



Higher nisin yield is reached with glutathione and pyruvate compared with heme in *Lactococcus lactis* N8

Zeynep Girgin Ersoy¹ · Ceyhun Kayihan² · Sedef Tunca¹

Received: 19 August 2019 / Accepted: 17 December 2019 / Published online: 2 January 2020
© Sociedade Brasileira de Microbiologia 2020

Abstract

There are different studies that aim to enhance the production of nisin by *Lactococcus lactis* since its chemical synthesis is not possible. In this study, glutathione (GSH) and pyruvate, which are known to reduce the oxidative stress of cells, have been shown to trigger the production of nisin at both transcriptional and translational levels in *L. lactis* cells grown under aerobic condition. Presence of GSH and pyruvate caused more nisin yield than the heme-supplemented medium. Moreover, the expression of genes that encode stress-related enzymes were apparently upregulated in the presence of GSH and pyruvate. It can be concluded that GSH and pyruvate contribute to the defense system of *L. lactis* cells and so that higher biomass was obtained which in turn enhance nisin production. Antioxidant effect of GSH and pyruvate was known; however, their stimulating effect on nisin production was shown for the first time in this study.

Keywords Glutathione · *Lactococcus lactis* · Nisin production · Oxidative stress · Pyruvate

Introduction

Lactococcus lactis is a member of the lactic acid bacteria having been used in food fermentation for centuries. As well as its usage as a starter culture for dairy products, *L. lactis* subsp. *lactis* produces a bacteriocin called nisin which is especially effective on Gram-positive bacteria [1]. Nisin is the only bacteriocin characterized in generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA) in 1988 [2], and its usage as a food preservative was approved by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) in 1969. Up to date, nisin has been used in the food industry as a

safe food preservative due to its toxicity similar to that of common salt (about LD₅₀ (kittens and rats) 7 g kg⁻¹) [3]. In 2017, the European Food Safety Authority (EFSA) announced that the level of nisin A which is used for the preservation of unripened cheese (at maximum level of 12 mg/kg) and heat-treated meat products (at maximum level of 25 mg/kg) is safe for human consumption [4]. Since nisin cannot be synthesized chemically, it is important to find different ways to increase the nisin production yield of *L. lactis* subsp. *lactis* strains.

Nisin production was shown to be directly related to the growth [5], and thus, attempts to increase the biomass will cause the enhancement of nisin production. Oxidative stress and acid stress are the main factors that affect the growth of *L. lactis* cells negatively. During cellular processes, reactive oxygen species (ROS: O₂⁻, OH, and H₂O₂) with high oxidizing potential are formed and cause damage to the cell. To cope with this oxidative stress, *L. lactis* cells have several enzymes. One of them is manganese superoxide dismutase (SOD) which converts superoxide anion radical to H₂O₂. NADH oxidase/NADH peroxidase system is also effective to prevent the cells from oxidative stress. Like SOD, the activity of NADH oxidase enzyme causes the production of H₂O₂, which is another toxic molecule as well. Inadequate H₂O₂ scavenging by NADH peroxidase and the absence of catalase enzyme cause H₂O₂ accumulation which in turn lead to the oxidative

Responsible Editor: Elaine Cristina Pereira de Martinis.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s42770-019-00216-w>) contains supplementary material, which is available to authorized users.

✉ Sedef Tunca
sgedik@gtu.edu.tr

¹ Department of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Gebze, Kocaeli, Turkey

² Department of Molecular Biology and Genetics, Faculty of Science and Letters, Başkent University, Etimesgut, Ankara, Turkey

stress in *L. lactis* cells. It was shown that the expression of *Bacillus subtilis* heme catalase gene (*katE*) in *L. lactis* alleviated oxidative stress of the cells; both survival after treatment with 4 mM H₂O₂ and long-term survival of aerated cultures were increased; moreover, DNA damage decreased [6].

It is known that the presence of antioxidant molecule glutathione (GSH) in the growth medium increased the survival rates of different *L. lactis* strains although they are treated with H₂O₂ [7]. The study revealed that some of the cells accumulate GSH intracellularly and this causes the elimination of H₂O₂ enzymatically by a GSH/GSH reductase system. In another study, the heterologous expression of *Escherichia coli* genes (*g*-glutamylcysteine synthetase and glutathione synthetase) responsible for GSH production resulted in an oxidative stress-resistant phenotype in *L. lactis* subsp. *cremoris* NZ9000. These recombinant cells producing GSH showed an increase in survival after exposure with H₂O₂ (150 mM) and with a superoxide generator molecule, i.e., menadione (30 μM) [8].

Interestingly, like glutathione, pyruvate was also shown to have antioxidant properties, since it interacts with H₂O₂ by a non-enzymatic process to produce H₂O and acetate and thus, it contributes to the resistance of oxidative stress of the cell [9]. Besides its H₂O₂-scavenging task, pyruvate can be a suitable candidate to prevent the decrease of the pH of the growth media since it is also proposed as an effective buffer for biological systems [10].

Papagianni and Avramidis [11] expressed the alternative oxidase gene (*aox1*) of *Aspergillus niger* in *L. lactis* and obtained a strain with an improved response to oxidizing stress. Lactate formation was found to be suppressed in this recombinant strain which reached a higher biomass and nisin yield in the conditions tested.

The growth of *L. lactis* is also inhibited by high lactic acid concentrations produced during fermentation. Overexpressing transcriptional regulator gene *ythA* was found to enhance the acid resistance of *L. lactis* F44, and it was shown that the survival rate and nisin yield of the recombinant strain were significantly improved [12]. Miao et al. [13] showed that *anti41* overexpression improved the survival rate of the cells under acid stress and increased the cell growth which in turn led to an enhanced production of nisin. In another study, a small non-coding RNA, *s015*, was shown to improve the acid tolerance of *L. lactis* and increase the nisin yield at low pH [14]. Zhang et al. [15] found that expressing different acid stress genes individually or in combination led to the increment of nisin production. Recently, the lower biomass of cells due to acid stress was found to be caused by the decrease in the expression of genes involved in the cell wall and cell membrane biosynthesis [16].

L. lactis membrane is equipped with all of the electron transfer chain (ETC) components for respiration. Yet, it is unable to respire because some of the genes are missing to

produce heme, the cofactor of cytochrome oxidase. However, the addition of heme into the growth medium is enough for respiration to begin, and there are studies that show the protective effect of heme against oxidative stress. In the presence of heme, long-term survival and higher biomass of *L. lactis* were achieved both by reducing the oxidative stress through elimination of ROS more effectively and by decreasing the acid stress through providing higher pH of the growth medium due to mixed acid fermentation [17–19]. It was also shown that in respiration-permissive conditions, the increase in *L. lactis* biomass caused an enhancement in nisin production [5]. From these studies, it could be concluded that reducing oxidative and acid stresses of *L. lactis* can enhance nisin production by increasing biomass.

The desire of the consumers for *natural* food preservatives in recent years makes researchers find different ways to enhance nisin production by changing the content of the growth medium instead of genetic manipulations. For this reason, in this study, the effects of GSH and pyruvate on growth, nisin production, and stress response of *L. lactis* cells were determined.

