Effect of *alsD* deletion and overexpression of *nox* and *alsS* on diacetyl and acetoin production by *Lactocaseibacillus casei* during milk fermentation

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ABSTRACT

Diacetyl and acetoin are key aroma components of fermented milk but are produced in low concentrations by starter cultures. In this study, we expressed NADH oxidase, acetolactate synthase, and inactivated acetolactate decarboxylase in *Lactocaseibacillus casei* TCS to generate recombinant *L. casei* strains, and investigated the effects of the genes encoding these enzymes on diacetyl and acetoin production during milk fermentation. In the single-gene recombinant strains tested, diacetyl concentrations were highest in milk fermented by *L. casei* TCSI-*nox* (*nox* gene overexpressed, 3.68 mg/kg), whereas acetoin concentrations were highest in milk fermented by *L. casei* TCS- Δ *alsD* (*alsD* gene deleted, 32.94 mg/kg). Moreover, diacetyl and acetoin concentrations were higher in the inducible strains than in the corresponding constitutive strains (e.g., TCSI-*nox* vs. TCSC-*nox*, and TCSI- Δ *alsD*-*nox* vs. TCSC- Δ *alsD*-*nox*). This phenomenon was also reflected in the protein expression levels and enzyme activities. In the double-gene recombinant strains tested, the highest concentrations of diacetyl and acetoin were produced by *L. casei* TCSI- Δ *alsD*-*nox* (*nox* overexpressed and *alsD* deleted, 4.66 mg/kg, 69.62 mg/kg, respectively). The triple-gene recombinant *L. casei* TCS- Δ *alsD*-*nox*-*alsS* produced the highest concentrations of diacetyl and acetoin, which were 2.38 and 11.19 times, respectively, the concentrations produced by the original strain. These results show that the *nox*, *alsS*, and *alsD* genes make key contributions to the biosynthesis of diacetyl and acetoin by *L. casei*. The modification of multiple genes had a synergistic effect, leading to greatly in-

creased synthesis of diacetyl and acetoin by *L. casei* during its fermentation of milk.

Key words: fermented milk, gene knockout, overexpression, diacetyl, acetoin

INTRODUCTION

Fermented milk is a dairy product made by fermenting raw bovine, caprine, or ovine milk, or the corresponding powdered milk, with lactic acid bacteria (LAB) as the starter cultures (Tamime, 2002). Fermented milk is one of the most popular fermented dairy products globally, due to its texture, nutrition, and flavor (Shiby and Mishra, 2013; Chen et al., 2017). Flavor plays a vital role in determining consumers' acceptance of and preference for fermented milk (Güler, 2007). The acknowledged and key compounds contributing to the flavor profile of fermented milk are diacetyl and acetoin (Sodini et al., 2004), as these contribute the characteristic milky and buttery flavors to fermented milk (Cheng, 2010; Chiocchetti et al., 2019).

Diacetyl and acetoin can be synthesized by microorganisms during fermentation (Sieuwerts et al., 2008; Chen et al., 2017; Figure 1). The biosynthetic precursors for diacetyl and acetoin are usually citric acid and glucose. Glucose is converted to pyruvate via glycolysis, whereas citric acid produces oxaloacetic acid by the catalytic action of citrate glucose and then pyruvate is generated by decarboxylation (Smit et al., 2005). Acetolactate synthase (ALS) then catalyzes the synthesis of α -acetolactate from pyruvate (Zhang et al., 2013). Diacetyl is generated from α -acetolactate through non-enzymatic oxidative decarboxylation under acidic conditions. Acetoin is generated either from α -acetolactate by α -acetolactate decarboxylase or from diacetyl by diacetyl reductase. Diacetyl and acetoin are either excreted as end products or partially reduced to butanediol catalyzed by acetoin reductase (Papagianni, 2012; Yang et al., 2017). The application and development of genomic and metabolomic technologies have enabled

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Figure 1. Simplified pathway of diacetyl and acetoin biosynthesis in lactic acid bacteria. NOX = NADH oxidase; ALS = acetolactate synthase; ALDC = acetolactate decarboxylase; LDH = lactate dehydrogenase; OAD = oxaloacetate decarboxylase; NOD = non-enzymatic oxidative decarboxylation. Red boxes represent the key flavor compounds; words in red represent key enzymes in the biosynthetic pathway.

study of the key genes involved in the biosynthesis of diacetyl and acetoin (Liu et al., 2008; Sieuwerts et al., 2010).

There is current interest in enhancing the flavor of food by the endogenous generation of flavor compounds rather than by the exogenous addition of such compounds. Because the flavor of fermented milk is mainly produced by metabolism of LAB, their production of diacetyl and acetoin has attracted much research attention (Lo et al., 2018). The genera *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* (Liu et al., 2011; D'Angelo et al., 2017; Wang et al., 2019b; Raveschot et al., 2020) have all shown potential to produce diacetyl and acetoin during fermented milk production. In a previous study, we

used a high-throughput screening method to identify *Lacticaseibacillus casei* TCS, an *L. casei* strain that has high diacetyl and acetoin production capacity (Tian et al., 2019). Thus *L. casei* TCS can be used as a parent strain for genetic modification and as a starter culture to improve the aromatic profile of fermented milk.

