Supplementary Material

Table S1. The Cre-*lox* system plasmids and knockout plasmid constructs

Plasmids	Relevant features	References
pNZ5319	Cm^r, Ery^r, containing *lox66*-P32-*cm*-*lox71* fragment	(Lambert et al., 2007)
pNZ5348	Ery^r, Cre-recombinase expression	(Lambert et al., 2007)
pNZ5319-Δ*menA*	Cm^r, Ery^r; pNZ5319 derivative containing homologous regions upstream and downstream of NZ9000 *menA*	This study
pNZ5319-Δ*menB*	Cm^r, Ery^r; pNZ5319 derivative containing homologous regions upstream and downstream of NZ9000 *menB*	This study
pNZ5319-Δ*menE*	Cm^r, Ery^r; pNZ5319 derivative containing homologous regions upstream and downstream of NZ9000 *menE*	This study
pNZ5319-Δ*menG*	Cm^r, Ery^r; pNZ5319 derivative containing homologous regions upstream and downstream of NZ9000 *menG*	This study

Table S2. Sequence of oligonucleotide primers

Primer	Nucleotides
up A F *Xho*I	CGTCTCGAGATTGTTCCTAATGGCTCAAAA
up A R *Swa*I	TGCATTTAAATTTCCTCTTTTAATGTGATTTATCAA
down A F *Sac*I	TCCGAGCTCATAAAAAACTCCAATTAATTAA
down A R *Bgl*II	CGAAGATCTTTTTATTTAAAGCTTATAACTG
up B F *Xho*I	GATCTCGAGTCGGCTCCCGTTGAAAAA
up B R *Swa*I	GAAATTTAAATGATTTCTCCTTTAAGATGTGAGGG
down B F *Ban*II	TCCGRGCYCATAAAGCGTCATTTTGGCGC
down B R *Bgl*II	CGAAGATCTATGTTTATTTTTCCAGAATTGACCACTTC
up E F *Xho*I	CCGCTCGAGACTGGGTTGCCCTTA
up E R *Swa*I	CGCATCATTTAAATAGCGCCAAAATGACG
down E F *Ban*II	TCCGAGCTCAAGGGCCTTTCCGGAT
down E R *Bgl*II	CGAAGATCTACCGGCCATAGCATTTCC
up G F *Xho*I	GATCTCGAGCCTGACGTCGAGTCTG
up G R *Swa*I	GTCATTTAAATACTGGTCACAAGGTCAGC
down G F *Sac*I	GCCGAGCTCAGTTTTGAACGCTTGGATAG
down G R *Bam*HI	TACGGATCCTCGAGCACGCTTTCTTTTAT
lox F	AAATCTACCGTTCGTATAATGTATGC
lox R	CTCATGCCCGGGCTGTACCG
menA F	GTTTCTGCTGGGCCTTTCGTAC

men A R	GACAATGGTTTTGGGCCTCCTG
men B F	TCGCAATGCTTTTCGTCCAA
men B R	TCCCGCAAATTGTTGCAGTC
men E F	ATGAAATGGTTAAAAAAACAGGCGG
men E R	TCATGCTTTGAGCTCTTT
men G F	AACGAAGAACGTGTGCAAGA
men G R	AAGCGTCTTGGCATCTGGAA
Ery F	CGATACCGTTTACGAAAT
Ery R	CTTGCTCATAAGTAACGG

*Underlined sequences indicate the restriction sites.

Table S3. Primers used for the functional verification of menaquinone biosynthesis genes study

Primers	Nucleotides
LpA1 F	GGAGTACTTTGAAACCAAAAGTGTTTCTTGAA
LpA1 R	CTAGAGCTCCTAGTTAAAAATCAGTCCTAAGCC
LpA2 F	GGAGTACTTTGTTGACGAACGAACCAAC
LpA2 R	CTAGAGCTCTTAAAAGAAAATACCCAGGATCAGTC
LpA3 F	GGAGTACTATGTTTAAAAAATGGCTAACTTG
LpA3 R	CTAGAGCTCTTACAACCAAGTCCCTAAAACAAA
LpG1 F	GGAGTACTATGACAGCTTTAGAAAGAAGTTG
LpG1 R	TACGAGCTCTCAATCACGACGAGCCA
LpG2 F	GGAGTACTATGGCAAATCGTTATTTAC
LpG2 R	CTAGAGCTCTTACTTGGCCTCCTTAG
LbA F	GGAGTACTATGAGTTTATCAACTTTTTCCCAG
LbA R	CTAGAGCTCCTAGTGGGCAATCAGGG
LbG F	GGAGTACTATGACGCTGACGAACAA
LbG R	CTAGAGCTCTCAGGGCTTCATTCCCCA
LbB F	GGAGTACTATGACTTCAGTTAAATGGGAATC
LbB R	CTAGAGCTCCTATGGGAACTTAGGAAATTG
LbE F	GGAGTACTATGAAAGTGGATAATTGGATTTTAA
LbE R	CTAGAGCTCTTATAACAACTGTTTCAGCTTGAATC
LLA F	GGAGTACTATGAATTTTAAAACATTCGCT