Materials and methods

Bacterial strains and growth conditions

Lactococcus lactis N8 and *Micrococcus luteus* NCIMB 8166 (National Collection of Industrial, Food and Marine Bacteria, UK) were used as the nisin producer and the indicator strain, respectively. Both strains were kindly obtained by Prof. Per Saris, Helsinki University, Finland. *L. lactis* was grown in GM17 medium (M17 containing 0.5% glucose) (Biolife, Milan, Italy) at 30 °C with 180 rpm agitation to provide aerobic conditions as in the study of Pedersen et al. [20] with some modifications. The culture volume was 100 ml in a 500-ml baffled Erlenmeyer flask. *M. luteus* cells were grown on Beef Luria Bertani (BLB) agar (10 g peptone (BD Biosciences, NJ, USA), 1 g yeast extract (BD Biosciences, NJ, USA), 5 g beef extract (meat extract, Merck, Darmstadt, Germany), 5 g NaCl (Merck, Darmstadt, Germany), and 15 g agar (Merck, Darmstadt, Germany) per liter, pH 7.2) for 20 h at 37 °C [21].

Preparation of growth curve

A single colony of *L. lactis* on GM17 agar was inoculated into 10 ml of GM17 broth to prepare the overnight culture. One hundred milliliters of GM17 broth supplemented with GSH or pyruvate was prepared in different baffled flasks. GM17 without any supplement was used as the control sample in the experiment. Around 10⁹ cells from the overnight culture (1 ml, OD₆₀₀ = 2.5) were inoculated into GM17 broth

in each flask and incubated under aerobic conditions with 180 rpm agitation at 30 °C. The optical density of the cultures at 600 nm (UV-1900 UV-Vis Spectrophotometer, Shimadzu, Kyoto, Japan) was monitored overtime to form the growth curve of *L. lactis* cells. The pH change during the growth was measured by using a pH meter (Hanna, RI, USA). The results were given as the average of three biological replicates.

Determination of nisin production

Nisin concentration in the supernatants of *L. lactis* cultures was measured by a bioassay method [21]. An inoculating loop of *M. luteus* grown on BLB agar for 20 h at 37 °C was suspended in 6 ml of sterile isotonic NaCl solution (0.9%). A total of 20 ml of S1 agar (8 g tryptone (Merck, Darmstadt, Germany), 5 g yeast extract (BD Biosciences, NJ, USA), 5 g glucose (Merck, Darmstadt, Germany), 5 g NaCl (Merck, Darmstadt, Germany), 2 g Na₂HPO₄ (Sigma-Aldrich, MO, USA), 1.5% Tween 20 (Merck, Darmstadt, Germany), and 16 g agar (Merck, Darmstadt, Germany) per liter) at 50 °C were mixed with *M. luteus* suspension and poured into a Petri dish. The supernatants of *L. lactis* cells were incubated at 70 °C for 10 min to inactivate the remaining viable cells. After heat inactivation, 80 µl of each supernatant was added into the wells on S1 agar and plates were incubated for 20–24 h at 30 °C. Zones of inhibition were measured, and nisin activity was determined according to a standard curve ($R^2 = 0.99$) plotted by using standard nisin solutions which were prepared at different concentrations, ranging from 0 to 80 IU ml⁻¹ nisin. Nisin stock solution was prepared according to Cheigh et al. [22] as 10,000 IU ml⁻¹ by using a commercial nisin (Sigma-Aldrich, MO, USA).

Detection of lipid peroxidation

Malondialdehyde (MDA) and thiobarbituric acid (TBA) form a pink-colored MDA-TBA complex whose absorbance at 532 nm was used for the detection of MDA level in the cells. For the detection of MDA quantity, 0.25 ml of the samples was mixed with 0.5 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 11,000 rpm for 15 min at 4 °C. After centrifugation, 0.25 ml of 0.1 M Tris/HCl (pH 7.6) and 0.5 ml of the indicator solution (15% TCA, 0.375% TBA, and 0.25 M HCl) were added to 0.25 ml of the supernatant and incubated at 95 °C for 45 min. Samples were transferred into the ice for cooling and then centrifuged at 11,000 rpm for 5 min. The absorbance of the supernatants was measured at 532 and 600 nm. The lipid peroxidation of the samples was expressed as nmol ml⁻¹ MDA per milligram (dry weight) of the cell [23].

Measurement of H₂O₂

To detect H₂O₂ concentration, the ferrous oxidation-xylenol orange (FOX) method was used. This method is based on the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-$) in which H₂O₂ reacts with the ferrous ion (Fe²⁺). The reaction between the ferric ion (Fe³⁺) and xylenol orange results in the formation of a complex which has the maximum absorbance at 560 nm [24]. A 950-µl supernatant of the samples was mixed with 50 µl of 20× FOX reagent containing ammonium ferrous sulfate (5 mM), H₂SO₄ (500 mM), sorbitol (2 M), and xylenol orange (2 mM) and incubated in the dark for 30 min. After incubation, the absorbance value of the samples at 560 nm was measured. The standard curve ($R^2 = 0.99$) was formed using H₂O₂ (Merck, Darmstadt, Germany) ranging from 0 to 4 µM. The H₂O₂ content of the samples was calculated according to the standard curve.

RNA extraction and cDNA synthesis

For RNA isolation, an approximately 10⁸ number of *L. lactis* cells were collected by centrifugation at 13,000 rpm for 5 min at 4 °C and then, they were washed with Tris-EDTA (TE) buffer (pH 8.0) twice. The cells were incubated in lysozyme solution at the concentration of 10 mg ml⁻¹ for 30 min at 37 °C. After the incubation time, total RNA was extracted with the innuSPEED Bacteria/Fungi RNA Kit (Analytic Jena, Jena, Germany) through the homogenization process according to the manufacturer's instructions. After isolation, the integrity of the RNA samples was controlled by agarose gel electrophoresis. The concentration of the samples (ng/µl) was detected by the measurement of the absorbance at 260 nm (A_{260}) using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, MA, USA). When the ratio of A_{260} to A_{280} ranges from 1.8 to 2.0, the samples were accepted as pure enough for complementary DNA (cDNA) synthesis. Genomic DNA contamination from the samples was eliminated by RNase-free DNase I (Thermo Fisher Scientific, MA, USA), and absence of DNA in each RNA sample was verified by PCR. After DNase treatment, RNA samples were used to synthesize cDNA by using the iScript cDNA Synthesis Kit according to the recommendations of the manufacturer (Bio-Rad, CA, USA).

Quantification of relative gene expression by using quantitative real-time PCR

Primers used for quantitative real-time PCR (RT-qPCR) were designed by using the PRIMER3 software [25, 26], and they were listed in Table 1. Primers were screened for possible self-dimer, heterodimer formation, and secondary structures by using the IDT OligoAnalyzer Tool (Integrated DNA Technologies Inc., IA, USA).

