine was investigated in this study. Nisin was found to be active against *Leuconostoc mesenteroides* CICC 9008, *Lactobacillus acidophilus* CICC 6241, *Oenococcus oeni* CICC 6066, and *Acetobacter pasteurianus* CICC 20874. However, *Saccharomyces cerevisiae* was not sensitive to nisin. The inhibitory activity of nisin against these four strains was tested by adding different concentrations of nisin (25, 50, 75, and 100 μg/ml) under different pH conditions (pH 3, 3.5, 4, and 4.5). The dynamic models of nisin action against these four strains were constructed. When nisin was added in the juicing process, the growth of indicator strains was not inhibited; indicating that components in tangerine juice might impact the activity of nisin. However, the addition of nisin would decrease the concentration of SO_2 added in tangerine wine production. The addition of nisin would increase the final concentration of malic acid and decrease the final concentration of lactic acid. The results indicated that nisin inhibited the natural fermentation of lactic acid.

Keywords: bacteriocin; lethality; time of addition; sensorial properties

China is one of the biggest producers of tangerines. Tangerine wine has been one of the most important products of the tangerine industry. However, microbial spoilage is among the most significant bottlenecks for tangerine wine production (Fugelsang & Edwards 2007). Lactic acid bacteria cause the fermentation of malic acid, which produces several unfavourable compounds such as butanediol, tartaric acid, and so on, resulting in the spoilage of tangerine wine (Arauz *et al*. 2009). These lactic acid bacteria include e.g. *Lactobacillus* spp., *Leuconostoc* spp., and *Pediococcus* spp. *Bacillus aceticus* was also found to be one of the spoilage bacteria in tangerine wine (Rojo-Bezares *et al*. 2007). Although sulphur dioxide is widely applied in the wine industry to control the fermentation of malic acid-tartaric acid (Costantini *et al*. 2009), its usage is subjected to very strict limitations.

Nisin is recognised as safe for use in foods by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives and was allowed to be applied as one of the food additives by more than 40 countries all over the world (Zacharof & Lovitt 2012). Nisin has an extremely strong inhibitory activity against most of the Gram-positive bacteria including *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Staphylococcus aureus*, and *Listeria* spp. However, it

Supported by Shaanxi Education Department's key laboratory, SCI Project No. 16JS021, Shaanxi Technology Department's key laboratory, Project No. 2015SZS-15-05, Shaanxi Technology Department's Agricultural Technology Innovation Project No. 2015NY025, and Shaanxi University of Technology Research Start Project for qualified scientists and technicians No. SLGKYQD2-18.

doi: 10.17221/545/2015-CJFS

was not found to be active against yeast (Zacharof & Lovitt 2012; Parapouli *et al.* 2013; Gharsallaoui *et al.* 2015). The application of nisin would help reduce the usage of sulphur dioxide in wine production. Due to its protein structure, it would be easily decomposed in the human body by stomach protein enzymes. Therefore, nisin is considered as one of the safest food preservatives. In this study, we investigated the application of nisin in tangerine wine.

MATERIAL AND METHODS

Production of tangerine wine. Tangerine wine was prepared following the diagram presented in Figure 1. After the juicing process of tangerine fruits, SO_2 was added immediately at 75 mg/l of fruit juice to rapidly inhibit the oxide present therein. Sugar was added to 20°Brix. After the tangerine juice sample was adjusted to pH value of 3.5, sterilisation (60°C, 30 min) was conducted. Specific yeast of tangerine wine cultured at Shaanxi University of Technology was then inoculated with the inoculum size of 7% followed by fermentation at 18°C for 8 days. The wine was filtered to remove the impurities. A pasteurisation method (80°C, 15 min) was used to sterilise the bacteria in the final product. The wine was stored at a constant temperature of 10°C. The total sugar content was tested by Fehling's Reagent and the content of SO_2 was tested by a pararosaniline hydrochloride method (Arauz *et al.* 2009). Contents of total acids and soluble solids were tested according to national standards (GB 2758-2012 and GB/T12456-2008).

