Bioengineering of a *Lactococcus lactis* subsp. *lactis* strain enhances nisin production and bioactivity.

Roxana Portieles¹#, Hongli Xu¹#, Feng Chen¹, Jingyao Gao¹, Lihua Du¹, Xiangyou Gao¹, Carlos Borroto Nordelo², Qiulin Yue³, Lin Zhao³, Nayanci Portal Gonzalez⁴, Ramon Santos Bermudez⁴, Orlando Borrás-Hidalgo¹,³*

¹ Joint R and D Center of Biotechnology, RETDA, YOTABIO-ENGINEERING CO., LTD., 99 Shenzhen Road, Rizhao, 276826, Shandong, P.R. China
² VBS Biotec SA, México
³ State Key Laboratory of Biobased Material and Green Papermaking, Shandong Provincial Key Lab of Microbial Engineering, Qilu University of Technology (Shandong Academic of Science), Jinan, People’s Republic of China
⁴ School of Biological Science and Technology, University of Jinan, No. 336, West Road of Nan Xinzhuang, Jinan, 250022, Shandong, People’s Republic of China

* Corresponding author
E-mail: orlando@yotabio.com (OBH)

# These authors contributed equally to this work.
Abstract

*Lactococcus lactis* subsp. *lactis* is a food bacterium that has been utilized for decades in food fermentation and the development of high-value industrial goods. Among these, nisin is produced by several strains of *L. lactis* subsp. *lactis* plays a crucial role as a food bio-preservative. The relative expression of the gene cluster involved in nisin production was evaluated using qPCR analysis. Additionally, a series of re-transformations of the strain introducing multi copies of the *nisA* and *nisRK* genes involved in nisin biosynthesis were developed. The simultaneous expression of *nisA* and *nisZ* genes was used to potentiate the effective inhibition of foodborne pathogens. Further, qPCR analysis revealed a low expression of the *nisA* and *nisRK* genes in wild-type *L. lactis* subsp. *lactis*. After several re-transformations of the strain with the *nisA* and *nisRK* genes, a high expression of these genes was obtained, contributing to improved nisin production. Also, the co-expression of *nisA* and *nisZ* genes results in highly efficient antimicrobial activity. Hence, this study would supply an approach to enhancing nisin production during industrial processes and antimicrobial activity.

Introduction
The control of foodborne pathogens constitutes an important issue in the food industry. Diseases produced by these kinds of pathogens are disseminated rapidly [1]. Although diverse actions were deployed to decrease the infections [2], foodborne diseases even remain prevalent worldwide [3]. To limit the potential risks, many ways of managing foodborne pathogens have been developed [2]. One option is to incorporate bacteriocins and bacteriocin-producing lactic acid bacteria (LAB) in food as effective alternatives to chemical preservatives [4, 5].

*Lactococcus lactis* is used to produce different fermented milk products. Originally, *L. lactis* was included in the *Streptococcus* genus. It was re-classified into the *Lactococcus* genus in 1985 [6]. *L. lactis* is divided into three subspecies, namely, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *hordniae* [7]. Phenotypically, it is classified as a Gram-positive, spherical, homolactate, non-sporulating, and facultative anaerobic gut bacterium with hundreds of strains and bio-variants published to date [8, 9]. *L. lactis* has been used for centuries in the fermentation of food. *L. lactis* is a lactic acid bacterium (LAB) that also produces acid that preserves food. Some strains further enhance this preservation property with the production of bacteriocins.

The antimicrobial peptide, nisin, is a peptide with 34 amino acid residues produced during the growth of several *L. lactis* strains [10]. Generally, nisin inhibits and prevents the spread of Gram-positive bacteria [11-13]. This constitutes an important element to be used as an excellent food preservative and additive in the food industry [14]. Due to its non-toxicity in animals, the World Health Organization in 1969 and the Food and Drug Administration in 1988 approved the consumption of nisin by humans as safe.
Other likely applications of nisin are related to the biomedical field [15]. Several studies highlight the multiple ways in which nisin could potentially be used as a therapeutic agent. For instance, it has been shown that nisin has immunomodulatory properties analogous to those described for many human defense peptides, giving rise to the suggestion of a possible role for nisin as a novel immunomodulatory therapeutic [16]. Additionally, nisin was shown to be effective in the treatment of head and neck squamous cell carcinoma [17].