Table 1 The primers used for RT-qPCR analysis

Gene	Primer		Amplicon size (bp)
	Direction	Sequence (5' to 3')	
<i>nisZ</i>	Forward	AGATTCAGGTGCATCACCAC	75
	Reverse	CCCATCAGAGCTCCTGTTTT	
<i>sodA</i>	Forward	CCAAACACTGACGGTTCTGA	128
	Reverse	CCTGAACCAAAAACGACCTGT	
<i>recA</i>	Forward	TGACTCAGTTGCAGCTTGG	80
	Reverse	TACGAGCTTGAAGCCCAACT	
<i>ahpF</i>	Forward	GGCGGTCAACCACTAGAAAC	85
	Reverse	CAAGCTGACGACCAAGTTGA	
<i>ahpC</i>	Forward	TATTGACCCAGACGGTGTGA	128
	Reverse	GACAAACTTCCCCTGGATGA	
<i>noxE</i>	Forward	ATCGATATCCAGGCCATGAG	87
	Reverse	TCCGACCCAAATAGCTGTC	
<i>noxB</i>	Forward	GTGGACATCGTCTTCAAGCA	190
	Reverse	CCACCATTGATGTCTGCAAG	
<i>trxBI</i>	Forward	GGTGTTCCTGGTGGTCAAAT	164
	Reverse	TCCCATGGTCTTCAATAGC	
<i>relA</i>	Forward	TTATGCCCTCTTGCTCATC	189
	Reverse	CCAGCTTTTTCGACTCGTTC	
<i>cydA</i>	Forward	TCCCACCAGTAACGGTTCTC	187
	Reverse	CCTGAGGTAATCGCAAGGAA	
<i>gshR</i>	Forward	AAATGGGGAGGAACTTGTC	100
	Reverse	CCTGCCCTTTAACTGTTCC	
<i>tuf</i>	Forward	CGTCACCTGGGAAGTCGTAT	180
	Reverse	CTGCAACTGATGGACCAATG	

RT-qPCR was performed in ABI 7500 StepOnePlus™ (Applied Biosystems, CA, USA) by using iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) according to the manufacturer's protocol. Thermal cycling program used was as follows: 95 °C for 30 s, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. To control the specificity and the homogeneity of the products, the melt curve analysis was conducted. The relative gene expression was calculated by $2^{-\Delta\Delta C_t}$ [27] using *tuf* gene, which encodes the elongation factor Tu, as the house-keeping gene. All experiments were performed at least three times, in replicate.

Statistical analysis

Two-way ANOVA analyses were performed to examine the differences between *L. lactis* cells grown in GM17 broth supplemented with GSH or pyruvate and the cells grown in GM17 broth without any supplement (control sample). The results of RT-qPCR were statistically analyzed by the Mann-Whitney test using the GraphPad Prism statistical program (GraphPad Software Inc., CA, USA) to signify the significant differences ($P < 0.05$).

Results and discussion

Growth and nisin production of *L. lactis* cells in the presence of glutathione and pyruvate

L. lactis cells were grown in GM17 medium supplemented with different concentrations of GSH (1.6, 3.2, 6.4 mM) and pyruvate (2.5, 5, and 10 mM) in the presence or absence of heme (2.5 $\mu\text{g ml}^{-1}$) under aerobic conditions at 30 °C. In the presence of heme, GSH was found to have a negative effect on growth and nisin production (Figs. 1a and 2a). On the other hand, both growth and nisin production increased with increasing GSH concentration in the absence of heme (Figs. 1b and 2b). A total of 6.4 mM GSH caused more nisin yield than the heme-supplemented medium.

The effect of different pyruvate concentrations on the growth was negligible in the presence of heme (Fig. 1c) whereas in the absence of heme, the growth was positively affected in parallel with increasing pyruvate concentration (Fig. 1d). With the addition of 10 mM pyruvate, higher production of nisin was achieved in heme-free medium (Fig. 2d) compared with that of heme-supplemented medium (Fig. 2c).

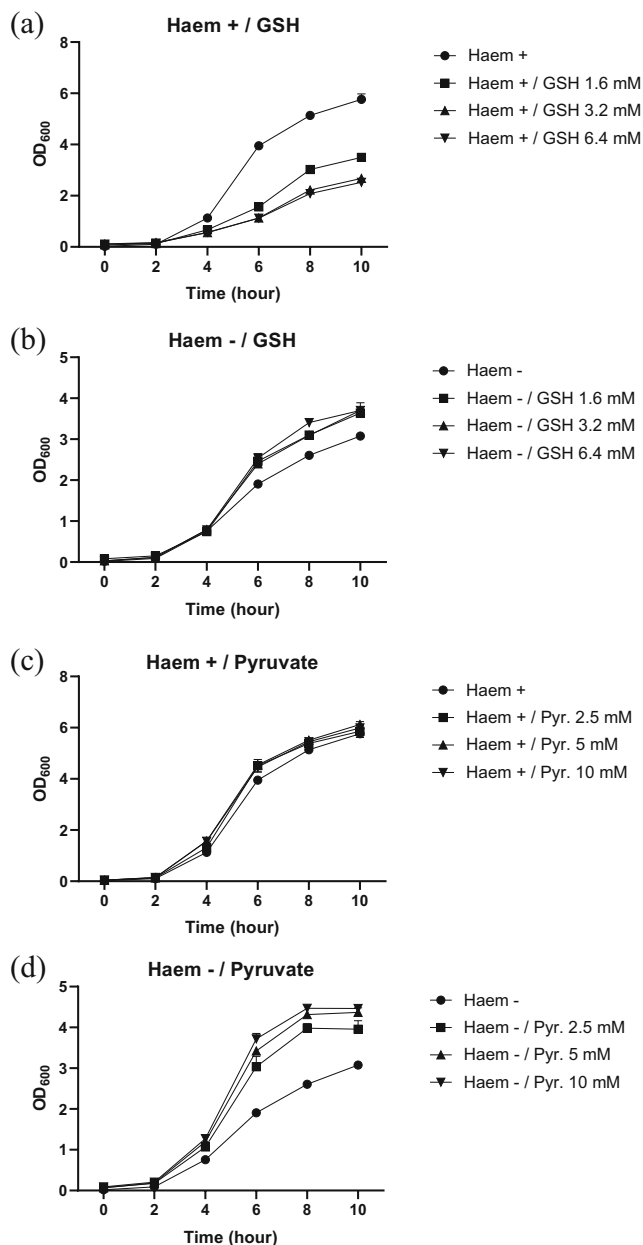


Fig. 1 The growth curves of *L. lactis* cells grown in GSH-supplemented GM17 medium in the presence (a) or absence (b) of heme and in pyruvate-supplemented GM17 medium in the presence (c) or absence (d) of heme under aerobic conditions at 30 °C. The concentration of heme was 2.5 μg ml⁻¹. The experiments were done in triplicate, and error bars indicate the standard deviations

In this study, the highest nisin production level was achieved with the highest concentration of GSH (6.4 mM) and pyruvate (10 mM). The finding of increased nisin production directly proportional to the increased concentration of GSH and pyruvate in the absence of heme is important, so it is of interest to see how nisin production will be affected at higher antioxidant concentrations. For the rest of the study, 6.4 mM GSH and 10 mM pyruvate concentrations were selected as the concentrations that caused the highest biomass