LLA R	GGCGAGCTCTTAAAATCTAATCAAACTAATAAAGA
LLB F	GGAGTACTATGTCAAAATTTAACTGGGTTGC
LLB R	GGCGAGCTCTTATGGGAATTTTGGAAATTGGTC
LLE F	GGAGTACTATGAAATGGTTAAAAAAACAGGCG
LLE R	AACGGYRCCTCATGCTTTGAGCTCTTTTCTTAA
LLG F	GGAGTACTATGACTAAAGTAAACGAAGAACGT
LLG R	GGCGAGCTCTTACTTTTTACCAATACGAATATTTGATTT
pnz8150 F	GCATAATAAACGGCTCTGAT
pnz8150 R	CAGCAATATCAGTAATTGCTTTATC

*Underlined sequences indicate the restriction sites.

Table S4. Plasmids and menaquinone expression plasmid constructs

Plasmids	Relevant features	References
pNZ8150	Cm^r, nisA promoter, expression vector	(Mierau and Kleerebezem, 2005)
pNZ8150-*lpmenA1*	Cm^r; nisA promoter, *lpmenA1* gene cloned to the MCS	This study
pNZ8150-*lpmenA2*	Cm^r; nisA promoter, *lpmenA2* gene cloned to the MCS	This study
pNZ8150-*lpmenA3*	Cm^r; nisA promoter, *lpmenA3* gene cloned to the MCS	This study
pNZ8150-*lpmenG1*	Cm^r; nisA promoter, *lpmenG1* gene cloned to the MCS	This study
pNZ8150-*lpmenG2*	Cm^r; nisA promoter, *lpmenG2* gene cloned to the MCS	This study
pNZ8150-*lbmenA*	Cm^r; nisA promoter, *lbmenA* gene cloned to the MCS	This study
pNZ8150-*lbmenB*	Cm^r; nisA promoter, *lbmenB* gene cloned to the MCS	This study
pNZ8150-*lbmenE*	Cm^r; nisA promoter, *lbmenE* gene cloned to the MCS	This study
pNZ8150-*lbmenG*	Cm^r; nisA promoter, *lbmenG* gene cloned to the MCS	This study
pNZ8150-*llmenA*	Cm^r; nisA promoter, *llmenA* gene cloned to the MCS	This study
pNZ8150-*llmenB*	Cm^r; nisA promoter, *llmenB* gene cloned to the MCS	This study
pNZ8150-*llmenE*	Cm^r; nisA promoter, *llmenE* gene cloned to the MCS	This study
pNZ8150-*llmenG*	Cm^r; nisA promoter, *llmenG* gene cloned to the MCS	This study

Table S5. *Lactococcus lactis* strains with menaquinone expression plasmid

Strains	Relevant features	References
Lactococcus lactis (ll)		
lpmenA1	NZ9000-Δ*menA* carrying pNZ8150-*lpmenA1*	This study
lpmenA2	NZ9000-Δ*menA* carrying pNZ8150-*lpmenA2*	This study
lpmenA3	NZ9000-Δ*menA* carrying pNZ8150-*lpmenA3*	This study
lpmenG1	NZ9000-Δ*menG* carrying pNZ8150-*lpmenG1*	This study
lpmenG2	NZ9000-Δ*menG* carrying pNZ8150-*lpmenG2*	This study
lbmenA	NZ9000-Δ*menA* carrying pNZ8150-*lbmenA*	This study
lbmenB	NZ9000-Δ*menB* carrying pNZ8150-*lbmenB*	This study
lbmenE	NZ9000-Δ*menE* carrying pNZ8150-*lbmenE*	This study
lbmenG	NZ9000-Δ*menG* carrying pNZ8150-*lbmenG*	This study
llmenA	NZ9000-Δ*menA* carrying pNZ8150-*llmenA*	This study
llmenB	NZ9000-Δ*menB* carrying pNZ8150-*llmenB*	This study
llmenE	NZ9000-Δ*menE* carrying pNZ8150-*llmenE*	This study
llmenG	NZ9000-Δ*menG* carrying pNZ8150-*llmenG*	This study