Testing of strains and activity spectrum. *Leuconostoc mesenteroides* CICC 9008, *Lactobacillus acidophilus* CICC 6241, *Oenococcus oeni* CICC 6066, *Acetobacter pasteurianus* CICC 20874 were bought from the China Centre of Industrial Culture Collection (CICC). *Saccharomyces cerevisiae* ATCC 9763 were bought from the American Type Culture Collection (ATCC).

Tangerine wine was inoculated with indicator strains from the exponential phase (2% inocula) as well as with nisin (100 µg/ml) (Yinxiang Bioengineering Co. Ltd., Zhejiang, China). The choice of a high concentration of nisin was made in order to confirm the inhibitory activity. Bacteriocin activity expressed as lethality is:

$$\text{Lethality \%} = (A_0 - A)/A_0 \times 100\%$$

where: A – OD_{600} of tangerine wine treated with nisin after 24 h incubation; A_0 – OD_{600} of tangerine wine without nisin treatment after 24 h incubation

Bacteriocin activity in tangerine wine. Doses (10^4 CFU/ml) of indicator bacteria from the mid-exponential phase were respectively inoculated into tangerine wine with pH 3.5. Nisin (50 µg/ml) was added to tangerine wine and cultivated at 30°C for 24 hours. OD_{600} and viable cells (by plate counts with MRS medium at 30°C for 48 h) were tested and the wine without nisin treatments was used as a control. Tangerine wines with 10^4 CFU/ml indicator bacteria and 50 µg/ml nisin, as described above, were adjusted to different pH (3.0, 3.5, 4.0, and 4.5, respectively) and cultivated (maintaining the set pH values) for 24 h to study the effect of pH on the activity of nisin in tangerine wine. The choice of the pH range was made to replicate the final pH of tangerine wine. The same experiments were also conducted with varied concentrations of nisin (25, 50, 75, and 100 µg/ml).

Application of nisin to tangerine wine. After the juicing process was over, nisin (25, 50, and 100 µg/ml) was added at rates of 0, 25, 50, and 75 mg/l of SO_2, respectively. Then the samples were inoculated with 10^4 CFU/ml of indicator strains and cultivated at

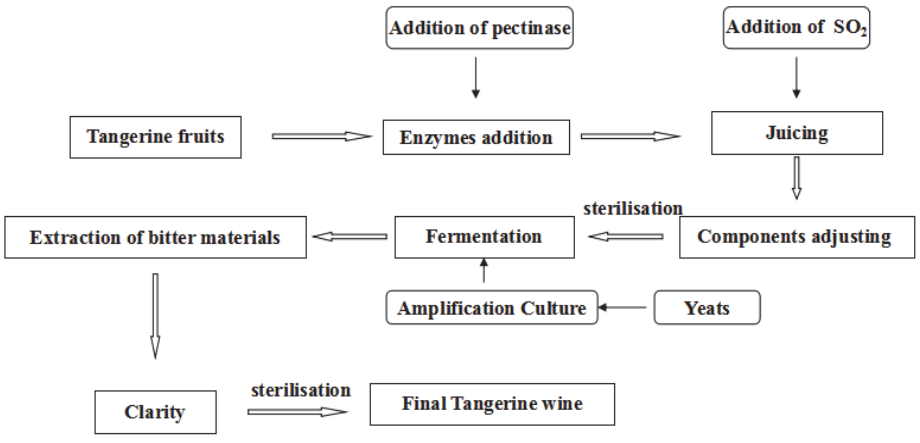

Figure 1. Process of making tangerine wine

doi: 10.17221/545/2015-CJFS

Table 1. Activity spectrum of nisin

Strains	Lethality (%)
L. mesenteroides CICC9008	85.37
L. acidophilus CICC6241	90.14
O. oeni CICC6066	87.25
A. pasteurianus CICC20874	88.69
S. cerevisiae ATCC9763	7.26

Lethality % = $(V_0 - V)/V_0 \times 100\%$; where: V_0 – viable cells before nisin treatment; V – viable cells after nisin treatment

18°C for 8 days for fermentation as described above. OD_{600} and viable cells of indicator strains were tested every day. On the other hand, nisin was added to the final wine product as well as the indicator strains of 10^4 CFU. OD_{600} and viable cells were also investigated.