A cluster of 11 genes has been involved in the complex biosynthesis of nisin. The gene cluster includes the structural gene (nisA); genes involved in post-translational modifications (nisB, nisC), transport (nisT), and extracellular precursor processing (nisP); genes encoding immunity to the producer strain (nisI, nisFEG); and the regulatory genes, which belong to the class of the two-component auto-regulatory system consisting of a response regulator (nisR) and a histidine kinase (nisK) [18, 19]. A better understanding of the nisin biosynthetic process and its metabolic regulation seems to be a prerequisite for achieving improved nisin yield. Increasing nisin resistance by the introduction of a plasmid containing immunity genes leads to a slight improvement in the rate and level of nisin production [20].

The evaluation of the nisin biosynthesis opens the road to carrying out genetic manipulations aimed at increasing the nisin yield in production stage. These genetic manipulations have focused on the problems that restrict nisin production. Innovative genetic approaches have increased nisin production with L. lactis strains expressing the aox1, pfk13, and pkaC genes [21]. These recombinant nisin producers induced oxidative respiration and glycolytic activity. Further, genome shuffling was used through protoplast
fusion to enhance the nisin Z production of *L. lactis* ssp. *lactis* YF11 [22]. The acidic tolerance of the producer *L. lactis* was enhanced through the simultaneous expression of the *hdeAB*, *Idh*, and *murF* genes, this strategy improved the nisin production [23]. Herewith, over-expression of the *asnH* gene increased the acidic tolerance in the nisin producer *L. lactis* [24].

Previous studies have shown that the increase in the copy number of the important genes involved in nisin biosynthesis allowed an increase in nisin production [25-29]. Besides, increasing the copy number of *nisRK* and *nisFEG* genes involved in the regulation and resistance in nisin biosynthesis improved notably the nisin production [29]. Most of the precedent works were done through a simple genetic transformation and selection of the most promising strains with high production of nisin. However, it is unknown about the impact of the genetic re-transformation on *L. lactis* subsp. *lactis* strains several times with genes involved in nisin synthesis. Previously, the genetic re-transformation was associated with the significant increase of proteins in bacteria and yeast. The re-transformation of *Clostridium acetobutylicum* bacterium with genes expressing stress proteins resulted in a more robust phenotype and improved n-butanol tolerance during fermentations [30]. While following the same strategy resulted in a significant increase in insulin synthesis in the yeast *Pichia pastoris* [31].

The use of bacteriocins could be impeded by the restricted activity, high concentration of foodborne pathogens, and antimicrobial resistance. Several studies have shown additive effects when the bacteriocins are in combination or with other antimicrobial agents. Thus, this strategy is a good alternative for effective pathogen inhibition. The combination of nisin, carvacrol, and citric acid inhibited Gram-positive and
Gram-negative foodborne pathogens [32]. The activity of nisin was enhanced with the addition of EDTA against *L. monocytogenes* and *Escherichia coli* [33]. The combination of polymyxin, colistin, and nisin increased the activity against the biofilm formation of *Pseudomonas aeruginosa* [34]. Also, a *L. lactis* strain that co-expressed nisin and leucocin C demonstrated a potent antibacterial activity. This strain had a broad activity range and a good bacteriostatic capacity against Gram-positive foodborne pathogens [35]. Curiously, the biological activity of a *L. lactis* strain co-expressing simultaneously *nisA* and *nisZ* genes was not previously evaluated.

Therefore, the aim of this study was the characterization and evaluation of serial genetic re-transformations (RT) to enhance the nisin production in the *L. lactis* subsp. *lactis*. Additionally, we constructed a *L. lactis* subsp. *lactis* strain co-expressing *nisA* and *nisZ* genes with enhanced antimicrobial activity. This work describes how is possible for a significant increase in nisin production through serial genetic re-transformations in the *L. lactis* subsp. *lactis* strain with high activity.

**Materials and Methods**

**Bacterial strains, plasmids, and culture conditions**

The *Escherichia coli* DH5α (Invitrogen, USA) was grown in an LB (Luria-Bertani) medium (Difco) at 37°C for cloning and the construction of plasmids. The *Lactococcus lactis* subsp. *lactis* CICC 6242 strain used in the experiments was supplied by the China Center of Industrial Culture Collection (Beijing, People’s Republic of China). The pGEM-T Easy Vector (Promega, Madison, USA) and pMG36e vector (Addgene, MA, USA) were
used in the cloning and recombinant expression experiments. The different fermentations of *L. lactis* subsp. *lactis* were done in 250-mL flasks containing 50 mL of M17 medium (Difco) containing 0.5 \% of glucose. The *L. lactis* subsp. *lactis* was incubated by shaking at 100 rpm at 30°C for 18 hours without control of pH. The erythromycin (Sangon, Shanghai, China) was used at 250 \( \mu \text{g}/\text{mL} \) for *E. coli* DH5α and 5 \( \mu \text{g}/\text{mL} \) for *L. lactis* subsp. *lactis* strains, respectively. The cell growth (O.D. 600), pH and nisin titers (IU/ml) parameters were evaluated every two hours until 18 hours of fermentation.