Table S6. Primers used for the reconstitution of menaquinone biosynthesis pathway in *Lactipl. plantarum* and *Lent. buchneri* strains

Primers	Nucleotides
Psip 409 BsaIKO F	CACGTTACTAAAGGAAATGGAGACCGGGGT
Psip409 BsaIKO R	CGGTCGCCATTCCCTTTAGTAACGTGTAACTTTCCAAAT
Psip409 F BsaI	AAGGGTCTCATGCGTCTAGACTCGAGGAATT
Psip409 R BsaI	CATGGTCTCCGATCGCTAAAATCTCCTTGTAATA
Golden Gate Assemble for *Lactipl. plantarum* WCFS1 menaquinone expression vector construction	
Psip409 backbone F BsaI	AAGGGTCTCATGCGTCTAGACTCGAGGAATT
Psip409 backbone R BsaI	CATGGTCTCCCGCCGCTAAAATCTCCTTGTAATA
GO menF Lp F	TATGGTCTCAGCGGATTACAAGGAGATTTTAGCCATGAAATATATAAAAAACGATTTAATATTAA
GO menF Lp R	CGCGGTCTCATCTTTCATAAGGCTTCTAAAA
GO menD Lp F	TATGGTCTCAAGATATTACAAGGAGATTTTAGCCATGACCAATGAATATTTAGCTCC
GO menD Lp R	CGCGGTCTCCCCTGATCAATTTTCATAAGCAGTATATTTTTTAT
GO menH Lp F	TATGGTCTCATCAGATTACAAGGAGATTTTAGCCATGAAAATTGATAAAAAAATAATGACGA
GO menH Lp R	CGCGGTCTCGTAAGCCTAAGCCAAAAATTCCTC
PsppQ LP F	TATGGTCTCGCTTAGGAGATCTACCGGTTTAATTTGAAA
PsspQ LP R	CGCGGTCTCGGCTCCGCGGCTAAAATCTCCTTGTAATAGT

GO menB Lp F	TATGGTCTCGGAGCATTACAAGGAGATTTTAGCCATGTCAAAATTTAACTGGGTTG
GO menB Lp R	CGCGGTCTCGAACCTTATGGGAATTTTGGAAATTGG
GO menE Lp F	TATGGTCTCAGGTTATTACAAGGAGATTTTAGCCATGAAATGGTTAAAAAAACAGGC
GO menE Lp R	CGCGGTCTCTGTAGTCATGCTTTGAGCTCTTT
GO menC LP F	TATGGTCTCACTACATTACAAGGAGATTTTAGCCATGAAAATTGAAAAAATCACAATGT
GO menC Lp R	CGCGGTCTCTCGCATCATTTCAAGGAGGTCAAGC

*Underlined sequences indicate the restriction sites

Ribosome binding site are highlighted in grey

Colored nucleotides represent the overhangs for Golden Gate Assembly and the same color showed the complementary bases