To increase the production of diacetyl and acetoin by *L. casei* during fermented milk production, this study examined the influences of 3 genes: *nox*, *alsS*, and *alsD*. Specifically, overexpression and gene knockout strategies were used to make single- and multiple-gene modifications to determine the influences and synergistic effects of these genes on diacetyl and acetoin production. The results of this study clarify the biosynthesis of diacetyl and acetoin by *L. casei* and provide a theoretical

basis for the future optimization of flavor production in fermented milk.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in Table 1. *Lacticaseibacillus casei* TCS was used as the host strain for genetic modification, and *Escherichia coli* TOP10 was used as the host for cloning. *Lacticaseibacillus casei* TCS and the recombinant strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth (Merck) at 37°C without aeration. The *E. coli* strains were cultured in Luria Bertani broth (Merck) at 37°C with aeration at 200 rpm. When appropriate, the following antibiotics were added: 200 µg/mL erythromycin and 50 µg/mL kanamycin for *E. coli*, and 10 µg/mL erythromycin for recombinant *L. casei* strains, as described in our previous studies (Chen et al., 2017, 2020). The primers used in this study are listed in Table 2.

Construction of Recombinant Strains Overexpressing the *nox* and *alsS* Genes

The *nox* gene was amplified by PCR using *Streptococcus mutans* chromosomal DNA as a template, due to the high NADH oxidase activity of this strain (Li et al., 2018). The *alsS* gene was amplified by PCR using *L. casei* TCS chromosomal DNA as a template. The constructed recombinant strains had either constitutive or inducible expression. In those with constitutive expression, the *nox* and *alsS* genes were amplified using the primer pairs *nox*-F_p/*nox*-R_p and *alsS*-F_p/*alsS*-R_p, respectively. The PCR products were purified using the TIANquick Midi Purification Kit (Tiangen Biotech Co. Ltd.) and then cloned into plasmid pIB184 using a One Step Cloning Kit (Vazyme Biotech Co. Ltd.) to construct a constitutive expression plasmid. After transformation into *E. coli*, the constructs were verified by PCR using the primers pIB184-F and pIB184-R, and their construction was confirmed by DNA sequencing. The constructed plasmids were named pIB184-*nox* and pIB184-*alsS*. The recombinant plasmids were then

Table 1. Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics ¹	Source ²
Strains		
<i>Escherichia coli</i>		
<i>E. coli</i> DH5α	Cloning host	Promega
<i>E. coli</i> Top10	Cloning host	Takara
Top/p1628-RFP	Kana-r, <i>E. coli</i> Top10 containing p1628-RFP	Song et al., 2017
DH5α/ <i>nox</i> -409	Em-r, <i>E. coli</i> DH5α containing pSIP409- <i>nox</i>	This work
DH5α/ <i>nox</i> -184	Em-r, <i>E. coli</i> DH5α containing pIB184- <i>nox</i>	This work
DH5α/ <i>alsS</i> -409	Em-r, <i>E. coli</i> DH5α containing pSIP409- <i>alsS</i>	This work
DH5α/ <i>alsS</i> -184	Em-r, <i>E. coli</i> DH5α containing pIB184- <i>alsS</i>	This work
Top10/Up-Down-gRNA	Kana-r, <i>E. coli</i> Top10 containing p-Up-Down-gRNA	This work
<i>Lacticaseibacillus casei</i>		
TCS	Wild-type <i>Lacticaseibacillus casei</i>	CCTCC no. M2017364
TCS-Δ <i>alsD</i>	In-frame deletion of <i>alsD</i> in <i>L. casei</i> TCS	This work
TCSI- <i>nox</i>	Em-r, <i>L. casei</i> TCS containing pSIP409- <i>nox</i> plasmid	This work
TCS- <i>nox</i>	Em-r, <i>L. casei</i> TCS containing pIB184- <i>nox</i> plasmid	This work
TCSI- <i>alsS</i>	Em-r, <i>L. casei</i> TCS containing pSIP409- <i>alsS</i> plasmid	This work
TCS- <i>alsS</i>	Em-r, <i>L. casei</i> TCS containing pIB184- <i>alsS</i> plasmid	This work
TCSI-Δ <i>alsD</i> - <i>nox</i>	Em-r, <i>L. casei</i> TCS-Δ <i>alsD</i> containing pSIP409- <i>nox</i> plasmid	This work
TCS-Δ <i>alsD</i> - <i>nox</i>	Em-r, <i>L. casei</i> TCS-Δ <i>alsD</i> containing pIB184- <i>nox</i> plasmid	This work
TCSI-Δ <i>alsD</i> - <i>alsS</i>	Em-r, <i>L. casei</i> TCS-Δ <i>alsD</i> containing pSIP409- <i>alsS</i> plasmid	This work
TCS-Δ <i>alsD</i> - <i>alsS</i>	Em-r, <i>L. casei</i> TCS-Δ <i>alsD</i> containing pIB184- <i>alsS</i> plasmid	This work
TCS-Δ <i>alsD</i> - <i>nox</i> - <i>alsS</i>	Em-r, <i>L. casei</i> TCS-Δ <i>alsD</i> containing pSIP409- <i>nox</i> - <i>alsS</i> plasmid	This work
Plasmids		
pSIP409	Em-r, cloning vector derived from the plasmid pSIP401	Sørvig et al., 2005
pIB184	Em-r, cloning vector	Li et al., 2015
pLCNICK-1628	Kana-r, cloning vector derived from the plasmid pIB184	Song et al., 2017
pSIP409- <i>nox</i>	Em-r, pSIP409 derivative, <i>gusA</i> replaced by <i>nox</i>	This work
pIB184- <i>nox</i>	Em-r, pIB184 derivative, <i>gusA</i> replaced by <i>nox</i>	This work
pSIP409- <i>alsS</i>	Em-r, pSIP409 derivative, <i>gusA</i> replaced by <i>alsS</i>	This work
pIB184- <i>alsS</i>	Em-r, pIB184 derivative, <i>gusA</i> replaced by <i>alsS</i>	This work
pSIP409- <i>nox</i> - <i>alsS</i>	Em-r, pSIP409 derivative, <i>gusA</i> replaced by <i>nox</i> and <i>alsS</i>	This work
p-Up-Down-gRNA	Plasmid derived from PCR inserts containing upstream and downstream <i>alsD</i> genes and targeting RNA	This work