**Preparation of recombinant strains**

Chromosomal DNA from *L. lactis* subsp. *lactis* CICC 6242 strain producing nisin A was extracted using a “Wizard Genomic DNA Purification” kit (Promega, Madison, USA). The *nisA* and *nisRK* genes were amplified through a polymerase chain reaction using the primers listed in Table 1 [29]. The PCRs were performed using the following reagents: 2.5 \( \mu \text{L} \) of DNA polymerase buffer, 2.5 \( \mu \text{L} \) of dNTPs, 0.5 \( \mu \text{L} \) of MgCl₂, 0.5 \( \mu \text{L} \) of each primer, 0.1 \( \mu \text{L} \) of DNA polymerase (Promega, Madison, USA) at 50 ng/\muL and enough water in a final volume of 12.5 \( \mu \text{L} \). Amplifications were performed in a Bio-Rad thermocycler T100TM (Bio Rad, USA) with the following program: an initial denaturation step at 94 °C for 5 min, followed by 35 denaturation cycles at 94 °C for 1 min, annealing (*nisA*: 62 °C and *nisRK* 60 °C), extension at 72 °C for 1 min and a final extension step at 72 °C for 7 min. The amplified products were stained with a mix of 2X Blue/Orange Loading Dye (Promega, Madison, USA), loading buffer (1X) and 1X SYBr green Nucleic Acid Gel Stain (Sangon Biotech, Shanghai) dye and were separated by electrophoresis on 1% agarose gels. The sequence integrity from *nisA* and *nisRK* genes were evaluated.
by sequencing. Herewith, the *nisZ* gene was synthesized (Shanghai RealGene Bio-Tech, Inc).

The genes were cloned one by one into pGEM-T Easy Vector (Promega, Madison, USA) and finally in pMG36e vector (Addgene, MA, USA) under p32 promoter to the recombinant expression using PCR Cloning Kit (Qiagen, Germany). The recombinant plasmids were named as pMG36e:*nisA* (*nisA*), pMG36e:*nisZ* (*nisZ*) and pMG36e:*nisRK* (*nisRK*). The recombinant plasmids were first introduced in *E. coli* DH5 cells and cultured on LB agar plates with 250 μg/mL of erythromycin. Restriction enzyme digestions and sequencing analyses were used to examine the DNA plasmids. To obtain the electro-competent *L. lactis* cells, the bacterium was cultivated overnight at 30 °C to an O.D.600 of 0.7. After the cells were washed twice with ice-cold washing solution (0.5 M sucrose, 10% glycerol), resuspended in 1/100 volume of electroporation solution (0.5 M sucrose, 10% glycerol) and stored on ice. Using a Gene Pulser device (Bio Rad, USA) set at 2.2 kV, 200, and 25°F, the verified recombinant plasmids were electro-transformed in *L. lactis* subsp. *lactis* strain. Likewise, the transformants pMG36e:*nisA*, pMG36e:*nisZ*, and pMG36e:*nisRK* were selected on M17 medium containing 5 μg/mL erythromycin. The plasmids from the transformant strains were further analyzed by DNA sequencing.

The strategies of re-transformation and expression of *nisA* and *nisRK* genes in the same strain were as follow: a) genetic re-transformation was done in the original *L. lactis* subsp. *lactis* strain with the *nisA* and *nisRK* genes and the colonies were selected by erythromycin resistance and the number of copies of the genes introduced regarding the number of copies of these genes in the original strain; b) independent clones of *L.
*Lactis* subsp. *lactis* resistant to erythromycin and with the highest copies numbers of *nisA* and *nisRK* genes were selected, respectively; c) two more subsequent transformations were carried out using in each step the clones with the highest copies numbers as selection criteria (pMG36e:*nisA* RT and pMG36e:*nisRK* RT), since the antibiotic resistance of the plasmid is the same (S1 Fig).

