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Synergistic Enhancement of Nisin Production through Metabolic and Regulatory Engineering of *Lactococcus lactis* ATCC 11454: Thermal and pH Stability and Application in Food Model System

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Abstract

Nisin is one of the most studied bacteriocins due to its broad antimicrobial spectrum and Generally Recognized as Safe status. In this study, *nisRK* was overexpressed in lactic acid-deficient *Lactococcus lactis* ATCC 11454, generating a recombinant *Lactococcus lactis* ATCC 11454 Δ*ldh* carrying pMG36e-P8-*nisRK* (*ldh*Δ*nisRK*^{OE}), to enhance nisin biosynthesis. Growth kinetics (OD600 monitoring) and antimicrobial activity agar (well diffusion assay) were compared among wildtype, *ldh*Δ, *nisRK*^{OE}, and double mutant strains. The *ldh*Δ*nisRK*^{OE} strain exhibited significantly higher antimicrobial activity than all other strains, with inhibition zones increased by up to 107% against Gram-positive and 78% against Gram-negative bacteria, compared with the wildtype. Thermal and pH stability tests showed that antimicrobial activity was well preserved at low temperatures (0 -4 °C) and across a wide pH range (2 - 10), with optimal performance near pH 6. Activity declined by 26% - 34% following heat treatment at 100 - 121 °C. Application in raw chicken demonstrated that CFS from the engineered strain slowed microbial growth and extended shelf life. After seven days at 4 °C, treated samples maintained lower bacterial counts (8.41 ± 0.02 log CFU/g) compared with controls (9.10 ± 0.07 log CFU/g). At 30 °C, untreated samples spoiled within 72 h, while treated meats remained stable until day 7. These findings highlight that combining metabolic redirection with regulatory overexpression can substantially improves nisin yield and antimicrobial activity. *L. lactis* ATCC 11454-*ldh*Δ*nisRK*^{OE} strain holds strong potential as a microbial cell factory for bacteriocin-based applications in food preservation and biotechnology.

Keywords: Antimicrobial activity, Engineered lactic acid bacteria, Nisin, Preservation, Stability

Introduction

Nisin, a ribosomally synthesized and post-translationally modified peptide (RiPP), is one of the most studied bacteriocins due to its broad antimicrobial spectrum and its Generally Recognized as Safe (GRAS) status [1,2]. Produced by *Lactococcus lactis*, nisin has been used in the food industry for a long time as a natural preservative. It helps inhibit spoilage microorganisms and foodborne pathogens [3,4]. In addition to food preservation, nisin and other lantibiotics are gaining interest in biomedical and biotechnological applications, including combating antibiotic-resistant pathogens and serving as model systems to study complex biosynthetic pathways [5].

The NisRK two-component system precisely controls how nisin is produced. NisK is a histidine kinase that is linked to the membrane and detects nisin outside the cell. NisR is the response regulator that turns on the transcription of the genes that make nisin [6,7]. Studies have demonstrated that overexpressing *nisRK* can increase the production of nisin by increasing the transcription of the structural gene *nisA* and other biosynthetic parts [8,9]. Another study reported that the simultaneous overexpression of *nisA, nisRK,* and *nisFEG* in *L. lactis* LS01 resulted in the highest enhancement of bioactive nisin production relative to the parent strain [10]. However, despite its essential regulatory role, the potential of *nisRK* overexpression as a strategy for yield enhancement has not been thoroughly examined in metabolically modified *L. lactis* strains.

Several genetic strategies have been explored to increase nisin yield, including redirecting carbon fluxes, overexpressing biosynthetic genes, and optimising fermentation processes [11-14]. For instance, introducing multicopy plasmids with *nisRK* increased the expression of the nisin pre-peptide, thereby boosting production [8,9]. Nevertheless, while these strategies improve yield individually, the synergistic effects of regulatory engineering (*nisRK* overexpression) and metabolic rewiring are inadequately elucidated, leaving an important gap in understanding how these approaches may collaboratively optimise bacteriocin biosynthesis.

Our earlier study addressed this question by applying CRISPR/Cas9-mediated deletion of the *ldh* gene in *L. lactis* ATCC 11454 and other LAB strains. Knockout *ldh* redirected carbon flux away from lactic acid formation towards the production of bacteriocin-like inhibitory substances (BLIS) and other antimicrobial metabolites. The resulting *ldh*Δ strains exhibited significantly higher antimicrobial activity, up to 78% greater than their wild-type counterparts, particularly against Gram-negative pathogens [15]. This metabolic rewiring not only reduced acidification of the culture environment but also promoted the biosynthesis of secondary metabolites with potential biotechnological applications.

In the present study, we extend our previous work by introducing *nisRK* overexpression into the *ldh*Δ background of *L. lactis* ATCC 11454. The aim of the dual alteration is to synergistically combine metabolic flux redirection with enhanced transcriptional activation of the nisin biosynthetic cluster. By comparing wild-type, *ldh*Δ, *nisRK*^{OE}, and *ldh*Δ-*nisRK*^{OE} strains, we examine how these genetic modifications affect cell growth, antimicrobial yield, and the physicochemical stability of the resulting bacteriocins at different pH and temperature levels. Finally, recognizing that bacteriocins may behave differently in complex food matrices, we assess their performance in a meat system, using raw chicken as a highly perishable model food prone to microbial spoilage. This method directly connects metabolic and regulatory engineering to functional results that are important for preserving food.

Materials and methods

Bacterial strains, plasmids, and culture media

Table 1 lists the plasmids and bacterial strains used in this study for *nisRK* overexpression. *Lactococcus lactis* ATCC 11454 was grown in M17 broth (Oxoid) at 30 °C. The genetically engineered *L. lactis* ATCC 11454-Δ*ldh* strain, generated from our previous study [15], was cultured in M17 broth with 5 µg/mL erythromycin and 10 µg/mL chloramphenicol at 30 °C. *Escherichia coli* TOP10 was grown in Luria-Bertani broth (Merck, Darmstadt, Germany) at 37 °C, with the addition of 150 µg/mL erythromycin for propagation of *E. coli* TOP10 harboring the *nisRK* plasmid. Pathogenic bacteria used as indicator strains in the antibacterial assays, namely *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 6538,

Bacillus subtilis ATCC 19659, *Vibrio parahaemolyticus* ATCC 17802, and *Escherichia coli* ATCC 8739, were grown overnight in Luria-Bertani broth at 37 °C. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a UV/Vis spectrophotometer (Techcomp UV1000, Thailand).