Sensorial properties. Different nisin concentrations of 20, 50, 100, and 200 μg/ml were separately added to the final tangerine wine. Twenty people were asked to taste the samples to tell whether the taste is acceptable or not. Transmittance was measured by $OD_{625.}$ Glucose, fructose, ethanol, glycerol, acetic acid, succinic acid, citric acid, tartaric acid, malic acid, and lactic acid were tested according to national standards (GBT 15038:2006 – Commen analysis methods for wine and other fermentation fruits wine, GB 2758:2012 – Quality of fermentation wine and their integrated alcoholic beverages, and GB/T12456:2008 – Determination of total acids in foods).

Statistical analysis. All data represent an average of three replications. The values recorded in each experiment did not vary by more than 5%. Single data points are therefore presented in the figures without standard deviation bars. SAS software (version??, year) was used for the statistical analysis.

RESULTS AND DISCUSSION

The final wine was golden in colour and clear. Sugar content was found to be 31.03 g/l, SO_2 was 53 mg/l, pH was 3.62, soluble solids content was 12.9%, and content of acids was 0.20 mol/l.

Leuconostoc mesenteroides CICC 9008, *Lactobacillus acidophilus* CICC 6241, *Oenococcus oeni* CICC 6066, and *Acetobacter pasteurianus* CICC 20874 were sensitive to nisin. However, *Saccharomyces cerevisiae* ATCC 9763 was resistant to nisin (Table 1). The assessment of the inhibitory spectrum is an important characteristic when evaluating possible applications of a bacteriocin (Aly *et al*. 2012). Neris *et al*. (2013) reported 11 strains of lactic acid bacteria to be sensitive to 1000 IU/ml of nisin in grape wine. In this study, nisin was able to inhibit the spoilage bacteria in the tangerine wine making process but it did not inhibit the growth of yeast. Similar results were reported by Rojo-Bezares *et al*. (2007), who observed that nisin MIC_{50} values for the tested isolates were as follows: 0.024, 12.5, 200, and ≥ 400 μg/ml for *oenococci*, *Lactobacilli–Pediococci–Leuconostoc*, acetic acid bacteria, and yeasts, respectively. This characteristic property makes nisin a promising ingredient in tangerine wine making to replace SO_2.

According to the results of single factor experiment, pH and bacteriocin concentration significantly affect the activity of nisin and further experimentations were carried out within the pH range of 3.0 to 4.5 and with concentrations ranging from 25 μg/ml to 100 μg/ml. The results of the action of nisin against *Leuconostoc mesenteroides* CICC 9008 are shown in Table 2. Under the same conditions of nisin ad-

Table 2. Lethality of *Leuconostoc mesenteroides* CICC9008 (*L.c.*), *Lactobacillus acidophilus* CICC6241, *Oenococcus oeni* CICC6066 (*O.o.*), and *Acetobacter pasteurianus* CICC20874 (*A.p.*) treated with nisin at different conditions

Nisin (μg/ml)	pH	Lethality (%)			
		L.c.	*L.a.*	*O.o.*	*A.p.*
25	4.5	57.9	62.3	56.4	58.2
	4.0	59.6	82.8	64.1	69.4
	3.5	94.3	92.8	90.3	84.4
	3.0	97.5	98.4	95.8	94.9
50	4.5	61.9	68.1	65.4	68
	4.0	71.3	75.8	74	72.2
	3.5	95.3	94.6	92.7	85.4
	3.0	96.8	97.8	97.4	95.1
75	4.5	79.6	74.4	79.6	86
	4.0	76.2	83.9	78.9	86.8
	3.5	95.1	96.8	95.5	92.3
	3.0	97.3	98.5	98.1	96.3
100	4.5	84.6	78.7	88.1	88.2
	4.0	88.2	88.3	89.1	91.4
	3.5	97.1	97.9	97.1	92.4
	3.0	98.2	99.1	97.9	98.9