¹Kana-r = kanamycin resistant; Em-r = erythromycin resistant.

²Promega, Madison, WI; Takara, Tokyo, Japan; CCTCC = China Center for Type Culture Collection, Wuhan, China.

transformed into competent *L. casei* TCS cells by electroporation. The recombinant strains were verified by PCR with the corresponding primers (pIB184-F and pIB184-R) and named *L. casei* TCSC-*nox* and *L. casei* TCSC-*alsS*.

Similarly, to generate recombinant bacteria with inducible expression, the *nox* and *alsS* genes were amplified using the primer pairs *nox*-F₄/*nox*-R₄ and *alsS*-F₄/*alsS*-R₄, respectively. Inducible expression plasmids were then constructed by cloning the PCR products into plasmid pSIP409 using the One Step Cloning Kit (Vazyme). Transformation was performed according to the method described previously. The resulting plasmids, named pSIP409-*nox* and pSIP409-*alsS*, were verified by PCR using the primers 403-F and 403-R. The recombinant plasmids were then transformed into *L. casei* TCS cells, and their transformation was verified by repeating the steps previously described. The resulting recombinant strains were named *L. casei* TCSI-*nox* and *L. casei* TCSI-*alsS*.

Construction of Recombinant Strain with the *alsD* Gene Knocked Out

A knockout vector pLCNICK-1628 was constructed using the clustered regularly interspaced short palin-

dromic repeats (CRISPR) gene editing system, according to the method described by Song et al. (2017), with some modifications.

The pLCNICK-1628 plasmid was double-digested with endonucleases *ApaI* and *XbaI*, and the resulting vector fragment was recovered by electrophoretic separation and gel excision. The upstream and downstream sequences of the *L. casei* TCS *alsD* gene were amplified by PCR using *L. casei* TCS DNA as a template. The sgRNA fragment was obtained by PCR amplification of pLCNICK-1628, using the primers 2013gRNA-F and 2013gRNA-R. The upstream and downstream sequences and the sgRNA fragment were ligated to the digested vector fragment using the One Step Cloning Kit (Vazyme). After transformation into *E. coli* TOP10, plasmids were extracted from positive transformants through colony PCR, and verified by enzymatic digestion and sequencing. The transformed plasmid was named p-Up-Down-gRNA, and the recombinant strain was named *E. coli* TOP10/Up-Down-gRNA. The recombinant plasmid, p-Up-Down-gRNA, was then electrotransformed into competent *L. casei* TCS cells. Colony PCR with the primer pairs 2013yz-F/2013yz-R and TCSyz-F/TCSyz-R was used to verify that the recombinant plasmid had been incorporated into *L. casei* TCS and that the *alsD* gene had been knocked out of