Table 1 Strains and plasmids used in *nis*RK overexpression.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli TOP10	Cloning host	Laboratory stock
L. lactis ATCC11454	Nisin-producing strain	Laboratory stock
L. lactis ATCC11454- Δ*ldh*	*ldh* knockout mutant of *L. lactis* ATCC11454	Laboratory stock
L. paracasei K2003- Δ*ldh*	*ldh* knockout mutant of *L. paracasei* K2003	Laboratory stock
L. plantarum Y1002- Δ*ldh*	*ldh* knockout mutant of *L. plantarum* Y1002	Laboratory stock
L. rhamnosus MK2003- Δ*ldh*	*ldh* knockout mutant of *L. rhamnosus* MK2003	Laboratory stock
E. faecalis GM1003- Δ*ldh*	*ldh* knockout mutant of *E. faecalis* GM1003	Laboratory stock
Plasmids		
pMG36e	*L. lactis* constitutively expressed plasmid, E^r	Laboratory stock
pMG36e-P8	pMG36e derived, P32 promoter replaced by the P8 promoter	Laboratory stock
nisRK-pMG36e-P8	pMG36e-P8 derived, carrying *nisRK*	This study

Construction of the nisRK-expressing plasmid

Plasmid P8-pMG36e was employed as the vector backbone to drive *nisRK* expression under the control of the strong constitutive P8 promoter [15]. Propagation was carried out in *E. coli* TOP10. The *nisRK* sequence (derived from the complete *Lactococcus lactis* subsp. *lactis* nisin biosynthetic gene cluster (GenBank: HM219853.1) was synthesized and assembled into P8-pMG36e via PstI and SphI restriction sites to generate *nisRK*-P8-pMG36e. The recombinant plasmid was verified by DNA sequencing (GenScript, China) and by restriction enzyme digestion (PstI and SphI in a 37 °C water bath for 40 min).

Preparation of electrocompetent cells and transformation

L. lactis ATCC11454 and *L. lactis* ATCC11454-*ldh*Δ were made electrocompetent [15]. Immediately prior to transformation, 40 µL of each electrocompetent strain was mixed with 5 µL of *nisRK*-P8-pMG36e, and incubated on ice for at least 10 min. Each suspension was transferred to an ice-cooled electroporation cuvette (2-mm electrode gap) and subjected to a single electrical pulse at 2.3 kV using a Bio-Rad Xcell Gene Pulser (Bio-Rad Laboratories). The setup had a capacitance of 25 µF and a resistance of 200 Ω.

The electrotransformed cells were diluted in 960 µL of ice-cold SGM17 supplemented with 20 mM MgCl2 and 2 mM CaCl2, and incubated at 30 °C. After 4 h, 200 µL of the *L. lactis* ATCC11454 suspension was spread plated onto antibiotic-free M17 agar and M17 agar with 5 µg/mL erythromycin; besides, 200 µL of the *L. lactis* ATCC11454-*ldh*Δ suspension was spread plated onto antibiotic-free M17 agar and M17 with 5 µg/mL erythromycin and 10 µg/mL chloramphenicol. The plates were incubated for 24 - 48 h at 30 °C. Transformants were picked, stored as glycerol stocks, and tested for antimicrobial activity assay. As a control, and to verify *nisRK* overexpression, *nisRK*-P8-pMG36e plasmid vector was also transformed into our collection of *ldh*-deficient non nisin-producer LAB including *L. paracasei* K2003-*ldh*Δ, *L. plantarum* Y1002-*ldh*Δ, *L. rhamnosus* MK2003-*ldh*Δ, and *E. faecalis* GM1003-*ldh*Δ. The effect of *nisRK* overexpression on the growth of the *nisRK*-overexpressed (*nisRK*^{OE}) and double mutant (*ldh*Δ*nisRK*^{OE}) strains of *L. lactis* ATCC11454

was determined by measuring OD_{600} of and medium pH over a 48-hours incubation. Meanwhile, the impact of the genetic modification on nisin production was examined by comparing the antimicrobial activity of the engineered strains with that of the wildtype (WT).

Preparation of cell-free supernatant (CFS) and antimicrobial activity assay

The wildtype, *ldh*Δ, *nisRK*^{OE}, and *ldh*Δ-*nisRK*^{OE} strain of *L. lactis* ATCC11454 were grown overnight up to 9 log CFU/mL. An aliquot (10%) of each suspension was added to new MRS broth, incubated for 24 h at 35 °C, centrifuged (4.000×g, 15 min), and filter sterilised using 0.22 μm membrane.

Antimicrobial activity was assessed using the agar well diffusion assay against Gram-positive and Gram-negative pathogens. Wells (6 mm diameter) were punched in the Mueller Hinton agar plates seeded with 100 µL of the test pathogens (OD_{600} 0.1). Each well was filled with 100 µL of CFS from the test strains, 100 µL of MRS broth (negative control), or 100 µL of ampicillin 150 µg/ml (positive control). Plates were incubated at 37 °C for 24 h, after which inhibition zone diameter (mm) surrounding the wells were measured with a caliper. The strength of activity was classified as strong for inhibition zone diameters ≥ 20 mm, moderate for diameters ranging from 10 to 19 mm and weak for diameters less than 9 mm [16].

Thermal and pH stability assessment of nisin antimicrobial activity

The effect of temperature on antimicrobial activity of nisin was evaluated by incubating 1 mL of CFS in eppendorf tube at different temperatures: 0, 4, 35, 60 and 100 °C for 2 h, and at 121 °C at 15 lbs (autoclave) for 15 min [17]. For pH stability assessment, the pH of CFS (initial pH 6.2) was adjusted to values ranging from 2 to 13 by adding either 1 M NaOH or 1 m HCl, followed by overnight incubation at 35 °C. After each treatment, the antimicrobial activity of nisin was assessed against *S. aureus* ATCC 6538, according to the method previously described in Section 2.4.

Stability of nisin antimicrobial activity in raw chicken meat

Chicken meat preparation

Raw chicken breast samples were purchased from a local supermarket (Jumbo Mart, Pekanbaru city, Indonesia), stored in an icebox, and transported on ice and cut into pieces of approximately 2.0 cm. Experimental samples were treated with CFS derived from *ldh*Δ-*nisRK*^{OE} strain, while the negative control was treated with sterile MRS *broth* by immersion in the respective treatment solutions (1:1, w/v) for 30 min, and then drained for 10 mins. To evaluate CFS stability in chicken meat environment under different storage conditions, 2 sets of treated and control samples were prepared: One stored at room temperature and the other under refrigeration. Samples were collected after 0, 1, 3, 5 and 7 days of incubation for physicochemical and microbiological analysis.

pH analysis

Following incubation, 5 g of chicken meat from each treatment group was homogenized in 45 mL of distilled water. The pH of the resulting suspension was measured using a calibrated pH meter at 0, 1, 3, 5, and 7 days of storage.