Lethality % = $(V_0 - V)/V_0 \times 100\%$; where: V_0 – viable cells before nisin treatment; V – viable cells after nisin treatment

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dition, the lethality of *Leuconostoc mesenteroides* CICC 9008 increased as the pH decreased. When the concentration of nisin is up to 75 μg/ml, the lethality is more than 94%. The maximum lethality was recorded to be 98.2% with the addition of nisin at 100 μg/ml and pH 3.

The dynamic model relating the lethality caused by nisin (Y) with pH (P) and nisin addition (C) on *Leuconostoc mesenteroides* CICC 9008 was obtained by SAS software as below:

$Y = 0.1022C - 0.503P$

$^{**}P < 0.0001$, C[25, 100], P[3, 4.5]

Lactobacillus acidophilus CICC 6241 is more sensitive to nisin than the other strains (Table 2). The maximum lethality of 99.1% was achieved at pH 3 with the addition of nisin at 100 μg/ml. The dynamic model relating the lethality caused by nisin (Y) with pH (P) and nisin addition (C) on *L. acidophilus* CICC 6241 was obtained by SAS software as below:

$Y = -70.14 + 0.42C - 0.00022C^2 + 0.001658PC$,

$^{**}P < 0.0001$, C[25, 100], P[3, 4.5]

The lethality of *Oenococcus oeni* CICC 6066 strain caused by nisin at different conditions is shown in Table 2. When the nisin concentration is over 75 μg/ml, the lethality of all treatments is over 90%. The maximum lethality is 98.1%, which was achieved with pH 3 and the addition of nisin at 75 μg/ml. The dynamic model of the lethality of *Oenococcus oeni* CICC6066 caused by nisin (Y) is as follows:

$Y = -89.094 + 0.276C - 2.7P + 0.00363PC$,

$^{**}P < 0.0001$, C[25, 100], P[3, 4.5]

where: C – concentration of nisin added; P – pH of experiment

For *Acetobacter pasteurianus* CICC 20874, the maximum lethality was 98.9% at pH 3 and nisin addition was 100 μg/ml (Table 2). The dynamic model relating the lethality caused by nisin (Y) with the pH (P) and the addition of nisin (C) on *Acetobacter pasteurianus* was obtained by SAS software as below:

$Y = 0.145C - 2.658P + 0.0033PC$,

$^{**}P < 0.0001$, C[25, 100], P[3, 4.5]

The pH value is one of the most important factors to affect the activity of nisin. Bacteriocins generated