Also, the original and multi copies *nisA* *L. lactis* subsp. *lactis* strains were transformed with the *nisZ* gene to obtain the expression of both nisin in the same strain. The selection of the clones was based on antibiotic resistance, the number of copies of the *nisA* and *nisZ* genes, relative expression, RNA sequencing, and LC-MS/MS analysis (S2 Fig). To test their biological activity, clones expressing nisin A, Z, or both in the same strain were selected. In addition, the genetic stability of expression plasmid vector pMG36e in *L. lactis* subsp. *lactis* was evaluated by culturing transformants pMG36e:*nisA*, pMG36e:*nisA* RT, pMG36e:*nisRK* and pMG36e:*nisRK* RT in 100 μl of GM17 broth without erythromycin selection. The *L. lactis* subsp. *lactis* was maintained at an exponential phase for 120 generations. The *L. lactis* subsp. *lactis* was grown until the OD600 was at the upper log phase, and 100 μl of culture was inoculated into fresh 100 ml GM17 broth. This method was repeated until the cells had achieved roughly 120 generations. The *L. lactis* subsp. *lactis* was then plated on GM17 agar and incubated at 30°C for 18 hours. A total of 100 colonies were selected at random and subcultured on GM17 agar with erythromycin. Finally, the percentage of colonies that retained the plasmid was calculated.

**Analysis of gene copy number of transformed strains**
Total DNA from all the transformed and wild-type *L. lactis* subsp. *lactis* strains were extracted using a “Wizard Genomic DNA Purification” kit (Promega, Madison, USA). Real-time PCR was done using a Rotor-Gene Q machine (Qiagen, Hilden, Germany) with the QuantiTect SYBR Green PCR Kit (Qiagen, Germany). For absolute quantification of the *nisA* and *nisRK* genes in transformed and wild-type *L. lactis subsp. lactis* strains, two standard curves at different dilutions (1:10; 1:100; 1:1000, and 1:10000) were established using the genes previously cloned in pGEM-T Easy Vector (Promega, Madison, USA). The data were determined in terms of copies of the *nisA* and *nisRK* genes / µL of qPCR reaction. The primers used in the experiments are listed in Table 1 [29]. Real-time PCR conditions were as follows: an initial 95°C denaturation step for 15 min followed by denaturation for 15 sec at 95°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C for 40 cycles. Data were analyzed by one-way analysis of variance using GraphPad Prism 5.0 (GraphPad Software, Inc, California). Significant differences among means were determined by Tukey’s Multiple Comparison Test least significant difference mean separation at *P* < 0.05.

**Transcriptional analysis by quantitative real-time PCR**

The original *L. lactis* subsp. *lactis* and transformed strains were incubated in shaking at 100 rpm at 30°C during the fermentation. The samples for RNA isolation were taken at 0; 12 and 18 hours of fermentation. Total RNAs were isolated with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The cDNA was synthesized using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). The primers used in the experiments are listed in Table 1) [29]. For each
condition, reactions were done in triplicate in three separate experiments. To standardize cycle threshold (Ct) results, the 16S rRNA gene was used as an internal control. Quantitative real-time PCR was done using a Rotor-Gene Q machine (Qiagen, Hilden, Germany) with the QuantiTect SYBR Green PCR Kit (Qiagen, Germany). Real-time PCR conditions were as follows: an initial 95°C denaturation step for 15 min followed by denaturation for 15 sec at 95°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C for 40 cycles. The relative levels of genes were expressed as ‘mean normalized expression’ data using Q-Gene software [36]. The Q-Gene software application is a tool for dealing with complex quantitative real-time PCR experiments on a large scale, significantly accelerating and rationalizing experimental setup, data analysis, and data management while ensuring maximum reproducibility. Data were analyzed by one-way analysis of variance using GraphPad Prism 5.0 (GraphPad Software, Inc, California). Significant differences among means were determined by Tukey’s Multiple Comparison Test least significant difference mean separation at $P < 0.05$.