Table 2. Primers used in this study; F = forward; R = reverse

Primer	Sequence (5'-3')
Primers for gene cloning	
<i>nox</i> -F ₄	ACAAGGAGATTTTAGCCATGGACATGAGTAAAATCGTTATTGTTGGAGC
<i>nox</i> -R ₄	CGGGGTACCGAATTCCTCGAGTCATTTAGCTTTTAATGCTGCTTTG
<i>nox</i> -F _p	AATGACAATGATGTTGGATCCATGAGTAAAATCGTTATTGTTGGAGC
<i>nox</i> -R _p	TTATCGATAGATCTCGAGCTCTCATTTAGCTTTTAATGCTGCTTTG
<i>alsS</i> -F ₄	ACAAGGAGATTTTAGCCATGGATGGCAGAAGAGAAGCGCTATG
<i>alsS</i> -R ₄	CGGGGTACCGAATTCCTCGAGAAATGCTTCTGCATACTTCACAAAG
<i>alsS</i> -F _p	AATGACAATGATGTTGGATCCATGGCAGAAGAGAAGCGCTATG
<i>alsS</i> -R _p	TTATCGATAGATCTCGAGCTCAAATGCTTCTGCATACTTCACAAAG
2013-up-F	ACATCTTTTTCTAACTAGGGCCCAACAACGAGAAAGACCAGCA
2013-up-R	GTAATAGGAGGAGACCATCTGTAAAGCCTTGTGAGAGGC
2013-down-F	GCCTCTCACAAGGCTTTAACAGATGGTCTCCTCCTATTAC
2013-down-R	GAGTCGGTGCTTTTTTTGAGATCATTTCTACCGTCAGAT
2013gRNA-F	CTCAAAAAAAGCACCGACTC
2013gRNA-R	AGAAAGGATGATATCACCTCTAGAACCGCACCCGCATAGAGATCGTTTTAGAGCTAGAA
<i>nox</i> -F ₄₋₁	ACAAGGAGATTTTAGCCATGGCTATGAGTAAAATCGTTATTGTTGGAGC
<i>nox</i> -R ₄₋₁	CCATCTAAAATCTCCTTGTCATTTAGCTTTTAATGCTGCTTTG
<i>alsS</i> -F ₄₋₁	GACAAGGAGATTTTAGATGGCAGAAGAGAAGCGCTATG
<i>alsS</i> -R ₄₋₁	CGGGGTACCGAATTCCTCGAGTCAAAATGCTTCTGCATACTTCACA
Primers for validation	
403-F	GAAATACCCGTCTAAGGAATTG
403-R	TGGTCATGAATTAGTCTCGGA
pIB184-F	TTACAATTGTCATCACCATTCTTTG
pIB184-R	TAGAATTAAATTAAGGGAGGCC
TCS-F	CTGGTTGCAAAATTTTGAAAACCGCTA
TCS-R	ACCGTTACATATCAAAGGGAAAACCTGTC
2013yz-F	CTACCAAGTGTGCGGTGTCAAATTT
2013yz-R	TGATCGGTTATATCAACATGGCACA
TCSyz-F	CAGTTCAGTCCCTGAAAAAGAAAAA
TCSyz-R	GTCCCAGTGATATGACGTATCTGGC

the genome of *L. casei* TCS. The knockout strain was named *L. casei* TCS- Δ *alsD*.

Construction of Recombinant Strains of *L. casei* with Multiple-Gene Modifications

To build recombinant strains with a single-gene overexpression based on *alsD* gene deletion, the recombinant plasmids (pSIP409-*nox*, pSIP409-*alsS*, pIB184-*nox*, and pIB184-*alsS*) described earlier were transformed by electroporation into competent cells of TCS- Δ *alsD*, an *L. casei* TCS-derived *alsD*-knockout strain. Recombinant strains were selected and named TCSI- Δ *alsD*-*nox*, TCSC- Δ *alsD*-*nox*, TCSI- Δ *alsD*-*alsS*, and TCSC- Δ *alsD*-*alsS*.

To coexpress the *nox* and *alsS* genes in a single vector based on *alsD* gene deletion, a ribosome-binding site was introduced into the 5'-terminus of the *alsS* gene, and the *nox* and *alsS* fragments (generated using the primer pairs *nox*-F₄-1/*nox*-R₄-1 and *alsS*-F₄-1/*alsS*-R₄-1, respectively) were then ligated into a vector (pSIP409) using the One Step Cloning Kit (Vazyme). The recombinant plasmid was electrotransformed into competent TCS- Δ *alsD* cells, as described previously, and the recombinant strain was verified by sequencing. The constructed strain that coexpressed the *nox* and *alsS* genes and had its *alsD* gene knocked out was named *L. casei* TCS- Δ *alsD*-*nox*-*alsS*.