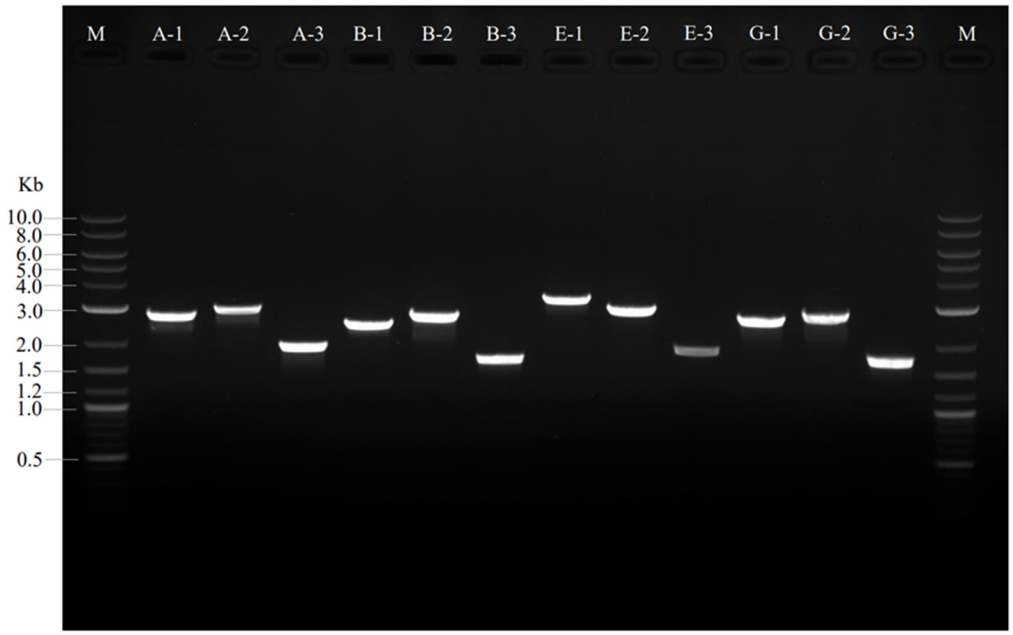

Figure S1. Diagnostic PCR of *L. lactis* NZ9000 *menA* (A-1), its gene replacement mutant *menA*:: *lox66*-P32-*cm*-*lox71* (A-2), *ΔmenA* (A-3); *menB* (B-1), *menB*:: *lox66*-P32-*cm*-*lox71* (A-2), *ΔmenB* (B-3); *menE* (E-1), *menE*:: *lox66*-P32-*cm*-*lox71* (E-2), *ΔmenE* (E-3); *menG* (G-1), *menG*:: *lox66*-P32-*cm*-*lox71* (A-2), *ΔmenG* (G-3); M is a 2 log DNA ladder

.

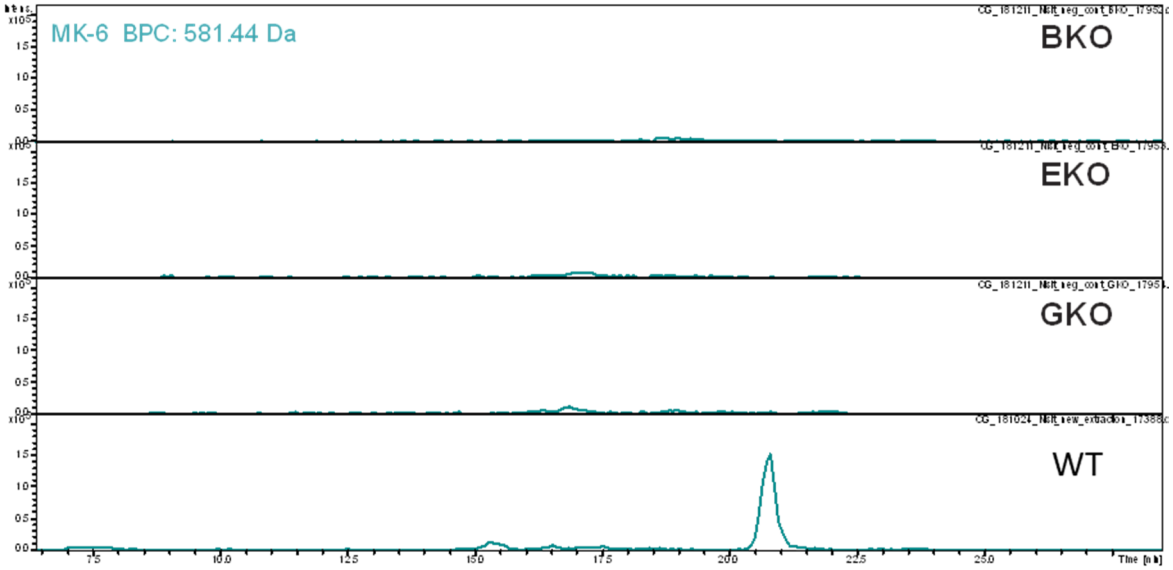

Figure S2. Base peak chromatogram of menaquinone extracts from *L. lactis* NZ9000 (WT) and its menaquinone deficient strains Δ*menB* (BKO), Δ*menE* (EKO), Δ*menG* (GKO) for MK-6.