**Nisin quantification and bioactivity assay.**

The nutrient broth (NB) medium (peptones 10 g/L, beef extract 1 g/L, yeast extract 2 g/L, sodium chloride 5 g/L, and pH 6.8) for *Micrococcus luteus* was used during the bioassay agar plates [37]. Before boiling and sterilizing, 0.75 % Bacto agar (Difco) and 1% of Tween 20 were added to the medium composition. After autoclaving, the medium was inoculated with 1% of the 24-h culture of *M. luteus*. The concentration of the microorganism was approximately 10$^8$ colony-forming units/mL. The bioassay agar was
placed aseptically into sterile Petri dishes (100 X 15 mm) and allowed to solidify for 3 hours. Four holes were bored on each plate using a 7-mm outer diameter stainless steel borer with a little suction applied. A well was filled with an aliquot (100 µL) of standard nisin solution and samples, and the bioassay agar plates were incubated at 30 °C for 24 hours. The diameter of the inhibitory zone was measured in the nisin-sensitive microorganism. Three replication were used in the bioassay. Multiple comparisons using Tukey's test were done.

Also, the nisin quantification was estimated by agar diffusion activity assays and expressed in international units / mL (IU/mL) [37]. A stock nisin solution (1,000 IU/mL) was prepared by adding 0.025 g of commercial nisin$10^6$ IU/g (Sigma Aldrich) into 25 mL of the sterile diluent solution of 0.02 N HCl: L. lactis fermentation medium (9:1 by volume), which consisted of (per L): 80 g of glucose, 10 g of peptone, 10 g of yeast extract, 10 g of KH$_2$PO$_4$, 2 g of NaCl, and 0.2 g of MgSO$_4$.7H$_2$O. Standard nisin solutions of 500, 400, 300, 200, 100, 50, 25, 10, 5, and 0 IU/mL were prepared using the 1,000 IU/mL nisin stock solution and diluent solution and utilized to construct the standard curve. Diameters of inhibition zones vs log10 nisin concentrations were plotted in order to obtain a standard curve. A line regression equation was determined for a standard curve. The sensitivity of the bioassay was correlated with the diameter of the inhibition zone obtained from the nisin standard solution [37].

**Chloroform extraction and LC-MS/MS analysis**

M17 medium (Difco) was inoculated with an overnight culture of the transformed L. lactis subsp. lactis and wild-type strains and incubated in shaking at 100 rpm at 30°C
for 18 hours. Bacterium cells were pelleted at 7500 g for 15 min in a refrigerated centrifuge (12 °C) and the supernatant was collected. Chloroform was added to the supernatant (1:1), stirred vigorously for 20 min, and centrifuged at 10400 g (12 °C) for 30 min. The precipitated and interface solids were collected. The solids were dried in a chemical hood overnight. Finally, the solids were suspended in 1 ml of Tris buffer (0.1 mol/L, 5±10 ml, pH 7.0) with agitation overnight at 8 °C. The extraction suspended in 1 ml of Tris buffer was filtered with a 0.20 μm membrane filter, and the filtered sample solution was used for LC-MS/ MS analysis. The identification and quantification of analyses were carried out using an HPLC system (1200 series, Agilent, Santa Clara, CA) and triple quadruple mass spectrometer (Agilent 6410), equipped with an electrospray ionization source, binary pump, degasser, and autosampler.

Chromatographic analysis was performed on a CW-C18 column (50 × 2 mm, 3 μm; Imtakt, Portland, OR), and the column oven temperature was held at 40°C. The mobile phase consisted of 0.1% formic acid (A) and 100% acetonitrile (B), and gradient elution was applied as follows: A:B (80:20) for 0.5 min; reaching A:B (10:90) over 6 min; A:B (10:90) for 2 min; reaching A:B (80:20) over 8.1 min; return to the initial conditions and re-equilibrate the column for 4 min. The injection volume was set at 10 μL, and the flow rate of the mobile phase was 0.3 mL/min. Instrumental source parameters, such as nebulization, drying gas temperature, drying gas flow, and spray voltage, were optimized by injecting nisin standard solution (1 μg/mL) in the mobile phase at a flow rate of 10 μL/min as follows: drying gas flow, 6 L/min; drying gas temperature, 300°C; spray voltage, 4,000 V; and nebulization, 40 psi. For quantitative purposes, LC-MS/MS analysis was performed with positive mode electrospray ionization and the multiple reaction-monitoring
parameters for the determination of nisin A and nisin Z content in the fermentation. The parent ions were initially determined by a full scan, and the optimal voltages of fragmentor were determined when the parent ions had the highest intensity. The fragment ions were also selected through scanning and the optimal settings of collision energy were achieved when fragment ions had the highest intensity. During MS/MS analysis, we used nitrogen as the nebulization and collision gas, and the run time was 12 min. Data were analyzed with Agilent 6410 Quantitative analysis version (B.02.01) analyst data-processing software.