Bacterial cell count analysis

The total viable cell count was determined to evaluate nisin antimicrobial effectivity and microbiological quality of treated/untreated chicken meat samples, following the method of Jo *et al*. [18] with minor modification. Briefly, 25 g of each sample was homogenised in 225 mL Buffered Peptone Water (pH 7.2), serially diluted up to 10^{-7}, and 100 µL of each suspension was spread plated onto *Plate Count Agar*. Plates were incubated at 37 °C for 24 h, after which colonies were enumerated and expressed as colony forming unit (CFU).

Data analysis

Antimicrobial activity was measured as the diameter of the inhibition zone, reported as mean ± standard deviation from triplicate experiments. One-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) were used to assess for statistical significance with Minitab 19 (Pennsylvania, USA). A two tailed t-test ($p < 0.05$) was applied to compare the antimicrobial

activity of the nisin control (without chicken meat) and the treatment group (with chicken meat).

Results and discussion

Construction of recombinant plasmids and the effect of nisRK overexpression

Figure 1 shows the digestion profile of the recombinant plasmid *nisRK*_P8-pMG36e (5569 bp) on 1% agarose gel. The observed fragment sizes matched the expected pattern, confirming successful insertion of the *nisRK* gene into the P8-pMG36e vector. Sequencing confirmed correct *nisRK* insertion, and transformants displayed stable growth on selective media. No growth differences were observed between control strains harboring the empty plasmid and those carrying *nisRK*, suggesting that plasmid maintenance imposed no major metabolic burden.

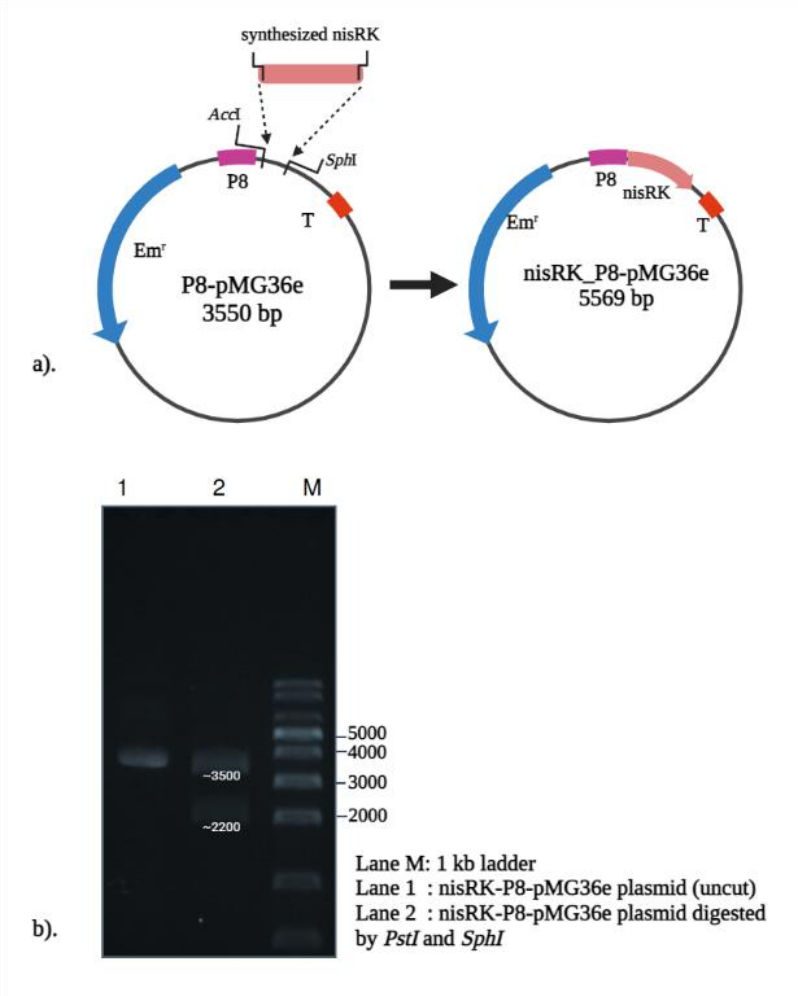

Figure 1 Construction of *nis*RK plasmid from P8-pMG36e as a vector backbone. (a) Insertion of synthesized *nis*RK gene, and (b) RE digestion to verify the construct.

The generated plasmid was introduced into both WT and *ldh*Δ-strains of *L. lactis* ATCC11454, generating *nisRK*-overexpressed (*nisRK*^{OE}) and double mutant (*ldh*Δ*nisRK*^{OE}). **Figure 2(a)** depicts medium pH changes over 48 h incubation. The pH of *nisRK*^{OE} and *ldh*Δ*nisRK*^{OE}-strains was not much different from the initial pH of the growth medium throughout the fermentation period. In WT cells, pH dropped to 4.7 due to lactic acid accumulation, reaching pH 4.6 after 48 h, which inhibited growth. By contrast, the *nisRK*^{OE} and *ldh*Δ*nisRK*^{OE} strains maintained a stable pH of ~ 6.0, close to their optimal growth range (6.3 - 6.9). This suggests that nisRK overexpression redirected carbon flux away from lactate production.