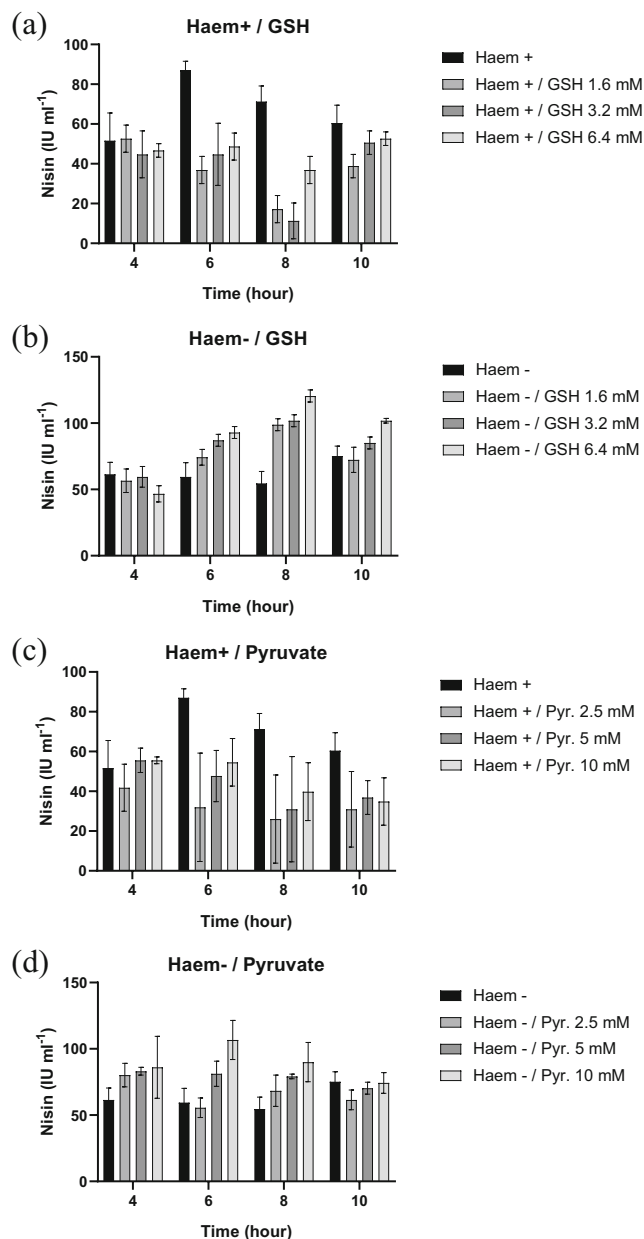
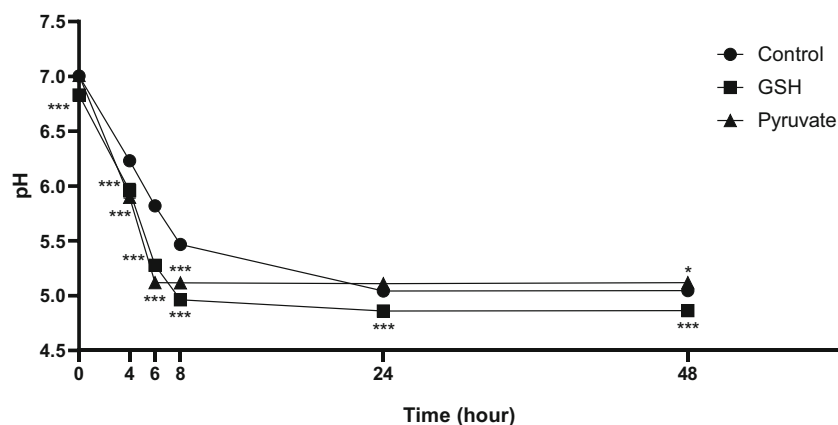


Fig. 2 The nisin production of *L. lactis* cells grown in GSH-supplemented GM17 medium in the presence (a) or absence (b) of heme and in pyruvate-supplemented GM17 medium in the presence (c) or absence (d) of heme under aerobic conditions at 30 °C. The concentration of heme was 2.5 μg ml⁻¹. The experiments were done in triplicate, and error bars indicate the standard deviations

and nisin production, and the experiments were conducted in the absence of heme.

pH change was measured during the growth of *L. lactis* cells in the presence of pyruvate and GSH. In the first 8 h of fermentation, the growth of *L. lactis* decreased the pH sharply and then, the pH of the medium supplemented with pyruvate showed a steady state. However, the pH of control medium and the GSH-supplemented one continued to decrease gradually until 24 h of growth (Fig. 3). The decrease in pH was

Fig. 3 pH changes during the growth of *L. lactis* cells under aerobic conditions at 30 °C. “GSH” indicates GM17 medium containing 6.4 mM GSH while “pyruvate” implies GM17 medium containing 10 mM pyruvate. The experiments were done in triplicate, and error bars show standard deviations. Statistical analyses were performed by two-way ANOVA; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$



expected because of the production of lactic acid. Without the addition of heme into the growth medium, *L. lactis* cells cannot shift their metabolism towards mixed acid fermentation; as a result, lactic acid production cannot be minimized even in the presence of oxygen. In this study, the decrease in pH was significantly more in the media supplemented with GSH and pyruvate compared with that of control. The growth of *L. lactis* was better in the pyruvate-supplemented media (Fig. 1d). When the number of cells increases, there will be more lactic acid accumulation which can explain the decrease in the pH. Although the growth profiles of *L. lactis* cells in control and GSH-supplemented media were similar, the decrease in pH was significantly more in the GSH-containing medium than the control. This difference can be explained by the presence of two carboxylic groups in the molecular structure of GSH that causes acidification of GM17 medium [28].

Evaluation of the oxidative stress level of *L. lactis* cells

Accumulation of H_2O_2 was measured as an indicator to determine the oxidative stress level of the cells grown under aerobic conditions. H_2O_2 concentration in the media decreased gradually until 8 h of growth, then showed a steady state (Fig. 4a). High H_2O_2 levels in the very early stages of the aerobic growth (up to 2 h) were also shown in *L. lactis* C10 by Grufferty and Condon [29]. The presence of GSH and pyruvate was very effective at the removal of H_2O_2 compared with that of the control medium without any supplement (Fig. 4a). The decrease in H_2O_2 concentration was statistically important until 8 h of growth in GSH-containing medium and until 24 h of growth in pyruvate-containing medium compared with that in the control sample. The long-lasting effect of pyruvate was striking.

Another method to determine the stress level of the cells is the detection of lipid peroxidation. Malondialdehyde (MDA) is produced during polyunsaturated fatty acid peroxidation. The attack of ROS to the cellular membrane causes fatty acid peroxidation which in turn brings about the increment in the MDA concentration. Measurement of MDA level of *L. lactis*

cells showed that the amount of MDA in the medium containing GSH and pyruvate was significantly lower than that of the control sample until 8 h of growth (Fig. 4b). These results are compatible with the results of H_2O_2 measurements in that GSH and pyruvate help *L. lactis* cells to cope with the oxidative stress. The accumulation of GSH in *L. lactis* is shown to be involved in the protection against various stresses such as oxidative stress [7], acid stress [30], and osmotic stress [31].