Results

Characterization of *L. lactis* subsp. *lactis* wild-type strain

The fermentation profiles of the *L. lactis* subsp. *lactis* wild-type strain were determined at different time points. The nisin titer was 324 IU/ml after 18 hours of culture. Further, the cell growth reached a maximum of 1.81 of optical density after 12 hours. Additionally, there was a strong reduction in pH which had a value of 4.5 upon finishing the fermentation process (Fig 1).

Further, the expression of the genes involved in nisin biosynthesis in the wild-type strain of *L. lactis* subsp. *lactis* were evaluated. The majority of the nisin biosynthetic genes had variable transcription levels. The *nisI*, *nisF*, and *nisG* genes showed higher levels of expression. However, the *nisR* and *nisK* genes had a low expression in the different time points analyzed. Also, the *nisA* gene showed a reduced expression (Fig 2). According to these results, the genes involved in nisin pre-peptide synthesis (*nisA*) and
two-component signal transduction (nisRK) were selected for further genetic transformation in the *L. lactis* subsp. *lactis* wild-type strain.

**Fig. 1. Evaluation of different parameters during the fermentation of the *L. lactis* subsp. *lactis* strain wild type.** (A) Temporal growth profile. (B) pH of the culture. (C) Nisin A production. The bars represent means of three independent replicates ± standard error (P<0.05).

**Fig. 2. Transcriptional analysis of genes involved in the biosynthesis of nisin in the *L. lactis* subsp. *lactis* strain using qRT-PCR.** For each condition, reactions were done in triplicate in three separate experiments. To standardize cycle threshold (Ct) results, the 16S rRNA gene was used as an internal control. The relative levels of genes were expressed as ‘mean normalized expression’ data using Q-Gene software [36]. The bars represent means of three independent replicates ± standard error (P<0.05).

**Serial genetic re-transformations to nisin production.**

We designed an approach based on a re-transformation of the *nisA* and *nisRK* genes into the wild-type *L. lactis* subsp. *lactis* strain. The transcription levels of *nisA* and *nisRK* genes were higher in modified *L. lactis* subsp. *lactis* strains generated following a single transformation than in the original strain (Fig 3). However, the relative expression was significant highest in re-transformed *L. lactis* subsp. *lactis* strains compared with the single transformed and wild-type strain, respectively (Fig 3).
Fig. 3. Transcriptional analysis of the *nisA*, *nisR*, and *nisK* genes in the wild-type and engineered *L. lactis* subsp. *lactis* strains using qRT-PCR. The relative expression values were determined regarding the 16S rRNA gene used as reference internal gene. The relative levels of genes were expressed as ‘mean normalized expression’ data using Q-Gene software [36]. The bars represent means of three independent replicates ± standard error (P<0.05).

The growth of the engineered *L. lactis* subsp. *lactis* strains was determined through optical density at 600 nm. The engineered *L. lactis* subsp. *lactis* pMG36e:*nisA*, pMG36e:*nisA* RT, pMG36e:*nisRK*, and pMG36e:*nisRK* RT strains displayed a similar cell growth density compared with the wild-type strain. There was no effect of the over-expression of these genes on cell growth (Fig 4A). A high significant number of copies of *nisA* genes were obtained in the engineered pMG36e:*nisA* RT and pMG36e:*nisRK* RT strains compared with single transformed and wild-type strain, respectively (Fig 4B).

Fig. 4. Characterization of the *L. lactis* subsp. *lactis* wild-type and engineered strains. (A) cell growth was evaluated through of optical density OD600 after 18 hours of fermentation. (B) the value data were determined in terms of copies of the *nisA* and *nisRK* genes / µL of qPCR reaction. (C) the nisin A quantification was determined by activity assay in the *L. lactis* subsp. *lactis* wild-type and engineered strains. The bars represent means of three independent replicates ± standard error (P<0.05).
The nisin productivity of the original strain and the engineered strains (pMG36e:nisA, pMG36e:nisA RT, pMG36e:nisRK, and pMG36e:nisRK RT) were evaluated in shake flasks after 18 h using agar diffusion activity assays. The wild-type and transformed strain single and three-fold transformations with empty plasmid exhibited similar nisin production, suggesting that the introduction of empty plasmid pMG36e did not affect nisin production. Nevertheless, the pMG36e:nisA and pMG36e:nisRK strains showed a high production of nisin, with 442.5 IU/ml and 369.2 IU/ml, respectively, after 18 hours, compared with the wild-type strain 169.3 IU/ml (Fig 4C).