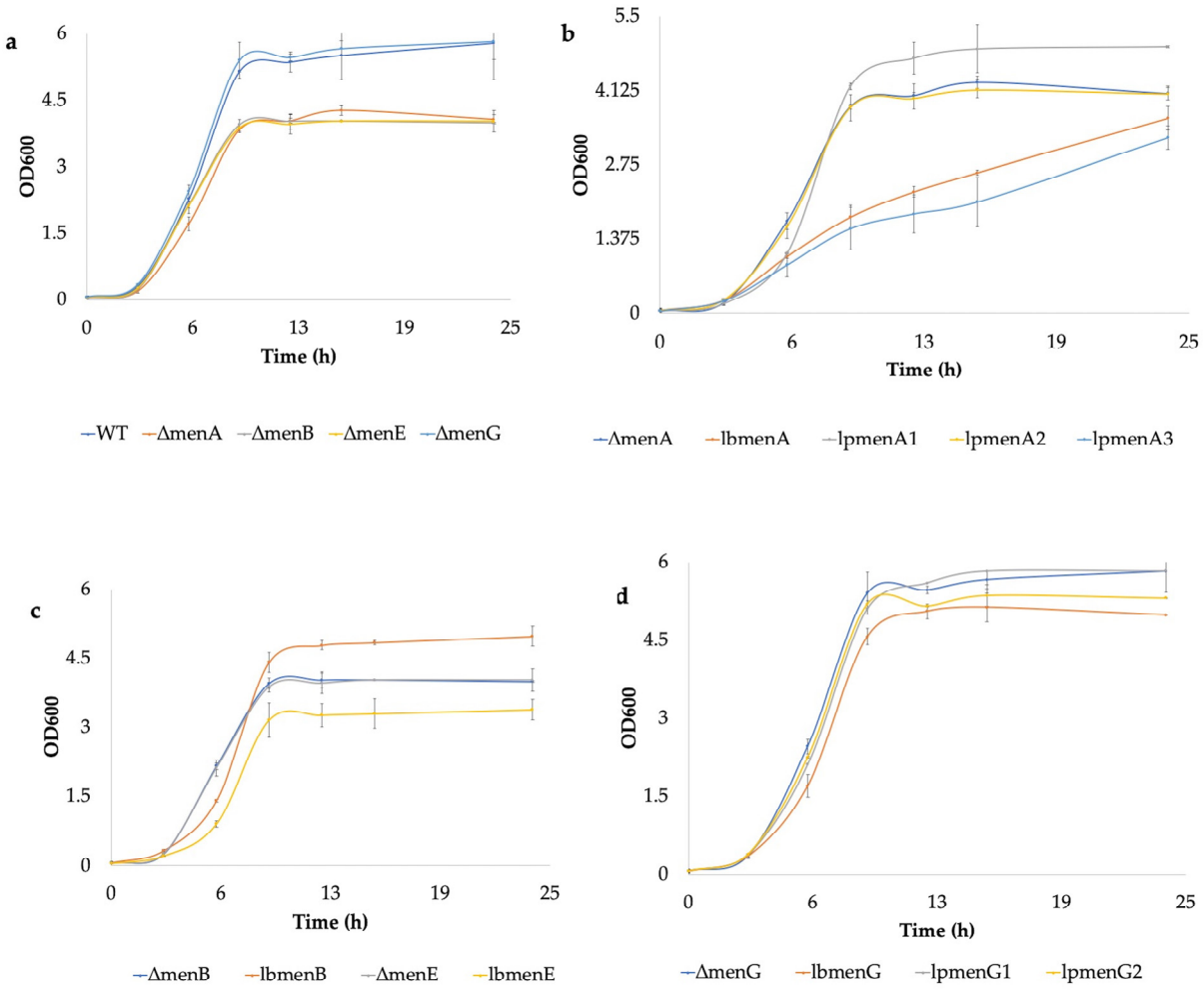

Figure S3. Growth profile analysis of (a) *L. lactis* NZ9000 (WT) and the knockout strains (Δ*menA*, Δ*menB*, Δ*menE*, Δ*menG*), (b) *L. lactis* Δ*menA* and its engineered derivatives carrying *lbmenA*, *lpmenA1*, *lpmenA2* and *lpmenA3*), (c) *L. lactis* Δ*menB*, *L. lactis* Δ*menE* and the engineered derivatives (carrying *lbmenB*, *lbmenE*) and (d) *L. lactis* Δ*menG* and its engineered derivatives carrying *lbmenG*, *lpmenG1* and *lpmenG2*. 300 mL of GM17 medium supplemented with heme (2µg/mL) were used, 1 ng/mL of nisin was added as an inducer at OD_{600} = 0.4.