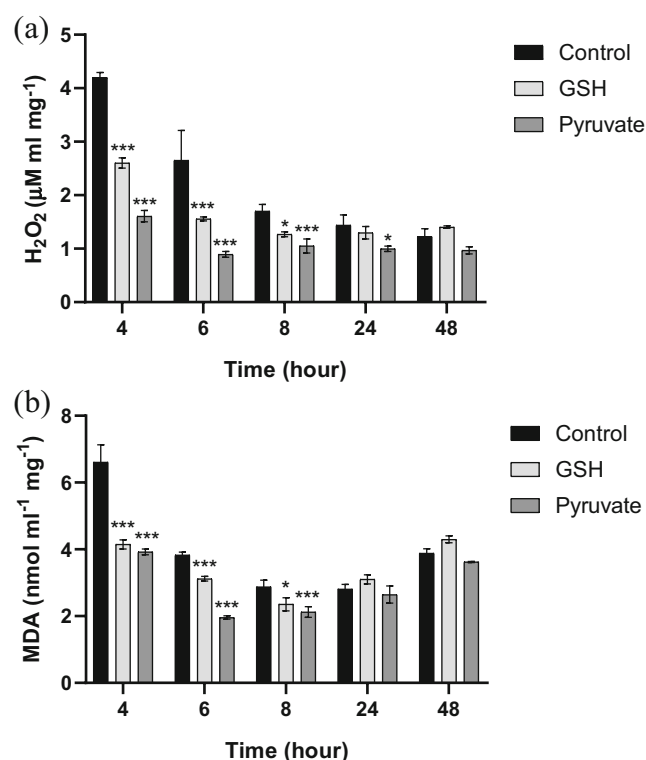


Fig. 4 Measurement of H_2O_2 (a) and MDA (b) of *L. lactis* cells grown in GM17 medium under aerobic conditions at 30 °C. “GSH” indicates GM17 medium containing 6.4 mM GSH while “pyruvate” implies GM17 medium containing 10 mM pyruvate. The experiments were done in triplicate, and error bars show standard deviations. Statistical analyses were performed by two-way ANOVA; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

The antioxidant activity of pyruvate was also evidenced by different studies [9, 32].

Effect of GSH and pyruvate on nisin production

Nisin production of *L. lactis* cells, grown in the presence of 6.4 mM GSH and 10 mM pyruvate, was measured at the transcriptional and protein levels. The expression of *nisZ*, which is one of the biosynthetic genes in nisin cluster, increased significantly in *L. lactis* cells grown in pyruvate-containing medium (Fig. 5a). Especially expression at the 6th hour of growth was remarkable; it was 7.8 times more than the control sample. *nisZ* expression in *L. lactis* cells that grew in GSH-supplemented medium was also higher at the 4th and 8th hours of growth.

Nisin concentration in the supernatants of *L. lactis* cultures was measured by using *M. luteus* cells as the indicator organism (Fig. 5b). Presence of GSH and pyruvate in the medium clearly increased the nisin production by *L. lactis* cells. The increment in nisin production in the cells grown in GM17 broth supplemented with GSH and pyruvate at the 6th and 8th hours of growth was statistically remarkable compared with that in the control sample. Although these two molecules

are known to reduce the oxidative stress of cells, they have been shown for the first time to trigger the production of nisin in *L. lactis* when they have been added as a supplement into the growth medium.

Transcriptional analysis of stress genes of *L. lactis* cells grown in the presence of GSH and pyruvate

The genes that encode stress-related enzymes were transcriptionally analyzed to describe the effect of GSH and pyruvate on the defense system of *L. lactis* cells. The genes selected for this part of the study are those associated with the stress response of *L. lactis* cells found in the literature (Table 2), namely *sodA* (superoxide dismutase), *ahpF* (H₂O₂-forming NADH oxidase), *ahpC* (NADH peroxidase), *gshR* (glutathione reductase), *trxB1* (thioredoxin reductase), *noxE* (H₂O-forming NADH oxidase), *recA* (recombinase), and *relA* (pppGpp synthetase).

The expression of *sodA* (superoxide dismutase) gene was found to be increased at the 4th and the 6th hours of growth in GSH-supplemented medium. In pyruvate-containing medium, *sodA* expression increased dramatically at the 6th hour of growth (7.5 times more than that of the control sample). At the 8th hour of growth, the expression of *sodA* decreased in the cells grown in both media compared with that of the control sample (Fig. 6a).

The increase in *ahpF* (H₂O₂-forming NADH oxidase) gene expression was statistically important in *L. lactis* cells at the 4th hour of growth in GSH-containing medium and at the 6th hour of growth in pyruvate-containing medium, whereas *ahpF* expression significantly increased in the cells grown in both media at the 8th hour of growth. Compared with that for the control sample, the difference at expression levels of *ahpF* genes at the 8th hour of growth was 6.2 and 10.1 times for GSH-containing and pyruvate-containing media, respectively (Fig. 6b).

The increase in the expression level of *ahpC* (NADH peroxidase) gene in *L. lactis* cells after the 4th hour of growth in GM17 medium containing GSH and the decrease in its expression after the 8th hour of growth in the medium containing pyruvate were statistically important (Fig. 6c).

Glutathione reductase enzyme encoded by *gshR* gene catalyzes the reaction in which glutathione disulfide is reduced to GSH. The opposite reaction, which is catalyzed by glutathione peroxidase enzyme, leads to the elimination of H₂O₂. In the presence of GSH, the expression of *gshR* gene increased 2.6 times than that of the control, whereas pyruvate caused 0.6 times decrease in the expression of the same gene at the 4th hour growth. Transcriptional level of *gshR* gene was lower in the cells grown in both media than in the control sample at the 8th hour of growth (Fig. 6d). Effective H₂O₂ removal by *L. lactis* cell in the presence of GSH (Fig. 4a) was probably

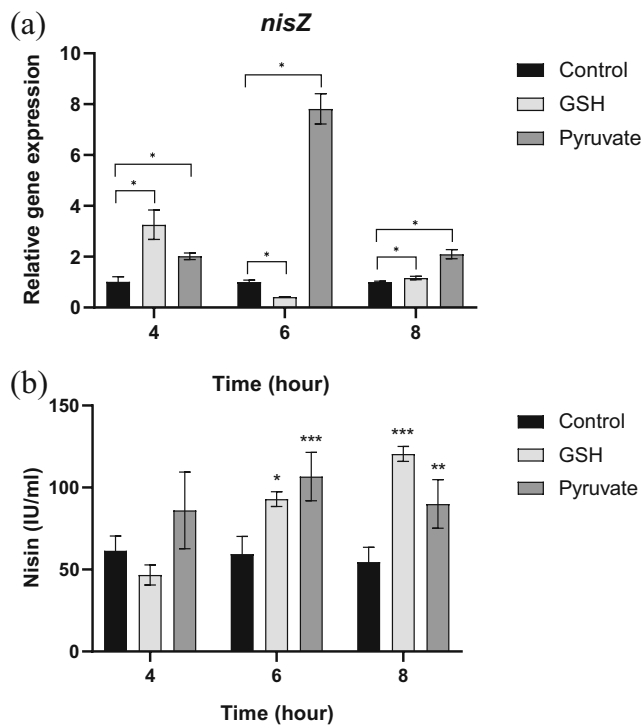


Fig. 5 Relative expression of *nisZ* gene (a) and nisin production (b) of *L. lactis* cells grown in GM17 medium at the 4th, 6th, and 8th hours of growth under aerobic conditions at 30 °C. “GSH” indicates GM17 medium containing 6.4 mM GSH while “pyruvate” implies GM17 medium containing 10 mM pyruvate. Statistical analyses were performed by the Mann-Whitney test (* $P < 0.05$) (a) and two-way ANOVA (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$) (b)