Expression of Recombinant Bacterial Proteins

Protein expression was performed according to our previously described method (Chen et al., 2014). Recombinant strains with inducible gene expression (TCSI-*nox*, TCSI- Δ *alsD*-*nox*, TCSI-*alsS*, TCSI- Δ *alsD*-*alsS*, and TCS- Δ *alsD*-*nox*-*alsS*) were inoculated into 100 mL of MRS broth containing 10 µg/mL erythromycin, and the resulting mixture was cultured at 37°C until it reached an optical density value at 600 nm (OD₆₀₀) of 0.3 to 0.5. An inducer (IP-673) was added to the culture to give a concentration of 25 ng/mL, and the culture was incubated at 37°C until an OD₆₀₀ value of 1.5 was reached. After induction, the cells were harvested by centrifugation (8,000 × *g*, 4°C, 5 min), washed with sterile buffer (50 mM Tris/HCl, pH 8.0), resuspended in the same buffer supplemented with 25 mg/mL lysozyme, and then sonicated. Cellular debris was removed by centrifugation (12,000 × *g*, 4°C, 10 min), and the supernatant containing the soluble proteins was collected. The whole culture, including the supernatant and cell pellets before and after induction, was collected, and the expression of the recombinant proteins NADH oxidase (NOX) and ALS, was analyzed via SDS-PAGE (12.0%, wt/vol). Various IP-673 concentrations (25 and

50 ng/mL), induction temperatures (16, 25 and 37°C), and induction times (OD₆₀₀ = 1.2, 1.5, and 1.8) were used to determine which conditions led to the optimal expression of NOX and ALS.

Enzyme Activity Determination

Activity of ALS was determined according to the method described by Holtzclaw and Chapman (Holtzclaw and Chapman, 1975), with some modifications. Briefly, the assay was performed in a mixture comprising 1 mL of 1 mM thiamine pyrophosphate, 5 mM MgCl₂, 0.1 mM flavin adenine dinucleotide, 100 mM sodium pyruvate, and 300 µL of crude enzyme solution. This mixture was heated at 37°C for 1 h, at which time 200 µL of 3 M H₂SO₄ was immediately added to terminate the reaction. After decarboxylation in a water bath at 60°C for 15 min, 8.5 mL of a coloring solution (5% α-naphthol, 0.5% creatine, 5% sodium hydroxide, and deionized water) was added. The resulting mixture was incubated for an additional 30 min at 37°C to allow for the acid hydrolysis of acetolactate to acetoin. Finally, the absorbance was measured at 520 nm using a spectrophotometer (Unico Instrument Co. Ltd.). One unit of enzyme activity is defined as the amount of enzyme that converts 1 nmol of substrate into product in 1 min under these conditions (Holtzclaw and Chapman, 1975).

Activity of NOX was measured using an NADH Oxidase Activity Detection Kit (Solarbio Science and Technology). One unit of enzyme activity is defined as a change in the 600-nm absorbance of a reaction system of 0.01 per min per 10,000 bacterial cells (Kawai et al., 2004).

Preparation of Fermented Milk

Fermented milk was produced using a previously described method, with some modifications (Tian et al., 2020). Various *L. casei* strains were revitalized in MRS broth and routinely cultured for 12 to 16 h at 37°C. The cells were then harvested by centrifugation at 6,000 × *g* for 10 min at room temperature, washed twice with sterile water, and then resuspended in a volume of sterile water equal to the volume of the medium. The resulting suspension was used as the starter culture.

A skim milk solution was prepared with 12% (wt/vol) skim milk powder (Fonterra), and the reconstituted milk was pasteurized according to the method described by Fernandez-Garcia and McGregor (1994). The milk was inoculated with 4% (wt/vol) starter cultures of *L. casei* strains and stirred thoroughly. Cells of the inducible recombinant strains (TCSI-*nox*, TCSI-*alsS*, TCSI- Δ *alsD*-*nox*, TCSI- Δ *alsD*-*alsS*, and TCS-

$\Delta alsD$ -*nox-alsS*) were induced by adding IP-673 to give a final concentration of 25 ng/mL. After inoculation, the samples were sealed in 100-mL glass bottles using polypropylene caps. The bottles were transferred to a thermostatically controlled water bath (37°C), where they were incubated until they reached a pH of 4.6. The resulting fermented samples were transferred to a refrigerator set to 4°C for ripening.

Determination of Diacetyl and Acetoin Concentrations

Gas chromatography spectrometry was used to measure diacetyl and acetoin concentrations, as described previously (Tian et al., 2017), with minor modifications. After ripening for 24 h, 5 g of a fermented milk sample was placed in a 15-mL extraction bottle equipped with a rotor, and 10 μ L of 2-octanol (internal standard, 19 mg/L) was added. The resulting mixture was balanced in a magnetic constant-temperature water bath at 55°C for 5 min, and the extraction head was then inserted for 40 min to enrich the volatile substances in the headspace. The chromatographic and mass spectrometric conditions for subsequent sample detection were the same as those previously described. All analyses were performed in triplicate.