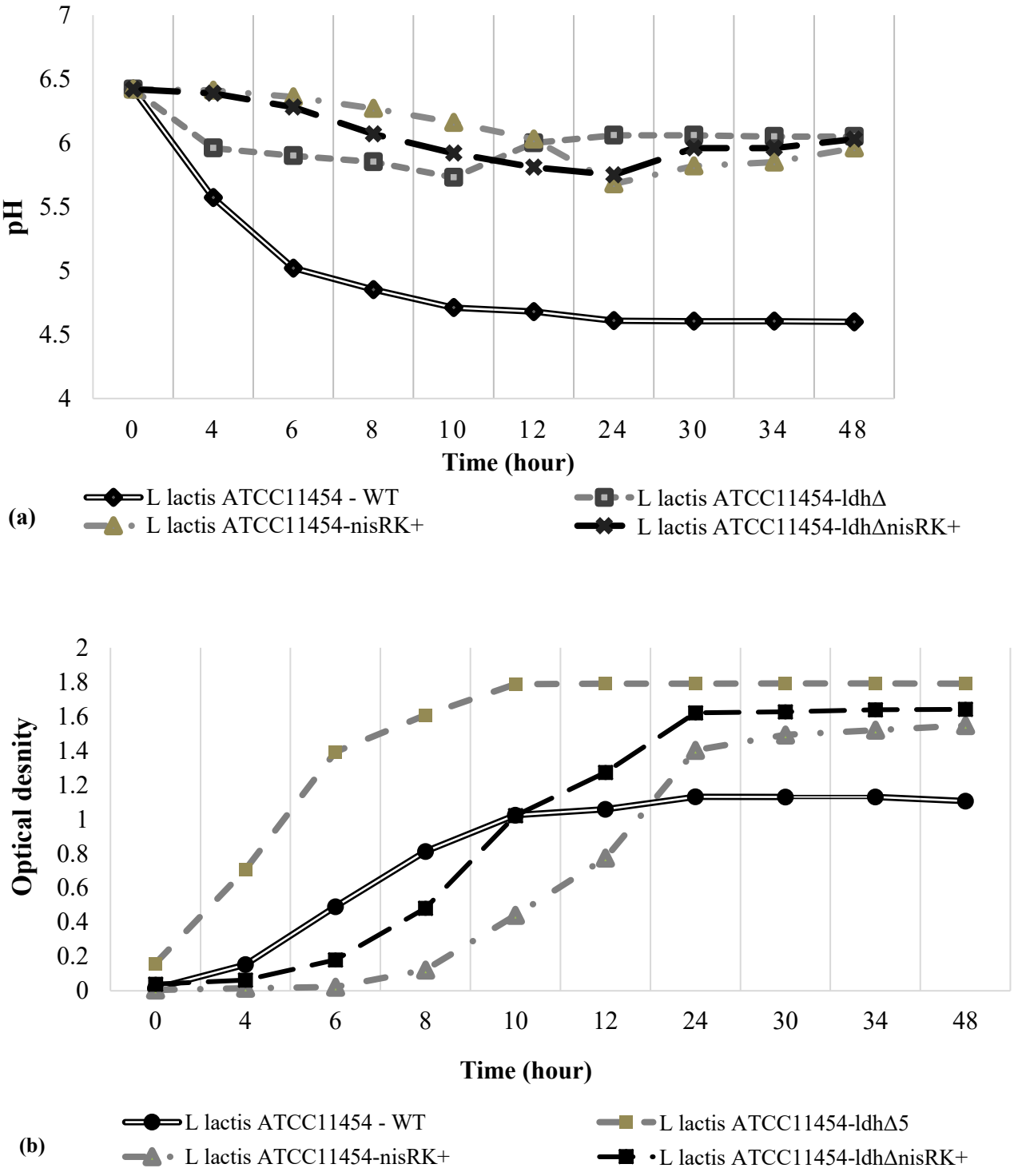

Figure 2 The effect of nisRK overexpression in the cell growth and pH of the growth medium.

The OD_{600} growth profile (**Figure 2(b)**) shows distinct growth dynamics among the strains. The *nisRK*^{OE}, and *ldhΔ-nisRK*^{OE} strains exhibited a noticeably prolonged lag phase lasting approximately 0 - 6 h, likely due to the additional metabolic load associated with *nisRK* overexpression. Despite the delayed onset, the *nisRK*^{OE} strains entered a robust exponential phase between 6 - 12 h and ultimately achieved comparable or higher final cell densities, indicating that *nisRK* overexpression did not exert a toxic effect. The WT and *ldhΔ* strains reached the stationary phase earlier, at approximately 10 h, whereas the *nisRK*^{OE} and *ldhΔnisRK*^{OE} strains reached stationary phase later, around 24 h. Since bacteriocin synthesis

typically peaks during the late logarithmic and early stationary phases [19], sample collection was aligned with these physiological stages: At 10 h for WT and *ldhΔ* strains, and at 24 h for the *nisRK*^{OE} and *ldh*Δ*nisRK*^{OE} strains [20], ensuring representative metabolite production profiles.

Antimicrobial activity of engineered *L. lactis* ATCC 11454

Nisin is known for its potent antimicrobial properties, low cell toxicity, and slow resistance development. Its biosynthesis is tightly regulated by the two-component NisRK system, which balances production and immunity [21,22]. The impact of overexpressed nisR and nisK proteins in nisin production was determined using an antibacterial assay against representative Gram-positive and Gram-negative indicator strains (**Table 2**). The results demonstrated clear differences among strains. Based on the established classification criteria, the WT strain exhibited only weak inhibition. The *ldh*Δ strain produced noticeably larger zones (up to 45% wider than WT), which can be classified as moderate inhibition, suggesting that redirecting pyruvate flux away from lactate enhances nisin synthesis. The *nisRK*^{OE} strain produced intermediate improvements as they showed intermediate to strong inhibition, indicating that upregulating the regulatory module alone positively affects bacteriocin output, albeit not to the same extent as ldh deletion. The strongest inhibition was recorded for the double mutant (*ldh*Δ*nisRK*^{OE}), which produced very strong and consistently well-defined clear zones, exhibited the strongest activity, increasing inhibition by 65% against *V. parahaemolyticus*, 78% against *E. coli,* and over 100% against *B. subtilis* and *S. aureus.* These trends indicate that although *nisRK* overexpression enhances nisin production compared to WT, deletion of *ldh* alone has a stronger effect, and the combination of both modifications yields the highest inhibition levels.

Table 2 Antimicrobial activity of the metabolically engineered *L. lactis* ATCC 11454.

No	LAB strains	Inhibition zone (mm)				
		Gram-positive			Gram-negative	
		SA *)	BS	LM	EC	VP
1	*L. lactis*-WT	8.17 ± 0.29^{d}	9.3 ± 0.58^{c}	10 ± 0^{c}	7.7±0.58^{b}	9.7±0.58^{c}
2	*L. lactis-ldh*Δ	14.2 ± 0.29^{b}	15.7 ± 0.58^{b}	14.2 ± 0.29^{ab}	13.3±0.58^{a}	14.8±0.29^{a}
3	*L. lactis-nisRK*^{ox}	13 ± 0^{c}	15 ± 0^{b}	13.3 ± 0.5^{b}	13±0^{a}	13.5±0.5^{b}
4	*L. lactis -ldh*Δ*- nisRK*^{ox}	16.8 ± 0.29^{a}	19.3 ± 0.58 ^{a}	14.5 ± 0^{a}	13.7 ± 0.58^{a}	16 ± 0.5^{a}

$^{a-d}$: Means in the same column with different lowercase letters differed significantly ($p < 0.05$)

*): SA = *Staphylococcus aureus* ATCC 6538; *Bacillus subtilis* ATCC 19659; LM = *Listeria monocytogenes* ATCC 7644; EC = *Escherichia coli* ATCC 8739; VP = *Vibrio parahaemolyticus* ATCC 17802.