Table 2 The genes examined in the RT-qPCR analysis

Function	Gene	Product	References
Nisin production	<i>nisZ</i>	Nisin Z precursor peptide	[38]
Related to stress response	<i>sodA</i>	Superoxide dismutase	[9]
	<i>ahpF</i>	H ₂ O ₂ -forming NADH oxidase	[34, 35]
	<i>noxE</i>	H ₂ O-forming NADH oxidase	[9, 34, 35]
	<i>ahpC</i>	NADH peroxidase	[34]
	<i>recA</i>	Recombinase	[36]
	<i>relA</i>	(p)ppGpp synthetase	[37]
	<i>gshR</i>	Glutathione reductase	[7]
	<i>trxBI</i>	Thioredoxin reductase	[33]
Components of the ETS	<i>cydA</i>	Cytochrome d oxidase	[17]
	<i>noxB</i>	Membrane NADH dehydrogenase	[35]

achieved due to the activity of the GSH/GSH reductase system.

Besides GSH/GSH reductase, another system contributing to the maintaining of redox balance in *L. lactis* is the thioredoxin/thioredoxin reductase system. This system provides also the elimination of H₂O₂ through the way like GSH/GSH reductase [33]. Thioredoxin peroxidase enzyme is encoded by *trxBI* gene, and the expression of this gene in the presence of GSH increased at the 4th hour of growth but decreased at the 6th and 8th hours of growth significantly compared with that of the control sample. In *L. lactis* cells grown in GM17 medium containing pyruvate, upregulation of *trxBI* gene at the 4th and 6th hours of growth and down-regulation of this gene at the 8th hour of growth were statistically important (Fig. 6e).

By using molecular oxygen as the substrate, *noxE* (H₂O-forming NADH oxidase) also plays an effective role in the oxidative defense system in *L. lactis* [9, 34, 35]. The expression of *noxE* increased at the 6th hour of growth in GSH-supplemented medium. However, when the growth reached to 8th hour, *noxE* expression decreased significantly in the cells grown in both media containing GSH or pyruvate (Fig. 6f).

Even though the activity of *sodA* leads to the removal of superoxide radicals, the reaction causes the production of another toxic substance, i.e., H₂O₂. The activity of *ahpF* also produces H₂O₂ inside the cell. Excess accumulation of H₂O₂ is the most challenging problem for *L. lactis* to cope with the oxidative stress conditions. According to our results, GSH and pyruvate induce the expression of *sodA* and *ahpF* genes at different time points of growth. Especially the positive effect of pyruvate on the transcription of both genes was impressive. It seems that the accumulated H₂O₂ is removed more efficiently from the medium by the action of *AhpC*, *GshR*, and *TrxB1* enzymes in *L. lactis*, and this removal was increased in the presence of GSH and pyruvate. Transcription of *ahpC* and

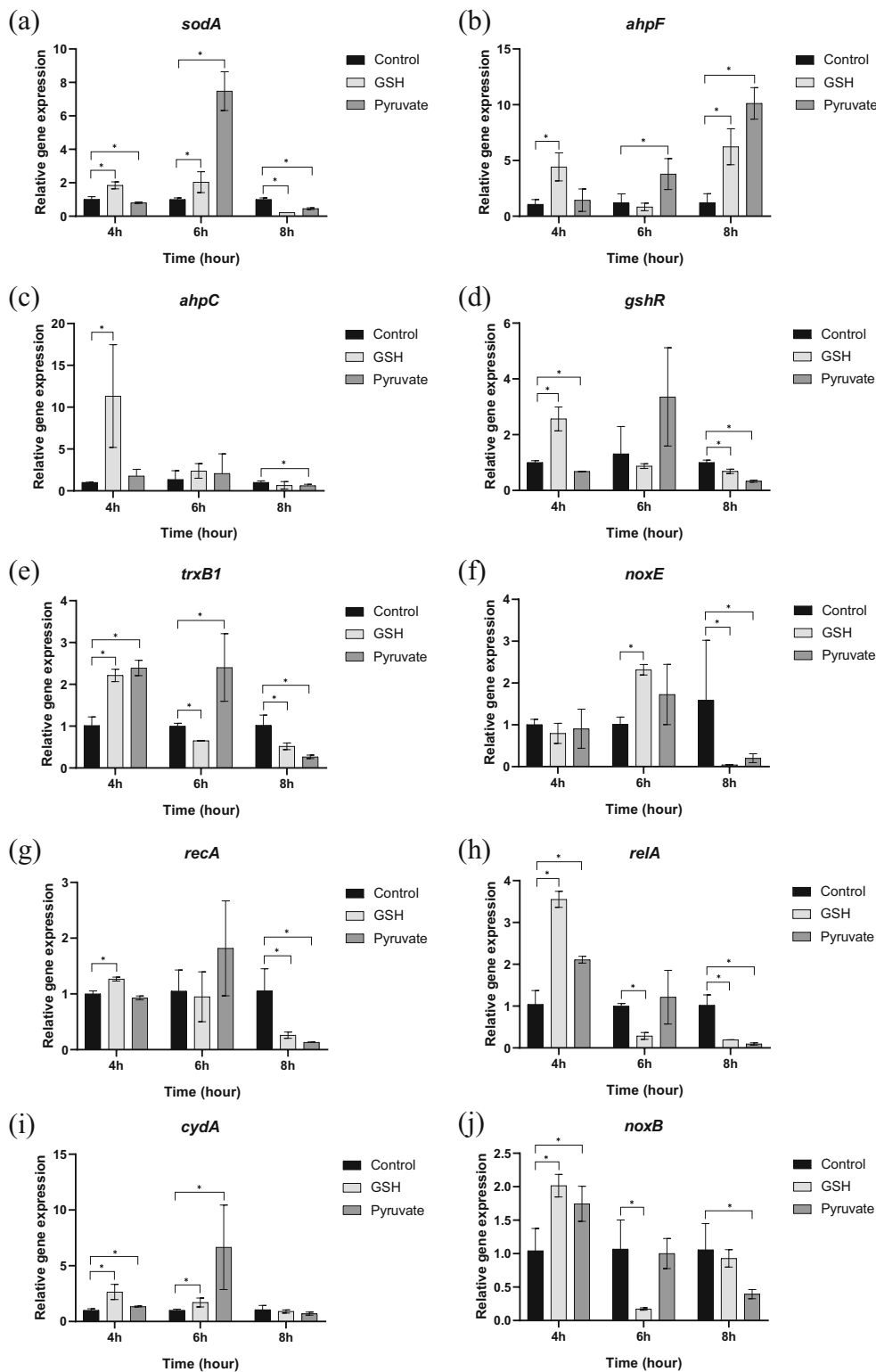
gshR was stimulated by the presence of GSH at the 4th hour of growth. *trxBI* expression, on the other hand, was stimulated by both GSH and pyruvate at the same hour. The positive effect of pyruvate on the *trxBI* expression continued also at the 6th hour growth. *ahpF* expression was still high in the presence of both GSH and pyruvate at the 8th hour of growth, although the expression of H₂O₂-scavenging enzyme-coding genes were lower than that of the control sample at the same hour.