Statistical Analysis

The presented data are representative of 3 independent experiments. Statistical analyses were performed by one-way ANOVA (with Duncan's multiple range method) using SPSS (version 19.0; IBM Corp.). Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Construction and Verification of Recombinant *L. casei* Strains

The inducible plasmid pSIP409 and the constitutive plasmid pIB184 were used to construct inducible and constitutive expression vectors, respectively, for the *nox* and *alsS* genes. Thus, 4 recombinant strains of *L. casei* with a single expressed gene were constructed; these were named TCSI-*nox*, TCSC-*nox*, TCSI-*alsS*, and TCSC-*alsS*. The *alsD* gene was knocked out using the clustered regularly interspaced short palindromic repeats (CRISPR) gene editing system, and the resulting recombinant *L. casei* strain was named TCS- $\Delta alsD$ (Table 1).

Recombinant strains with multiple genetic modifications were then constructed based on *L. casei* TCS-

$\Delta alsD$. Four strains of recombinant *L. casei* were constructed with double genetic modifications; these were named TCSI- $\Delta alsD$ -*nox*, TCSC- $\Delta alsD$ -*nox*, TCSI- $\Delta alsD$ -*alsS*, and TCSC- $\Delta alsD$ -*alsS* (Table 1). A recombinant strain that coexpressed *nox* and *alsS* and had its *alsD* gene knocked out was also constructed; this was named *L. casei* TCS- $\Delta alsD$ -*nox-alsS*. In total, 10 mutant strains were constructed, and the enzyme activity and diacetyl and acetoin yields of these mutant strains were measured and compared with those of the wild-type strain.

Protein Expression Levels of Recombinant *L. casei* Strains

The recombinant strains with inducible expression vectors, TCSI-*nox*, TCSI- $\Delta alsD$ -*nox*, TCSI-*alsS*, TCSI- $\Delta alsD$ -*alsS*, and TCS- $\Delta alsD$ -*nox-alsS*, were cultured in MRS broth until their OD₆₀₀ values reached 0.6. Induced and non-induced cells were harvested in the late stationary phase, and the cell extracts were analyzed by SDS-PAGE. The optimal induction conditions for the 4 recombinant strains were a temperature of 16°C and a final IP-673 concentration of 50 ng/mL. However, the optimal induction time varied between different strains. When *L. casei* TCSI-*nox* was induced for 59 h and *L. casei* TCSI- $\Delta alsD$ -*nox* was induced for 42 h, an intense protein band was seen at 45 to 60 kDa (Figure 2a, b), which is close to the expected size of recombinant NOX. The optimal induction times for *L. casei* TCSI-*alsS* and *L. casei* TCSI- $\Delta alsD$ -*alsS* were 60 h and 44 h, respectively (Figure 2c, d). The 2 recombinant strains expressing ALS also showed bands close to their expected size (58.87 kDa). The optimal induction conditions for the recombinant *L. casei* coexpressing NOX and ALS (TCS- $\Delta alsD$ -*nox-alsS*) were 37°C and a final IP-673 concentration of 25 ng/mL. This recombinant strain also showed unique bands at the expected sizes of 47.80 kDa (NOX) and 63.76 kDa (ALS; Figure 2e).

Enzyme Activity of Wild-Type and Recombinant Strains

The results of NOX and ALS activity measurements are shown in Figure 3. The NOX activities of the recombinant strains, TCSI-*nox*, TCSC-*nox*, TCSI- $\Delta alsD$ -*nox*, TCSC- $\Delta alsD$ -*nox*, and TCS- $\Delta alsD$ -*nox-alsS*, were 139.34, 13.66, 95.08, 9.78, and 8.63 times those of the wild-type strain, respectively. Activity of NOX was significantly higher in the recombinant strains with inducible NOX overexpression (TCSI-*nox* and TCSI- $\Delta alsD$ -*nox*) than in those with constitutive NOX expression (TCSC-*nox* and TCSC- $\Delta alsD$ -*nox*; $P < 0.05$). The ALS activity was also higher in the recombinant

Figure 2. Expression of recombinant proteins in various *Lactocaseibacillus casei* TCS. (1) NADH oxidase (NOX) expression in TCSI-*nox* (a) and TCSI- Δ *alsD*-*nox* (b), and acetolactate synthase (ALS) expression in TCSI-*alsS* (c) and TCSI- Δ *alsD*-*alsS* (d), as analyzed by SDS-PAGE. The optimal induction conditions for the 4 recombinant strains were 16°C and a final IP-673 concentration of 50 ng/mL. (2) NOX and ALS expression in TCS- Δ *alsD*-*nox*-*alsS* (e), as analyzed by SDS-PAGE. The optimal induction conditions for the recombinant strain coexpressing NOX and ALS (TCS- Δ *alsD*-*nox*-*alsS*) were 37°C and a final IP-673 concentration of 25 ng/mL. M = protein marker; 1, 2, and 3 = whole culture, supernatant, and cell pellets before induction, respectively; 4, 5, and 6 = whole culture, supernatant, and cell pellets after induction with IP-673, respectively; red box indicates the expected band. Strains are described in Table 1.

strains than in the original strain. However, although some differences were observed between the mutant and wild-type strains, enzyme activity did not significantly differ between the 5 recombinant strains (TCSC-*alsS*, TCSI-*alsS*, TCSC- Δ *alsD*-*alsS*, TCSI- Δ *alsD*-*alsS*, and TCS- Δ *alsD*-*nox*-*alsS*; $P > 0.05$).