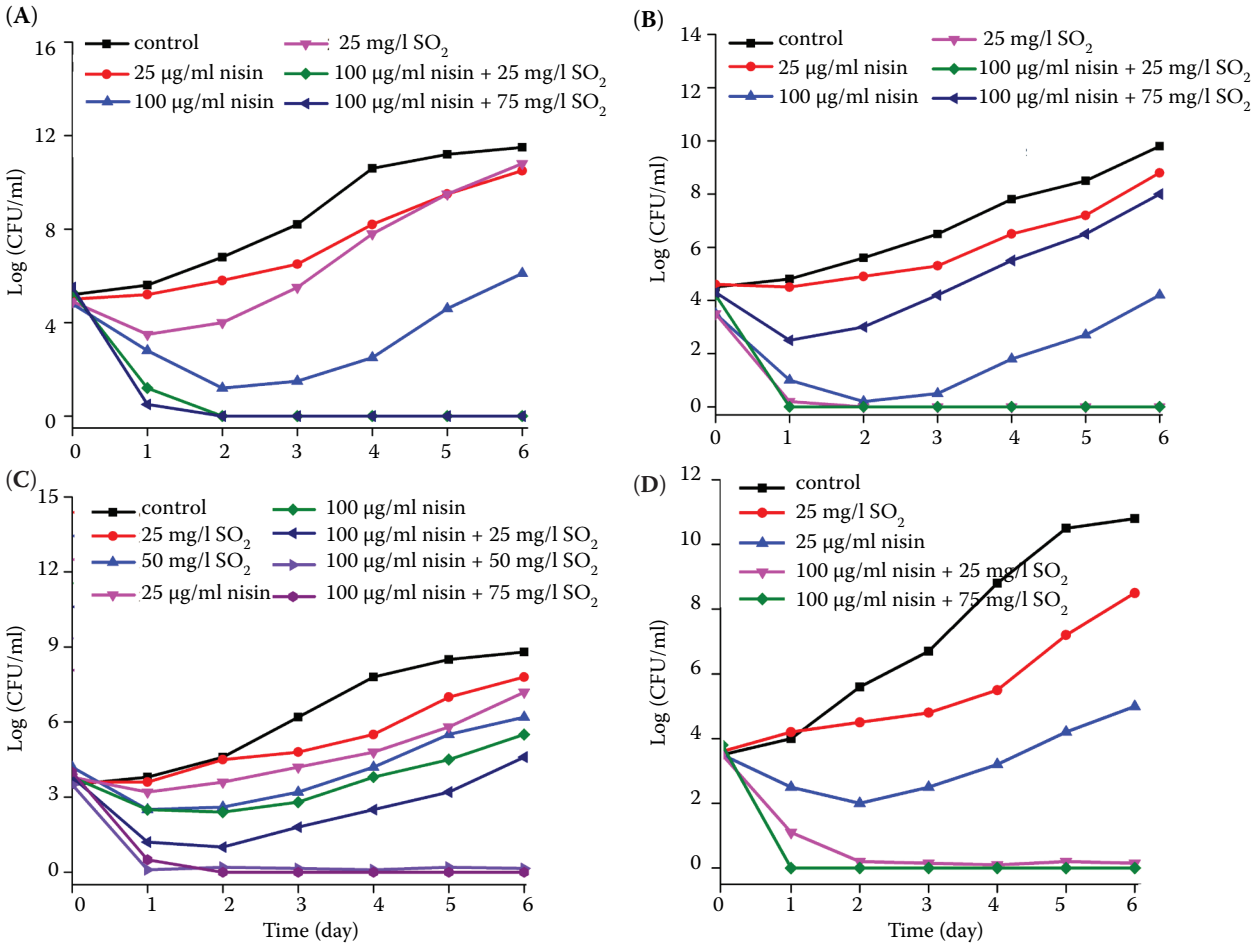

Figure 2. The cell growth of indicator strains with the addition of nisin alone, SO_2 alone, and combination of nisin and SO_2: (**A**) *Leuconostoc mesenteroides*, (**B**) *Lactobacillus acidophilus*, (**C**) *Oenococcus oeni*, and (**D**) *Acetobacter pasteurianus*

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by lactic acid strains are generally active at acid or neutral pH. Optimal activity of buchnericin LB to *Lactobacillus plantarum* was recorded at pH 5.0–8.0, but at pH 5–6 the activity was higher (Pingitore *et al*. 2012). Abrams *et al*. (2011) reported that bacteriocin was active over a wider pH range (2.0–10.0), but the best pH is 4. This pH dependence may be due to specific interactions between bacteriocins and target cells or structural pH dependent modifications of the bacteriocin receptors on the target cell surface. In this study, nisin (100 μg/ml) showed a good activity in the pH range from 3.0 to 4.5, which covered the pH of natural tangerine wine, meaning that nisin is promising in application of tangerine wine making.