The recombinase *RecA* is the main DNA repair enzyme. The involvement of *RecA* under heat and oxidative stress was shown in *L. lactis* [36]. Presence of GSH and pyruvate in the medium decreased the expression level of *recA* gene remarkably at the 8th hour of growth. This points out the alleviation of oxidative stress possibly due to the presence of antioxidant molecules in the media. Increase in *recA* expression was statistically significant at the 4th hour of growth in the presence of GSH (Fig. 6g).

The stringent response is regulated by the intracellular level of (p)ppGpp molecules which are synthesized by *RelA* protein. It was shown that (p)ppGpp level in the cell is related not only to nutritional stress but also to heat, acid, and oxidative stresses [37]. The increment in the expression of *relA* gene at the 4th hour of growth was statistically important in *L. lactis* cells grown in both GSH- and pyruvate-supplemented medium. The expression level of *relA* gene was 3.6- and 2.1-fold higher in *L. lactis* cells grown in the presence of GSH and pyruvate compared with that of the control, respectively. This increase of *relA* gene in the first hours of growth of *L. lactis* cells could be attributed to taking the precaution of the cells against stress conditions. Presence of GSH and pyruvate caused a significant decrease in *relA* expression at the 8th hour of growth most probably due to their contribution to reducing the stress level of the cells (Fig. 6h).

We also measured the transcription levels of *cydA* and *noxB* genes which are encoding the enzymes functioning in the

Fig. 6 Relative expression of stress-related genes (*sodA* (a), *ahpF* (b), *ahpC* (c), *gshR* (d), *trxB1* (e), *noxE* (f), *recA* (g), and *relA* (h)) and the genes coding the components of ETC (*cydA* (i) and *noxB* (j)) of *L. lactis* cells grown in GM17 medium under aerobic conditions at 30 °C. “GSH” indicates GM17 medium containing 6.4 mM GSH while “pyruvate” implies GM17 medium containing 10 mM pyruvate. RT-qPCR experiments were performed at least three times, in replicate. Statistical analyses were performed by the Mann-Whitney test (* $P < 0.05$)



ETC. GSH and pyruvate stimulated the expression of both genes at the early stages of growth (Fig. 6i, j). Upregulation of these genes may not be expected due to the non-functionality of cytochrome oxidase enzyme (*CydA*) without heme. However, Duwat et al. [17] compared the expression of

cydA gene in *L. lactis* cells grown under aerobic (without heme) and respiration (with heme) conditions. The expression of this gene was found to be similar in both conditions; only at the late exponential phase (approximately at the 6th hour of growth), the expression of *cydA* gene was shown to be

induced in the presence of heme. Therefore, it was concluded that *cydA* gene is expressed in *L. lactis* cells independently of the growth condition in the early exponential phase. In that case, our results that show the upregulation of *cydA* gene at the 4th and 6th hours of growth in the presence of GSH or pyruvate are intriguing (Fig. 6i).

In *L. lactis*, NADH dehydrogenase enzyme encoded by *noxB* gene was shown to be associated with the decrement of the oxidizing compounds originating from oxygen [35]. The significant increase in the expression level of *noxB* gene at the 4th hour of growth in GM17 medium supplemented with GSH or pyruvate (Fig. 6j) could be relevant to the response against oxidative stress in line with the results of the expression of other defense genes examined in this study.

Conclusion

The increase in consumer awareness about the clean ingredients in food leads the manufacturers to use *safe* food preservatives. Due to its organic production and negligible toxic effect, nisin has been used widely for this purpose by numerous countries. New studies are needed to find different ways to increase the nisin production yield of *L. lactis*.

It is known that the shift from fermentation to respiration in the presence of heme stimulates the genes related to the oxidative stress response and leads to higher biomass and nisin yield in *L. lactis*. According to our results, without shifting the metabolism to respiration, GSH and pyruvate can also activate stress-related genes as heme does and their presence in the medium clearly stimulate nisin production in *L. lactis*. Interestingly, the yield of nisin obtained in the presence of GSH and pyruvate was better than that of the heme-supplemented medium. The results of this study showed that supplementation of growth medium of *L. lactis* with GSH and pyruvate instead of heme is effective to trigger nisin production in flask culture. Whether the same positive effect of GSH and pyruvate on nisin production will be seen in a larger culture volume or not is the question to be answered.

Funding information This research was supported by Gebze Technical University, grant numbers 2013-A-09 and 2014-A-10.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