In the WT strain, strong acidification suppressed growth. Furthermore, under low pH conditions, the energy of sugar metabolism is mostly utilised to produce different proteins associated with different acid tolerance mechanisms [23]. These conditions affected the lower flux toward nisin production, explaining its weak antimicrobial activity. By comparison, the near-neutral pH of *nisRK*^{OE} strain supports their normal growth rate during the late stationary phase of fermentation. Since nisin biosynthesis is regulated in a cell-density-dependent manner [8], the elevated growth rate of *nisRK*^{OE} strain promoted higher nisin production and consequently enhanced inhibitory activity against the tested organisms.

Additionally, at this pH, *nisRK*^{OE} cells obtained a greater amount of energy from sugar catabolism, as they did not require the synthesis of various proteins associated with acid tolerance mechanisms [23]. This extra energy might be redirected toward other metabolites, including the synthesis of serine and threonine, the precursors for nisin biosynthesis. NisB dehydrates serine and threonine residues within the core

peptide during post-translational modification to generate dehydroalanine and dehydrobutyrine, respectively. NisC subsequently catalyses intramolecular addition reactions between cysteine residues and the double bonds of dehydroalanine and dehydrobutyrine, resulting in the formation of lanthionine and methyllanthionine. The modified nisin, still containing the leader peptide, is transported by NisT, after which the N-terminal leader sequence is cleaved by NisP, an extracellular serine protease, releasing the mature and active nisin (21). Thus, increased precursor availability could enhance the production of mature nisin, which in turn induces transcription of the nisin biosynthetic gene cluster through the NisR-NisK system [24,25].

Furthermore, the overexpression of *nisR* and *nisK* which are central regulators of nisin biosynthesis, might be responsible for the increased antibacterial activity. Nisin-inducible promoters, including the nisZBTCIPRK and nisFEG operons, are activated by the nisRK system [26]. Thus, overexpression of these genes holds strong potential for improving overall nisin yield. The *ldh*Δ*nisRK*^{OE} strain exhibits a significantly greater inhibitory effect in the cell-free supernatant (CFS) due to the synergistic contribution of *ldh* disruption and *nisRK* overexpression.

The double mutant strain demonstrated the most significant enhancement in inhibition against the Gram-positive *S. aureus* ATCC 6538 and *B. subtilis* ATCC 19659, showing increases of 106.12% and 107.14%, respectively. In comparison to the WT, significant increases were also observed against Gram-negative isolates, including *E. coli* ATCC 8739 (78.26%), and *V. parahaemolyticus* ATCC 17802 (65.52%). Nisin's potency against Gram-positive bacteria was greater than that against Gram-negative bacteria, as anticipated, in accordance with its established mechanism of action [15]. Nonetheless, the enhanced activity of the double mutant against both groups underscores the potential of integrating metabolic and regulatory engineering as a novel strategy to optimise bacteriocin production.

Thermal and pH Stability of Nisin antimicrobial activity

The stability of CFS from *L. lactis* ATCC 11454-*ldh*Δ*nisRK*^{OE} under different thermal and pH conditions was evaluated to determine the robustness of nisin in variable environments.

Thermal stability

The antimicrobial activity of the CFS was effectively maintained during storage at low temperatures (0 and 4 °C), as demonstrated in **Figure 3(a)**. The inhibition zones remained close to those of the untreated control (n-CFS; pH 6.0; 17.9 mm). In contrast, heating resulted in a substantial decrease in activity: the inhibition zone was reduced by 26% at 100 °C (11.63 mm) and by up to 34% at 121 °C (10.3 mm). This suggests that activity was more effectively retained at 100 °C than at 121 °C, indicating a threshold effect when exposure exceeded the boiling point. This thermal stability is in accordance with published reports that describe nisin as capable of maintaining antimicrobial activity after short-term high-temperature treatments, such as autoclaving and high-temperature short-time pasteurisation [27]. The sharper decline observed above 100 °C supports previous findings that nisin remains stable between 0 °C and 100 °C but loses activity under harsher conditions [28].

Disrupting *ldh* alters cellular metabolism, which can affect the post-translational modifications, folding and maturation processes, as well as the local chemical environment of nisin, thereby altering its stability. Nisin is a lantibiotic, a small antimicrobial peptide comprised of unusual amino acids, including lanthionine and dehydroalanine. Compared to larger proteins, its small size, simple structure, and strong covalent bonds confer a higher resistance to denaturation. Furthermore, the presence of lanthionine and β-methyllanthionine residues results in the formation of thioether bridges, which provide a rigid and stable conformation that prevents denaturation and unfolding at high temperatures [29].