Comparison of Diacetyl and Acetoin Yield in Milk Fermented by Various Recombinant *L. casei* Strains

Ten recombinant *L. casei* strains (TCS- Δ *alsD*, TCSI-*nox*, TCSC-*nox*, TCSI-*alsS*, TCSC-*alsS*, TCSI- Δ *alsD*-*nox*, TCSC- Δ *alsD*-*nox*, TCSI- Δ *alsD*-*alsS*, TCSC- Δ *alsD*-*alsS*, and TCS- Δ *alsD*-*nox*-*alsS*) and the wild-type strain (TCS) were used as starters for fermented milk production. Aromatic compounds were extracted from these samples, and diacetyl and acetoin were quantified by GC (Figure 4).

In general, the milk samples fermented by the mutant strains had higher concentrations of diacetyl and acetoin than those fermented by the wild-type strain. Of the strains with a single-gene modification, *L. casei* TCSI-*nox*, which had inducible *nox* gene overexpression, produced the highest diacetyl concentration (1.20 times the diacetyl concentration compared with strain TCS, Figure 4a). The acetoin concentration (Figure 4b) was highest in milk fermented by the recombinant *L. casei* TCS- Δ *alsD*, which had *alsD* knocked out. Milk fermented by this strain had 3.99 times the acetoin concentration of milk fermented by strain TCS. Milk fer-

mented by either of the 2 strains with inducible expression (TCSI-*nox* and TCSI-*alsS*) had higher diacetyl and acetoin concentrations than milk fermented by either of the 2 strains with constitutive expression (TCSC-*nox* and TCSC-*alsS*). For example, the diacetyl and acetoin concentrations in milk fermented with the recombinant *L. casei* TCSI-*nox* (with inducible *nox* gene expression) were 1.15 times and 1.28 times, respectively, those in milk fermented by *L. casei* TCSC-*nox* (with constitutive *nox* gene expression).

In strains with a double-gene modification, the diacetyl and acetoin concentrations were highest in milk fermented by *L. casei* TCSI- Δ *alsD*-*nox*, which had inducible *nox* gene expression and the *alsD* gene knocked out. The diacetyl and acetoin concentrations in milk fermented by this recombinant strain were 1.52 and 8.43 times, respectively, those in milk fermented by strain TCS. Furthermore, the acetoin concentrations were significantly higher in milk fermented by double-gene recombinant strains than in milk fermented by single-gene recombinant strains. For example, the acetoin concentration in milk fermented by *L. casei* TCSI- Δ *alsD*-*nox*, which had inducible *nox* gene expression and the *alsD* gene knocked out, was 1.31 times the sum of the acetoin concentration in milk fermented by *L. casei* TCS- Δ *alsD* (with *alsD* gene deletion) and *L. casei* TCSI-*nox* (with inducible *nox* gene expression). *Lactocaseibacillus casei* TCS- Δ *alsD*-*nox*-*alsS*, which had 3 genetic modifications, yielded the highest concentrations of diacetyl and acetoin of all tested strains, with

Figure 3. Activities of NADH oxidase (a) and acetolactate synthase (b) of wild-type and mutant strains of *Lactocaseibacillus casei* TCS. Strains are described in Table 1. One unit (U) of acetolactate synthase enzyme activity is defined as the amount of enzyme that converts 1 nmol of substrate into product in 1 min. One unit (U) of NADH oxidase enzyme activity is defined as a change in the absorbance at 600 nm (A_{600}) of a reaction system of 0.01 per min per 10,000 bacterial cells. Error bars represent SD based on 3 replicates. Values marked with different letters (a–d) are significantly different ($P < 0.05$).

Figure 4. Diacetyl (a) and acetoin (b) production in milk fermented by wild-type and mutant strains of *Lactocaseibacillus casei* TCS. Strains are described in Table 1. Error bars represent SD based on 3 replicates. Values marked with different letters (a–i) are significantly different ($P < 0.05$).

concentrations 2.38 times and 11.19 times higher, respectively, than those yielded by strain TCS.

DISCUSSION

Three genes, *nox*, *alsS*, and *alsD*, are known to be important for the biosynthesis of diacetyl and acetoin during the production of fermented milk (Wang et al., 2019a). However, the effects of these genes on diacetyl and acetoin production by *L. casei* have been unclear. In this study, we analyzed the synergistic effects of various modifications of these key genes in *L. casei* TCS on the yield of diacetyl and acetoin in fermented milk.