When nisin was added in the juicing process, the growth of indicator strains was not completely inhibited (Figure 2), indicating that components in tangerine juice might impact the activity of nisin. Previous reports have suggested that the activity of bacteriocins might be affected by some components in the food matrix. However, the addition of nisin would decrease the concentration of SO_2 in tangerine wine production (Figure 2). These results are interesting because currently there is a concern over the development of super-resistant strains in wineries where cultures are routinely exposed to sulphur compounds, thus the susceptibility of LAB to other inhibitory compounds is appreciated. The survival of a viable population in the bottled product is the most worrying contamination, responsible for the known 'second growth' which can make use of residual L-maleate as a carbon source (Fugelsang & Edwards 2007). An effective control of *O. oeni* by alternative antimicrobial compounds is really needed if we consider that it can survive in a concentration of 100 mg/l of free SO_2 (Neris *et al*. 2013). Our results suggested that nisin plus SO_2 exhibited better inhibitory activity. This may be so because SO_2 could change the physicochemical characterisations of the cell membranes of sensitive bacteria to allow nisin to make pores in the cell membranes.

Table 3. Sensorial properties of tangerine wine with nisin treatment

Sensorial properties	Without nisin	With nisin
Taste (20 μg/ml nisin)	palatable	palatable
Taste (50 μg/ml nisin)	palatable	palatable
Taste (100 μg/ml nisin)	palatable	palatable
Taste (200 μg/ml nisin)	palatable	unfavourable
Transmittance (%)	95.82	90.63
Glucose (g/l)	1.43	1.42
Fructose (g/l)	0.28	0.21
Ethanol (g/l)	78.6	78.3
Acetic acid (g/l)	0.23	0.23
Succinic acid (g/l)	0.22	0.21
Citric acid (g/l)	0.48	0.52
Tartaric acid (g/l)	2.35	2.45
Malic acid (g/l)	3.64	5.17
Lactic acid (g/l)	1.58	0.63

Transmittance, glucose, fructose, ethanol, acetic acid, succinic acid, citric acid, tartaric acid, malic acid, lactic acid were tested with or without 100 μg/ml nisin treatment

When nisin was added to the final wine, 50 μg/ml nisin was able to control four sensitive strains below 20 CFU/ml during the next 7 days (Figure 3). When added during the juicing process, nisin did not work very well. However, when added after the sterilisation process, nisin was able to inhibit spoilage bacteria. This might be so that the sterilisation process denatured the enzymes, polyphenol, or other food components in fresh tangerine wine, which could affect the activity of nisin (Knoll *et al*. 2008).

These results confirmed previous findings indicating that the inhibitory activity of bacteriocins in culture media was not always reproducible in food systems (*in situ*) (Collins *et al*. 2011). Several factors present in the food can influence the inhibitory effect, such as interaction with additives/ingredients, adsorption to food components, and inactivation by food enzymes and pH changes in the food (Pei *et al*. 2013). Low solubility and uneven distribution in the food matrix and limited stability of bacteriocins during the food shelf-life are additional factors that influence the activity of bacteriocins in foods.

There was no unfavourable smell when the concentration of added nisin solution was lower than 100 μg/ml. However, when the concentration of nisin solution was higher than 100 μg/ml, the flavour of tangerine wine was not palatable. Although transmittance was reduced after the addition of nisin (Table 3), it reached the national standard (GBT 15038-2006). The addition of nisin would increase the final concentration of maleic acid and decrease the final concentration of lactic acid (Table 3). The results indicated that nisin did not affect the components and sensorial characteristics of tangerine wine, but inhibited the natural fermentation of lactic acid. The effect on sensorial properties was important for application of food additives, such as vitamin C, ginger powder, and so on (Balestra *et al*. 2010; Gamboa-Santos *et al*. 2013). The effect of