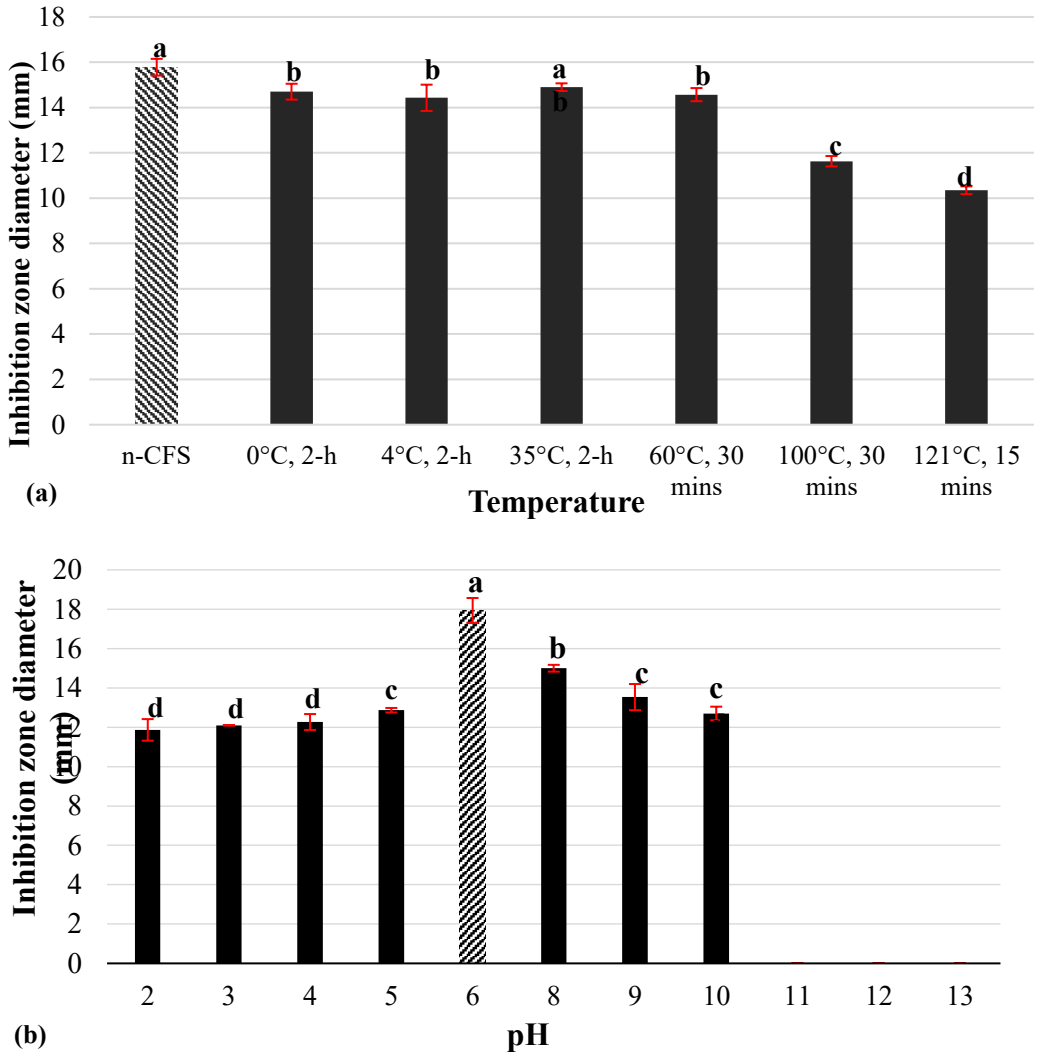

Figure 3 Effect of temperature (a) and pH (b) on antimicrobial activity of CFS from *L. lactis* ATCC 114545-*ldh*Δ*nisRK*^{OE}.

pH stability

The effect of pH variation on CFS activity is presented in **Figure 3(b)**. Antimicrobial activity was retained over a wide range from pH 2 to pH 10, although the inhibition zones varied significantly. The highest activity was observed at pH 6 (17.9 mm), corresponding to the fermentation medium, consistent with the optimal conditions for nisin synthesis [29]. At strongly acidic pH (1 - 3), inhibition zones were notably smaller (9 - 12 mm), indicating partial inactivation under extreme acidity. Activity improved at pH 4 - 5 (12 - 13 mm) and remained moderate under alkaline conditions at pH 8 - 10 (13 - 15 mm), although inhibition declined relative to neutral pH. Interestingly, the relatively high activity at pH 10 contrasted with earlier reports that alkaline environments reduce nisin solubility due to nucleophilic attack on dehydro residues by hydroxide ions, deprotonated amines, and hydroxyl groups, leading to aggregation [30]. This observation suggests that nisin and other metabolites present in the CFS from the double mutant strain may act protectively, minimizing modification of the nisin dehydro residues and thereby maintaining antimicrobial activity.

Stability and antimicrobial performance of nisin in raw chicken meat

Antimicrobial stability during storage

The antimicrobial activity of CFS against *S. aureus* ATCC 29213 was evaluated during seven days of storage at 4 °C, both in the absence and presence of chicken meats (**Table 3**). Over the storage period, a gradual decline in inhibition zones was observed in both

treatments. The decline in antibacterial activity of nisin-producing LAB CFS during storage at 4 °C can be attributed to aggregation, reduced solubility, conformational changes, and chemical degradation. Nisin molecules tend to aggregate at low temperatures, reducing their capacity to inhibit bacterial growth and compromising their stability [31]. At 4 °C, the solubility also decreases, which further diminishes the efficacy. Antimicrobial activity is dependant upon structural integrity, however, conformational modifications affecting bioactivity and stability may results from low temperatures. Although refrigeration delays degradation, it does not entirely prevent it; nisin undergoes chemical breakdown over time, even when stored in cold conditions [32,33].

Table 3 Changes in nisin/BLIS antimicrobial activity during seven days of storage at 4 °C in the presence and absence of chicken meats.

Storage time (Days)	Inhibition zones (mm)[*)]	
	Without chicken meat	With chicken meats
0	14.67 ± 0.15[a]	12.93 ± 0.12[a]
1	14.45 ± 0.30[ab]	12.43 ± 0.18[ab]
2	14.10 ± 0.05[b]	12.37 ± 0.21[b]
3	12.40 ± 0.05[c]	11.45 ± 0.22[c]
4	12.38 ± 0.08[c]	11.08 ± 0.29[c]
5	12.32 ± 0.15[c]	9.58 ± 0.13[d]
6	12.20 ± 0.10[c]	9.20 ± 0.17[de]
7	12.13 ± 0.13[c]	8.82 ± 0.20[e]

[*]Mean values ± standard deviation from three replicates. Superscript letters within the same column indicate no significant differences (α = 0.05) according to Tukey's test.

The antimicrobial activity of the control CFS, which was devoid of chicken meat, decreased moderately by 17.31%. However, the reduction in the chicken meat environment was more pronounced, reaching 31.79%. Significant reductions ($p < 0.05$) were observed in the control after day 2 and in the chicken meat samples after day 1, indicating that the inactivation process was faster when interacting with the meat matrix. The sharper decline in antimicrobial activity within the chicken meat environment can be attributed to physicochemical interactions between nisin and food components. Proteins and lipids in meat may bind or inactivate nisin through hydrophobic and electrostatic interactions, thereby decreasing its solubility and bioavailability. Previous studies have reported that fat globules adsorb nisin, thereby decreasing its capacity to penetrate bacterial membranes [34,35]. Furthermore, lipids may establish protective barriers around bacterial cells, thereby protecting them from the pore-forming action of nisin [36,37]. These results are consistent with previous observations that nisin stability and activity decrease more swiftly in complex food matrices than in simple laboratory media. This is attributed to the presence of proteins, fats, and other macromolecules [38]. The present results highlight the importance of considering food composition when applying bacteriocins as natural preservatives. Although nisin retained partial activity after seven days in chicken meats, the reduced inhibition zone suggests the need for formulation strategies such as encapsulation or combination with other antimicrobials to improve stability and efficacy in meat products.