- Punyaappa-path S, Phumkhachorn P, Rattanachaiakunsopon P (2015) Nisin: production and mechanism of antimicrobial action. *Int J Curr Res Rev* 7(2):47
- Dey BC, Rai N, Das S, Mandal S, Mandal V (2019) Partial purification, characterization and mode of action of bacteriocins produced by three strains of *Pediococcus* sp. *J Food Sci Technol* 56(5):2594–2604. <https://doi.org/10.1007/s13197-019-03744-3>
- Hurst A (1981) Nisin. In: *Advances in applied microbiology*, vol 27. Elsevier, pp 85–123
- Additives EPanel oF, Food NSat, Younes M, Aggett P, Aguilar F, Crebelli R, Dusemund B, Filipič M, Frutos MJ, Galtier P, Gundert-Remy U, Kühnle GG, Lambré C, Leblanc J-C, Lillegaard IT, Moldeus P, Mortensen A, Oskarsson A, Stankovic I, Waalkens-Berendsen I, Woutersen RA, Wright M, Herman L, Tobback P, Pizzo F, Smeraldi C, Tard A, Papaioannou A, Gott D (2017) Safety of nisin (E 234) as a food additive in the light of new toxicological data and the proposed extension of use. *EFSA J* 15(12):e05063. <https://doi.org/10.2903/j.efsa.2017.5063>
- Kördinkanlıoğlu B, Şimşek Ö, Saris PEJ (2015) Nisin production of *Lactococcus lactis* N8 with hemin-stimulated cell respiration in fed-batch fermentation system. *Biotechnol Prog* 31(3):678–685. <https://doi.org/10.1002/btpr.2075>
- Rochat T, Miyoshi A, Gratadoux J, Duwat P, Sourice S, Azevedo V, Langella P (2005) High-level resistance to oxidative stress in *Lactococcus lactis* conferred by *Bacillus subtilis* catalase KatE. *Microbiology* 151(9):3011–3018
- Li Y, Hugenholtz J, Abee T, Molenaar D (2003) Glutathione protects *Lactococcus lactis* against oxidative stress. *Appl Environ Microbiol* 69(10):5739–5745
- Fu R-Y, Bongers RS, Van Swam II, Chen J, Molenaar D, Kleerebezem M, Hugenholtz J, Li Y (2006) Introducing glutathione biosynthetic capability into *Lactococcus lactis* subsp. *cremoris* NZ9000 improves the oxidative-stress resistance of the host. *Metab Eng* 8(6):662–671
- van Niel EW, Hofvendahl K, Hahn-Hägerdal B (2002) Formation and conversion of oxygen metabolites by *Lactococcus lactis* subsp. *lactis* ATCC 19435 under different growth conditions. *Appl Environ Microbiol* 68(9):4350–4356
- Zhou FQ (2005) Pyruvate in the correction of intracellular acidosis: a metabolic basis as a novel superior buffer. *Am J Nephrol* 25(1):55–63
- Papagianni M, Avramidis N (2012) Cloning and functional expression of the mitochondrial alternative oxidase gene (*aox1*) of *Aspergillus niger* in *Lactococcus lactis* and its induction by oxidizing conditions. *Enzym Microb Technol* 50(1):17–21
- Wu H, Liu J, Miao S, Zhao Y, Zhu H, Qiao M, Saris PEJ, Qiao J (2018) Contribution of YthA, a PspC family transcriptional regulator of *Lactococcus lactis* F44 acid tolerance and nisin yield: a transcriptomic approach. *Appl Environ Microbiol* 84(6):e02483–e02417
- Miao S, Wu H, Zhao Y, Caiyin Q, Li Y, Qiao J (2018) Enhancing nisin yield by engineering a small noncoding RNA *anti41* and inhibiting the expression of *glnR* in *Lactococcus lactis* F44. *Biotechnol Lett* 40(6):941–948
- Qi J, Caiyin Q, Wu H, Tian K, Wang B, Li Y, Qiao J (2017) The novel sRNA *s015* improves nisin yield by increasing acid tolerance of *Lactococcus lactis* F44. *Appl Microbiol Biotechnol* 101(16):6483–6493
- Zhang J, Caiyin Q, Feng W, Zhao X, Qiao B, Zhao G, Qiao J (2016) Enhance nisin yield via improving acid-tolerant capability of *Lactococcus lactis* F44. *Sci Rep* 6:27973
- Tian K, Li Y, Wang B, Wu H, Caiyin Q, Zhang Z, Qiao J (2019) The genome and transcriptome of *Lactococcus lactis* ssp. *lactis* F44 and G423: insights into adaptation to the acidic environment. *J Dairy Sci* 102(2):1044–1058
- Duwat P, Sourice S, Cesselin B, Lamberet G, Vido K, Gaudu P, Le Loir Y, Violet F, Loubière P, Gruss A (2001) Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. *J Bacteriol* 183(15):4509–4516

18. Gaudu P, Vido K, Cesselin B, Kulakauskas S, Tremblay J, Rezaïki L, Lamberet G, Sourice S, Duwat P, Gruss A (2002) Respiration capacity and consequences in *Lactococcus lactis*. In: Lactic Acid Bacteria: Genetics. Springer, Metabolism and Applications, pp 263–269
19. Rezaïki L, Cesselin B, Yamamoto Y, Vido K, Van West E, Gaudu P, Gruss A (2004) Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. Mol Microbiol 53(5):1331–1342
20. Pedersen MB, Garrigues C, Tuphile K, Brun C, Vido K, Bennedsen M, Møllgaard H, Gaudu P, Gruss A (2008) Impact of aeration and heme-activated respiration on *Lactococcus lactis* gene expression: identification of a heme-responsive operon. J Bacteriol 190(14):4903–4911
21. Wu Z, Xuanyuan Z, Li R, Jiang D, Li C, Xu H, Bai Y, Zhang X, Turakainen H, Saris P (2009) Mu transposition complex mutagenesis in *Lactococcus lactis*—identification of genes affecting nisin production. J Appl Microbiol 106(1):41–48
22. Cheigh C-I, Choi H-J, Park H, Kim S-B, Kook M-C, Kim T-S, Hwang J-K, Pyun Y-R (2002) Influence of growth conditions on the production of a nisin-like bacteriocin by *Lactococcus lactis* subsp. *lactis* A164 isolated from kimchi. J Biotechnol 95(3):225–235
23. Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125(1):189–198
24. Rhee SG, Chang T-S, Jeong W, Kang D (2010) Methods for detection and measurement of hydrogen peroxide inside and outside of cells. Mol Cell 29(6):539–549
25. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40(15):e115–e115
26. Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23(10):1289–1291
27. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408
28. Huang X, Zhang T, Goswami A, Luo F, Asefa T (2015) Glutathione-triggered release of model drug molecules from mesoporous silica nanoparticles via a non-redox process. RSC Adv 5(36):28836–28839
29. Grufferty RC, Condon S (1983) Effect of fermentation sugar on hydrogen peroxide accumulation by *Streptococcus lactis* C10. J Dairy Res 50(4):481–489
30. Zhang J, Fu R-Y, Hugenholtz J, Li Y, Chen J (2007) Glutathione protects *Lactococcus lactis* against acid stress. Appl Environ Microbiol 73(16):5268–5275
31. Zhang Y, Zhang Y, Zhu Y, Mao S, Li Y (2010) Proteomic analyses to reveal the protective role of glutathione in resistance of *Lactococcus lactis* to osmotic stress. Appl Environ Microbiol 76(10):3177–3186
32. Olek RA, Antosiewicz J, Popinigis J, Gabbianelli R, Fedeli D, Falcioni G (2005) Pyruvate but not lactate prevents NADH-induced myoglobin oxidation. Free Radic Biol Med 38(11):1484–1490
33. Vido K, Diemer H, Van Dorselaer A, Leize E, Juillard V, Gruss A, Gaudu P (2005) Roles of thioredoxin reductase during the aerobic life of *Lactococcus lactis*. J Bacteriol 187(2):601–610
34. Jiang R, Riebel BR, Bommaris AS (2005) Comparison of alkyl hydroperoxide reductase (AhpR) and water-forming NADH oxidase from *Lactococcus lactis* ATCC 19435. Adv Synth Catal 347(7–8):1139–1146
35. Tachon S, Brandsma JB, Yvon M (2010) NoxE NADH oxidase and the electron transport chain are responsible for the ability of *Lactococcus lactis* to decrease the redox potential of milk. Appl Environ Microbiol 76(5):1311–1319
36. Duwat P, Ehrlich SD, Gruss A (1995) The *recA* gene of *Lactococcus lactis*: characterization and involvement in oxidative and thermal stress. Mol Microbiol 17(6):1121–1131
37. Rallu F, Gruss A, Ehrlich SD, Maguin E (2000) Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. Mol Microbiol 35(3):517–528
38. Mulders JW, Boerrigter IJ, ROLLEMA HS, SIEZEN RJ, de VOS WM (1991) Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. Eur J Biochem 201(3):581–584

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.