Recombinant strains with a single-gene modification produced more diacetyl and acetoin than the original strain during milk fermentation, and this was accompanied by increased expression levels and activities of key enzymes involved in the synthesis of diacetyl and acetoin. Of these recombinant strains, *L. casei* TCSI-*nox*, which had inducible *nox* gene overexpression, gave the highest yield of diacetyl in fermented milk and had the highest level of NOX activity. Glycolytic flux in LAB is affected by the redox balance between NAD^+ and NADH. It is known that NOX uses NADH to provide an additional pathway for the regeneration of NAD^+ under aerobic conditions, resulting in the diversion of pyruvate from the NADH-independent pathway and the formation of diacetyl (Lopez de Felipe and Gaudu, 2009; Guo et al., 2012). The overexpression of *nox* regulates the ratio of NADH/NAD^+ , which inhibits other pathways of pyruvate metabolism. Thus, *nox* gene overexpression directs a greater metabolic flow to pathways of diacetyl and acetoin biosynthesis, greatly increasing their production (Lopez de Felipe and Hugenholtz, 2001). In a similar study, Hugenholtz et al. (2000) changed an NADH/NAD^+ ratio by overexpressing the *nox* gene, which resulted in 80% of pyruvate being redirected toward the production of diacetyl and acetoin.

Furthermore, the recombinant *L. casei* TCSI-*alsS* (with inducible *alsS* gene expression) had higher levels of ALS activity and acetoin production than the original strain and the constitutive *L. casei* TCSC-*alsS*. In similar studies, Dorau et al. (2019) expressed ALS in a metabolically optimized *L. lactis* strain, which led to the efficient biotransformation of pyruvate into high titers of α -acetolactate. Gao et al. (2014) overexpressed the *alsS* gene in *Bacillus subtilis*, which led to increased production of diacetyl (0.57 g/L, compared with 0.01 g/L by non-overexpressed *B. subtilis*). In the current study, the highest acetoin concentration was found in milk fermented by the recombinant *L. casei* TCS- Δ *alsD*, which had its *alsD* gene knocked out. It is possible that knocking out the *alsD* gene inhibited the

reaction of α -acetolactate decarboxylase, which may have increased the metabolic flux of diacetyl (Zhang et al., 2015). This would have resulted in more diacetyl being reduced to the downstream product, acetoin (Xiao and Lu, 2014).

Diacetyl and acetoin concentrations were higher in milk fermented by double-gene recombinant strains than in milk fermented by the original strain or single-gene recombinant strains. For example, *L. casei* TCSI- Δ *alsD*-*nox*, which had inducible *nox* gene expression and the *alsD* gene knocked out, yielded the highest concentrations of diacetyl and acetoin. This indicates that the adjusted NADH/NAD^+ ratio resulting from knocking out *alsD* effectively increased metabolic flux for diacetyl and acetoin production. Hao et al. (2017) knocked out the *alsD* gene and simultaneously overexpressed the *alsS* gene in a *Bacillus* sp., resulting in a diacetyl yield of 1.94 g/L. Furthermore, similar to the results of single-gene modification, the recombinant strain with inducible expression produced higher concentrations of diacetyl and acetoin than the corresponding recombinant strains with constitutive expression, as evidenced by increased NOX and ALS activities.

When the 3 genes *nox*, *alsS*, and *alsD* were co-modified, the yields of diacetyl and acetoin in milk fermented with the resulting recombinant strain were much higher than the yields in milk fermented with strains with single or double genetic modifications. Thus, multiple genetic modifications had a synergistic effect on flavor enhancement. Consistent with previous reports (Hugenholtz et al., 2000; Hao et al., 2017), the overexpression of *nox* or *alsS*, as a result of knocking out *alsD*, led to recombinant strains that exhibited greatly increased diacetyl concentrations. Thus, changes in a single gene may not be sufficient to direct the metabolic flux toward diacetyl and acetoin production, because their precursors, such as α -acetolactate and pyruvate, are at the center of the metabolic pathway (Chen et al., 2017).

Notably, our previous study found that the concentrations of diacetyl and acetoin in fermented milk have optimum ranges (Tian et al., 2020), and that excessive concentrations can be perceived by consumers as imparting undesirable flavors. Thus, an optimal flavor is generated by an appropriate concentration of these 2 compounds, which should be accessible by LAB fermentation.

CONCLUSIONS

In summary, different recombinant strains of *L. casei* TCS were constructed by overexpressing *nox* and *alsS* and knocking out *alsD*. The concentrations of diacetyl and acetoin were higher in milk fermented by the recom-

binant strains than in milk fermented by the original strain, as determined by various indicators. The modification of multiple genes had a synergistic effect, greatly increasing the biosynthesis of diacetyl and acetoin by *L. casei* during fermented milk production. Our work will support further advances in flavor production during milk fermentation. In particular, although genetically engineered strains are not yet available for food production, this study provides guidance for the subsequent screening and application of relevant strains.

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