Total bacterial counts and shelf-life extension

Table 4 illustrates the impact of CFS coating on the total bacterial counts of chicken meats stored at 4

and 30 °C. In comparison to untreated controls, bacterial proliferation was significantly reduced in CFS-treated samples at refrigeration temperature (4 °C). The untreated controls reached 8.58 ± 0.03 log CFU/g by day 3, whereas the treated samples remained significantly lower at 7.52 ± 0.07 log CFU/g ($p < 0.05$). After seven days, bacterial counts in controls were 9.10 ± 0.07 log CFU/g, while in treated samples they were 8.41 ± 0.02 log CFU/g. This represents a 0.69 log reduction that can be attributed to the CFS. These findings indicate that CFS application effectively slowed microbial proliferation, thereby extending the microbiological shelf life of chicken meats in chilled storage.

Table 4 Total bacterial counts of chicken fillets with and without CFS treatment during storage at 4 °C and 30 °C

Storage time (Days)	Total bacterial count (log CFU/g)[1)]			
	Stored at 4 °C		Stored at room temperature (~30 °C)	
	Untreated sample	CFS-treated sample	Untreated sample	CFS-treated sample
0	7.82 ± 0.07[a]	7.16 ± 0.11[a]	7.82 ± 0.07[a]	7.16 ± 0.11[a]
1	7.87 ± 0.02[a]	7.38 ± 0.01[b]	8.64 ± 0.03[b]	7.96 ± 0.04[b]
3	8.58 ± 0.03[b]	7.52 ± 0.07[b]	NA[2)]	8.84 ± 0.10[c]
5	8.87 ± 0.06[c]	7.97 ± 0.00[c]	NA	9.78 ± 0.02[d]
7	9.10 ± 0.07[d]	8.41 ± 0.02[d]	NA	10.18 ± 0.01[e]

[1)] Mean values ± standard deviation from three replicates. Superscript letters within the same column indicate no significant differences (α = 0.05) according to Tukey's test.
[2)] NA = data not available (chicken fillet samples spoiled).

At room temperature (30 °C), spoilage occurred much more rapidly. The bacterial counts of control meats increased significantly, reaching 8.64 ± 0.03 log CFU/g after only 24 h. By day 3, visible deterioration was observed, rendering further measurements unavailable. On the other hand, CFS-treated samples demonstrated delayed microbial growth, rising from 7.16 ± 0.11 log CFU/g on day 0 to 7.96 ± 0.04 log CFU/g on day 1, and progressively reaching 10.18 ± 0.01 log CFU/g by day 7. The treatment effectively extended the utilisation of the product in comparison to the untreated controls, despite the fact that microbial growth was not entirely suppressed. In the untreated controls, spoilage occurred within 72 h, indicated by the strong odor, surface slime formation, and loss of structural integrity. At this stage, the meat matrix became highly degraded due to the activity of spoilage-associated microorganisms, and excessive microbial overgrowth, resulting in confluent colonies on agar plates and colony numbers exceeding the countable range. Because the samples no longer met the criteria for reliable enumeration, further quantitative measurements were not possible. This type of matrix breakdown and uncountable growth are typical indicators of advanced spoilage in high-protein perishable foods such as poultry.

The activity of nisin present in the CFS is responsible for the antimicrobial effect that was observed. Nisin inhibits cell wall biosynthesis by binding to lipid II and forming cavities in bacterial membranes, resulting in the leakage of cytoplasmic contents [39,40]. Nevertheless, the progressive deterioration of efficacy over time underscores the intricacy of implementing bacteriocins in actual food systems. For instance, glutathione, naturally present in meat, can inactivate nisin via glutathione S-transferase [35].

The relationship between nisin antibacterial activity and bacterial proliferation in chicken meats stored at 4 °C is illustrated in **Figure 4**. The inhibition zone was 12.93 mm in diameter at day 0, and the total bacterial count was 7.2 log CFU/g. Over the storage

period, inhibition zones slowly decreased, dropping to 8.9 mm by day 7, whereas bacterial counts increased steadily to 8.4 log CFU/g. This inverse relationship indicates that the gradual loss of antimicrobial activity directly corresponded with the rise in bacterial populations.

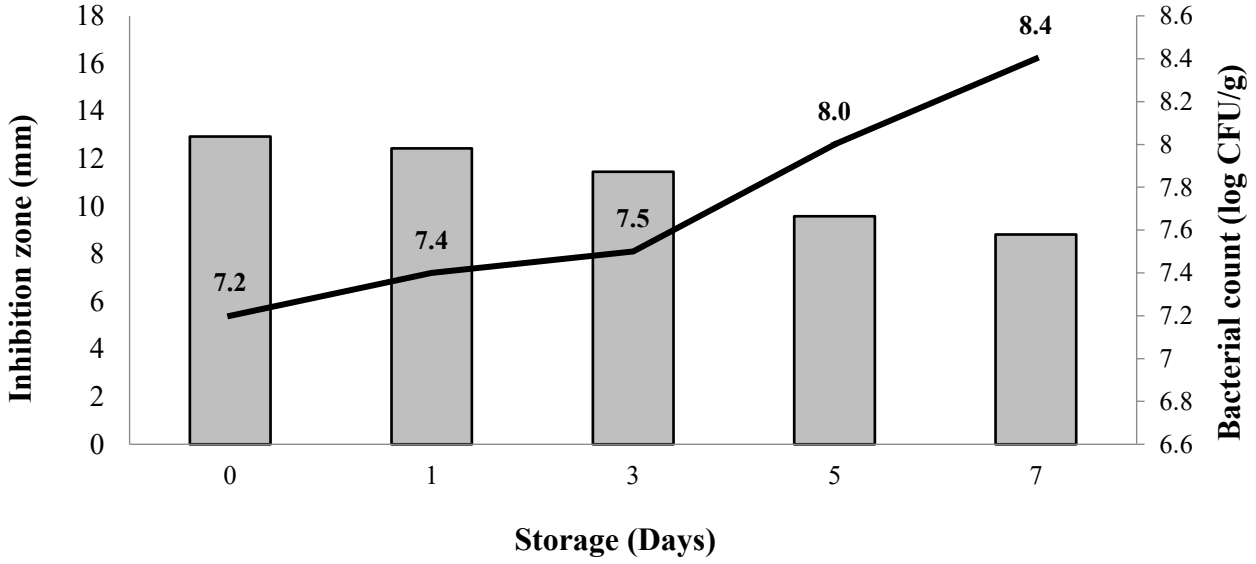

Figure 4 Relationship between nisin/BLIS antibacterial activity and bacterial proliferation in chicken fillets stored at 4 °C