Among these engineered strains, pMG36e:nisA RT (1111.5 IU/ml) and pMG36e:nisRK RT (1041.5 IU/ml) had the greatest increments in nisin production (Fig 4C). These results indicated that the strains engineered through re-transformation had a positive effect on nisin yield. Also, with or without erythromycin selection pressure, the pMG36e genetic stable percentage of retention in L. lactis subsp. lactis was about 98 % after 120 generations of continuous incubation (S3 Fig).

Co-expressing nisA and nisZ genes.

To verify the advantages of the co-expression of nisA and nisZ genes in terms of biological activity, we compared them with the original strain. Antimicrobial assays were done with engineered L. lactis subsp. lactis pMG36e:nisA, pMG36e:nisZ and pMG36e:nisA + pMG36e:nisZ constructs. The L. lactis subsp. lactis strain producing nisin A and nisin Z was verified by RNA sequencing and mass spectrometry, respectively. For biological activity, a strain selected with similar transcript expression levels from
the nisA and nisZ genes was used. Additionally, LC-MS/MS analysis showed the production of nisin A and Z in the same selected strain at similar concentration. All the strains inhibited the \textit{M. luteus} indicator strain through antagonism bioactivity assays. However, the strain expressing nisA and nisZ genes had the highest inhibitory effect on indicator strain (Fig 5). In terms of bacteriostatic activity against \textit{M. luteus}, co-expression of nisin A and Z was superior to the original strain.

\textbf{Fig. 5. Antimicrobial activity of \textit{L. lactis} subsp. \textit{lactis} strains expressing nisA, nisZ and nisA + nisZ genes against \textit{M. luteus}.} The plates' holes were filled with 150 ml of pasteurized supernatant from overnight cultures of \textit{L. lactis} subsp. \textit{lactis} strains. (A) Inhibition area; (B) inhibition diameter in millimeters. The bars represent means of three independent replicates ± standard error (P<0.05).

\section*{Discussion}

The parameters of cell growth and pH during fermentation remained adequate in the wild-type \textit{L. lactis} subsp. \textit{lactis} strain. A reduction in pH typically associated with lactic acid production was observed at the end of fermentation, which is closely related to nisin production. Although the wild-strain of \textit{L. lactis} subsp. \textit{lactis} produced certain levels of nisin at the end of fermentation by shake flask culture, the levels were low compared to previous work where other wild-strain of \textit{L. lactis} subsp. \textit{lactis} were used \cite{29, 38, 39}. To look for the causes of these low levels, a molecular characterization through qPCR was
developed with the aim of determining how the set of genes involved in nisin production were expressed.

Different approaches for understanding nisin biosynthesis and its metabolism have been evaluated [18, 39, 40, 41]. It is important to note that each of these genes should not be analyzed in a separate context. However, the *nisR* and *nisK* gene expression was significantly low in the wild-type *L. lactis* subsp. *lactis* strain. The *nisA* and *nisRK* genes play an important role in the synthesis and regulation of nisin [29, 38, 42]. It was evident that the expression of these genes was limited and could explain in some way the low production of nisin obtained. This has been demonstrated in other strains of *L. lactis* subsp. *lactis* how the increasing of these genes could enhance nisin production [39]. Specifically, when the produced nisin induces the transcription of the nisin biosynthetic gene cluster using two-component signal transduction where *nisRK* genes display an important role [18, 19, 40].

Given this, increasing the expression of these genes could in principle improve the production of nisin in the wild-type strain. Consequently, we increased the expression of these genes by introducing multi copies of *nisA* and *nisRK* genes in *L. lactis* subsp. *lactis*. To increase the expression of nisin, the *nisA* and *nisRK* genes were cloned in a high-copy-number vector. The resulting plasmids, pMG36e:*nisA* and pMG36e:*nisRK*, were introduced into *L. lactis* subsp. *lactis* through single- and three-fold genetic re-transformation. The results showed how it is possible to first increase the gene expression and then the production of nisin. The highest expression profiles were detected in the engineered strains obtained through the re-transformation of the wild-type strain compared with the single transformation wild-type strain. Our data support the idea that
high expression levels of the nisA and nisRK genes in the engineered strains increase nisin production. We successfully achieved a high production of nisin without any influence over the growth and stability of the engineered L. lactis subsp. lactis strains in the shake flasks. Interesting, in L. lactis subsp. lactis, pMG36e demonstrated high genetic stability. The genetic stability of pMG36e was not affected by erythromycin selection pressure. Previously, the stability of pMG36e plasmid was demonstrated in L. lactis M4 strain [43].