doi: 10.17221/545/2015-CJFS

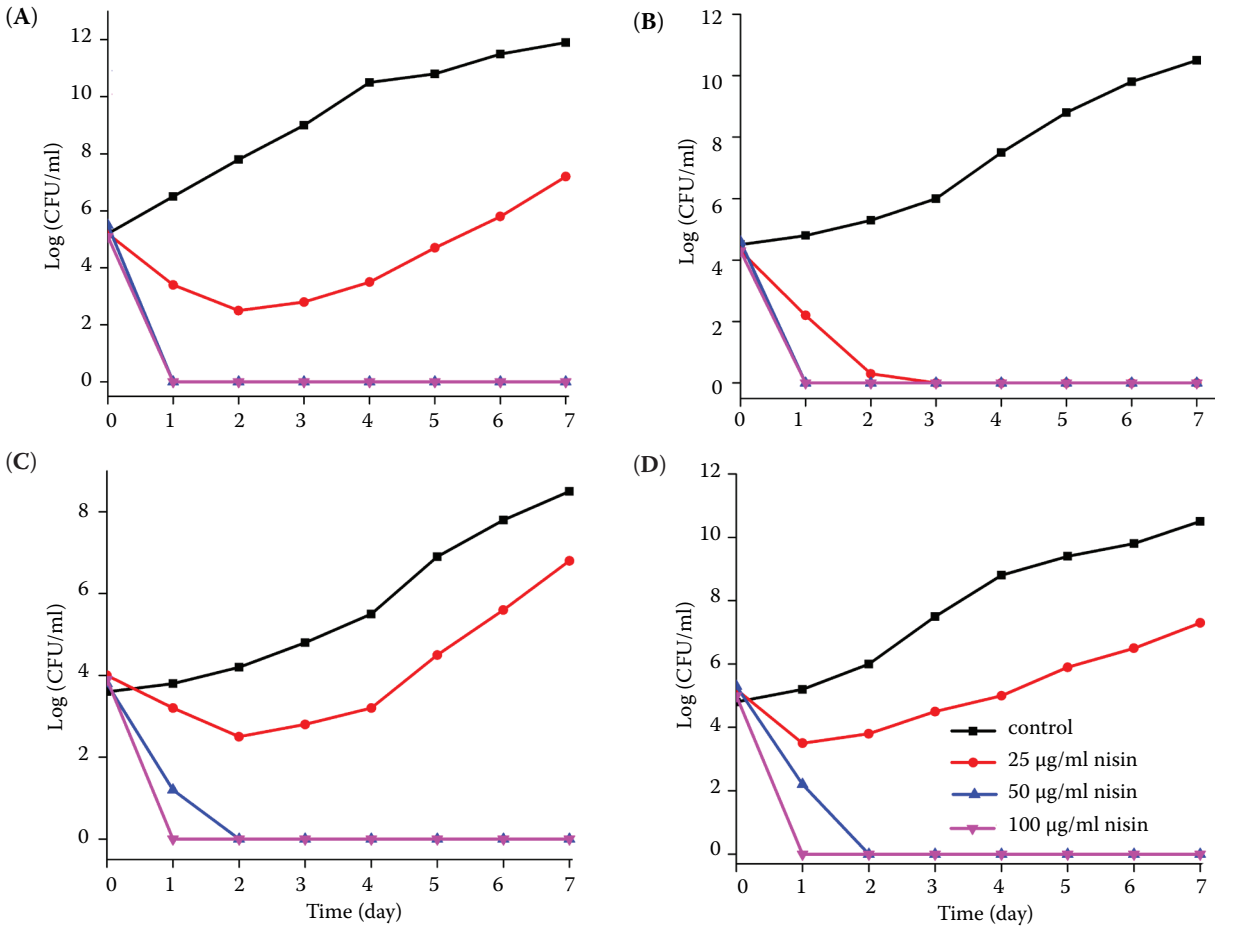

Figure 3. Cell growth of indicator strains with the addition of nisin to the final wine: (**A**) *Leuconostoc mesenteroides*, (**B**) *Lactobacillus acidophilus*, (**C**) *Oenococcus oeni*, and (**D**) *Acetobacter pasteurianus*

bacteriocins on the sensorial properties of foods was little studied. Fortunately, nisin application would not affect the sensorial properties of tangerine wine because of the low concentration of nisin addition.