The reduction in inhibition zones suggests a progressive decline in the bioactivity of nisin. Several mechanisms may contribute to this loss, including adsorption of nisin to meat proteins and lipids, enzymatic inactivation by endogenous compounds such as glutathione, and decreased bioavailability as storage progressed [34,35]. As a result, bacterial growth was only partially suppressed, particularly after day 3 when inhibition zones fell below 12 mm. Despite this decline, CFS treatment effectively slowed bacterial growth compared with untreated controls (**Table 4**).

pH changes during storage

The pH of chicken meats fluctuated over the storage period, as shown in **Figure 5**. Meats stored at 4 °C consistently exhibited lower pH values than those at 30 °C, mirroring microbial counts. At both 4 and 30°C, pH increased up to day 5, correlating with bacterial growth and the accumulation of biogenic amines produced by amino acid decarboxylation [41,42]. The pH increase was more pronounced at 30 °C, which is consistent with the higher rate of microbial proliferation. By day 7, a slight pH decrease was observed, which is likely the result of the accumulation of acidic metabolites and the onset of spoilage [43].

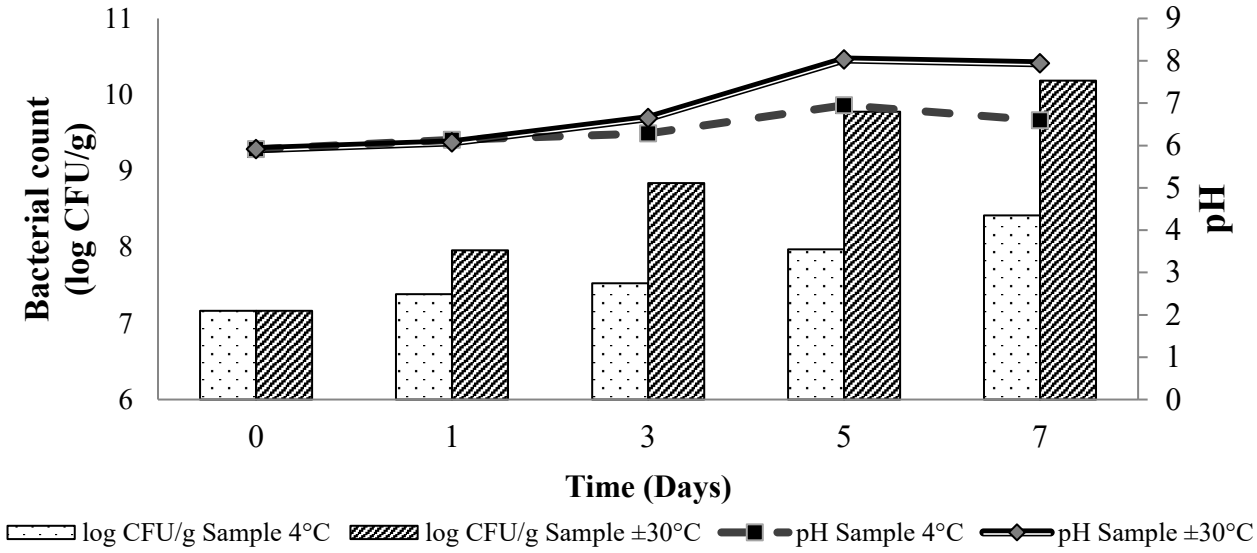

Figure 5 Changes in bacterial count and pH of chicken meats during storage at 4 and 30 °C

Overall, chicken meats coated with CFS demonstrated a longer shelf life and delayed microbial growth in comparison to uncoated controls, particularly under refrigeration. Given that raw chicken is highly susceptible to contamination by pathogens such as *Salmonella* spp., *Campylobacter* spp., *Staphylococcus aureus*, *Escherichia coli*, and *Listeria* spp. [44], these findings underscore the potential of nisin as a biopreservative in meat systems. Additionally, the broader antimicrobial spectrum observed with the engineered *L. lactis* ATCC 11454 *ldh*Δ*nisRK*^{OE} compared with wild-type strains emphasizes its promise for food applications. Beyond direct application, nisin can also be incorporated into active packaging systems, where it has been shown to inhibit microbial growth and extend shelf life in dairy and meat products [45,46].

Conclusions

This study demonstrates that combining lactate dehydrogenase knockout with *nisRK* overexpression in *Lactococcus lactis* ATCC 11454 significantly enhances nisin biosynthesis and antimicrobial activity. The double mutant (*ldh*Δ*nisRK*^{OE}) exhibited superior inhibitory effects against both Gram-positive and Gram-negative bacteria, outperforming strains engineered with single modifications. Stability tests confirmed that nisin produced by the engineered strain retained substantial antimicrobial activity across a wide pH spectrum and under short-term heat stress, highlighting its robustness for industrial applications.

Importantly, application of the engineered CFS to raw chicken meats revealed its capacity to reduce microbial proliferation and extend shelf life, particularly under refrigeration. These findings bridge laboratory-scale engineering with practical food preservation outcomes, underscoring the value of integrating metabolic and regulatory approaches to optimize bacteriocin production. Future studies should focus on scaling up fermentation, characterizing molecular interactions in complex food matrices, and exploring delivery systems such as active packaging to further expand the utility of engineered nisin-producing strains in food safety and preservation.

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Declaration of generative AI in scientific writing

Generative AI tools were used solely for editorial assistance, including grammar correction, phrasing improvements, and formatting refinement. All intellectual content, experimental design, data

interpretation, and scientific conclusions were developed entirely by the authors. After using these tools, the authors carefully reviewed and thoroughly edited the entire manuscript to ensure accuracy, clarity, and proper scientific representation. The authors take full responsibility for the content, integrity, and conclusions presented in this publication.

CRediT author statement

Yuli Haryani: Supervision; Conceptualization; Software; Original draft preparation. **Hanan Hasan**: Supervision; Conceptualization; Methodology; Data Curation. **Rudi Hendra:** Formal analysis; Funding acquisition. **Benni Iskandar**: Visualization; Project administration. **Yulia Andriana**: Resources; Investigation. **Yuwanda**: Investigation; Validation. **Eliza Khoirunnisa**: Resources; Investigation. **Nadrah Abdul Halid**: Writing - Reviewing and Editing. **Suriana Sabri**: Methodology; Validation. **Mahmud Ab Rashid Nor-Khaizura**: Writing - Reviewing and Editing. **Muhammad Asyraf Md Hatta**: Formal analysis; Validation.

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