On the other hand, the cell growth of high-yield engineered strains had no differences compared with the control strains. There was no toxicity effect produced by the presence of the multi copies of these genes, including the engineered strains obtained by re-transformation. The results provide an approach for improving nisin production. Additional studies related to the optimization of fermentation conditions in engineered strains in scale production should be developed.

Diverse efforts have been developed to increase nisin activity [35, 44, 45]. Combinations with other bacteriocins have allowed an increase in activity [35, 45]. Additionally, the combination with compounds that weaken the cell wall of Gram-negative bacteria such as E. coli has increased the spectrum of action [35, 46, 47]. In this context, the differences between nisin A and nisin Z are minimal [14]. Within these differences, different amino acids in position 27 and the ability of nisin Z to achieve a higher level of inhibition with respect to nisin A, mainly due to its ability to have a greater degree of diffusion in agar with respect to nisin A, are the most important [14].

An interesting point was how the behavior of L. lactis subsp. lactis would be expressing nisin A and nisin Z simultaneously. The results suggest that there is a
potentiation of activity when the two nisin are produced in the same strain of *L. lactis* subsp. *lactis*, compared to those that are expressed separately. There was a positive effect between them, which contributes to better activity in terms of inhibition activity.

Several analyses have shown that nisin has additive or synergistic effects when used with other antimicrobial compounds [33, 34]. However, there was no evidence available about the antibacterial activity of the *L. lactis* subsp. *lactis* strain co-expressing nisin A and nisin Z. Our findings show that co-expression of these two bacteriocins with minor variations resulted in extremely effective antibacterial action against *M. luteus*.

Finally, many efforts have been made to seek strategies that allow the increase in the production of nisin. Within these, the increase in the expression of genes related to the biosynthesis of nisin were extensively addressed. Our work, although focused on this issue, introduces the strategy of genetic retransformation for an increase in the number of significant copies, without affecting stability and with a marked increase in the yield of nisin. On the other hand, it was interesting to evaluate the biological effect of the expression of nisA and nisZ genes in the same strain of *L. lactis*. It is well known as the amino acid difference at position 27 and the greater agar diffusion capacity of nisin Z compared to nisin A. This was demonstrated, however it was curious to observe how this strain producing the same concentration of nisin A and Z, had a greater inhibitory effect. Further studies will be necessary to understand the reason for this increase in activity and behavior during scaled-up in fermenters.

References


[8] Garrigues C, Loubiere P, Lindley ND, Cocaign-Bousquet M. Control of the shift from homolactic acid to mixed-acid fermentation in Lactococcus lactis: predominant role...


**Supporting information**

S1 Fig. Schematic representation of the strategies of re-transformation and expression of \textit{nisA} and \textit{nisRK} genes in \textit{L. lactis}.

S2 Fig. Schematic representation of the strategies of co-expression of \textit{nisA} and \textit{nisZ} genes in \textit{L. lactis}.

S3 Fig. Evaluation of genetic stability of the pMG36e plasmid expressing of \textit{nisA} and \textit{nisRK} genes in \textit{L. lactis}.
A cell growth (OD 600)

B pH

C nisin titer (IU/ml)
Figure

A. Cell growth (OD 600) for control, pMG36e:nisA, pMG36e:nisA RT, pMG36e:nisRK, and pMG36e:nisRK RT.

B. Copies/μl for control, pMG36e:nisA, pMG36e:nisA RT, pMG36e:nisRK, and pMG36e:nisRK RT. Significance levels: ***P<0.05.

C. Nisin titer (IU/ml) for control, pMG36e:nisA, pMG36e:nisA RT, pMG36e:nisRK, and pMG36e:nisRK RT. Significance levels: ***P<0.05.
A

\[ \text{nisA} \quad \text{nisZ} \quad \text{nisA + nisZ} \]

\[ 21.486 \quad 22.228 \text{ mm} \quad 23.712 \text{ mm} \]

B

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<tr>
<th>diameter of the inhibition zone</th>
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<tr>
<td>30</td>
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\[ \text{nisA} \quad \text{nisZ} \quad \text{nisA + nisZ} \]

Figure