References

Abrams D., Barbosa J., Albano H., Silva J., Gibbs P.A., Teixeira P. (2011): Characterization of bacPPK34 a bacteriocin produced by *Pediococcus pentosaceus* strain K34 isolated from "Alheira". Food Control, 22: 940–946.

Aly S., Floury J., Piot M., Lortal S., Jeanson S. (2012): The efficacy of nisin can drastically vary when produced in situ in model cheeses. Food Microbiology, 32: 185–190.

Arauz L.J., Jozala A.F., Mazzola P.G., Penna T.C.V. (2009): Nisin biotechnological production and application: a review. Trends in Food Science and Technology, 20: 146–154.

Balestra F., Cocci E., Pinnavaia G.G., Romani S. (2010): Evaluation of antioxidant, rheological and sensorial properties of wheat flour dough and bread containing ginger powder. LWT-Food Science Technology, 44: 700–705.

Collins B., Cotter P.D., Hill C., Ross R.P. (2011): The impact of nisin on sensitive and resistant mutants of *Listeria monocytogenes* in cottage cheese. Journal of Applied Microbiology, 110: 1509–1514.

Costantini A., Garcia-Moruno E., Moreno-Arribas M.V. (2009): Biochemical transformations produced by malolactic fermentation. In: Moreno-Arribas M.V., Polo M.C. (esd): Wine Chemistry and Biochemistry. New York, Springer Science+Bussiness Media.

Fugelsang K.C., Edwards C.G. (2007): Wine Microbiology: Practical Applications and Produres, 2nd Ed. New York, Springer.

Gamboa-Santos J., Soria A.C., Perez-Mateos M., Carrasco J.A., Montilla A., Villamiel M. (2013): Vitamin C content and sensorial properties of dehydrated carrots blanched conventionally or by ultrasound. Food Chemistry, 136: 782–788.

Gharsallaoui A., Joly C., Oulahal N., Degraeve P. (2015): Nisin as a food preservative: part 2: antimicrobial polymer materials containing nisin. Critical Reviews in Food Science and Nutrition, 56: 1275–1289.

doi: 10.17221/545/2015-CJFS

Knoll C., Divol B., Du Toit M. (2008): Influence of phenolic compounds on activity of nisin and pediocin PA-1. American Journal of Enology and Viticulture. 59: 418–421.

Neris D.J.G., Bordignon-Junior S.E., Baratto C.M., Gelinski J.M.L.N. (2013): Nisin in the biopreservation of Bordo (Ives) and Niagara table wines from Santa Catarina. Brazil Journal of Biotechnology and Biodiversity, 4: 176–183.

Parapouli M., Delbès-Paus C., Kakouric A., Koukkou A.I., Montel M.C., Samelisc J. (2013): Characterization of a wild, novel nisin A-producing *Lactococcus* strain with an *L. lactis* subsp. *cremoris* genotype and an *L. lactis* subsp. *lactis* phenotype, isolated from greek raw milk. Applied and Environmental Microbiology, 79: 2476–2484.

Pei J.J., Yuan Y.H., Tue T.L. (2013): Characterization of bacteriocin bificin C6165: a novel bacteriocin. Journal of Applied Microbiology, 114: 1273–1284.

Pingitore E.V., Todorov S.D., Sesma F., Franco B.D.G. (2012): Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese. Food Microbiology, 32: 38–47.

Rojo-Bezares B., Saenz Y., Zarazaga M., Torres C., Ruiz-Larrea F. (2007): Antimicrobial activity of nisin against *Oenococcus oeni* and other wine bacteria. International Journal of Food Microbiology, 116: 32–36.

Zacharof M.P., Lovitt R.W. (2012): Bacteriocins produced by lactic acid bacteria: a review article. APCBEE Procedia, 2: 50–56.

Received: 2015–11–25
Accepted after corrections: 2016–10–25

Corresponding author:

Dr. Jinjin Pei, Shaanxi University of Technology, Shaanxi Key laboratory of Bioresource and Biology, Hanzhong, Shaanxi, 723001, P.R. China; E-mail: jinjinpeislg@163.com