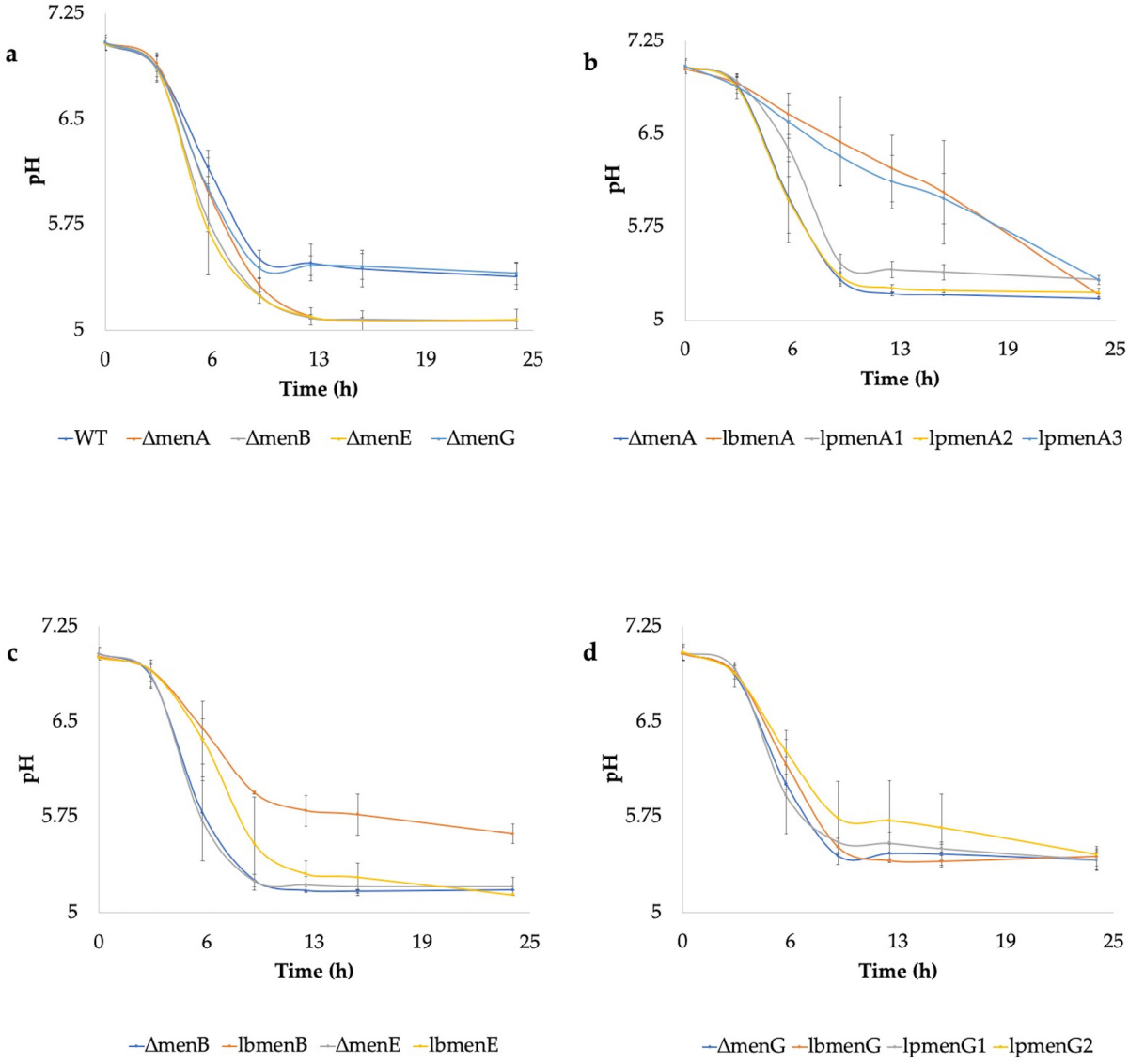

Figure S4. pH profile of (a) *L. lactis* NZ9000 (WT) and its menaquinone deficient strains (Δ*menA*, Δ*menB*, Δ*menE*, Δ*menG*), (b) *L. lactis* Δ*menA* and its engineered strains (lbmenA, lpmenA1, lpmenA2, lpmenA3), (c) *L. lactis* Δ*menB*, *L. lactis* Δ*menE* and its engineered strains (lbmenB, lbmenE) and (d) *L. lactis* Δ*menG* and its engineered strains (lbmenG, lpmenG1, lpmenG2). 300 mL of GM17 medium supplemented with heme (2μg/mL) was used, 1 ng/mL of nisin was added as inducer at OD_{600} = 0.4.

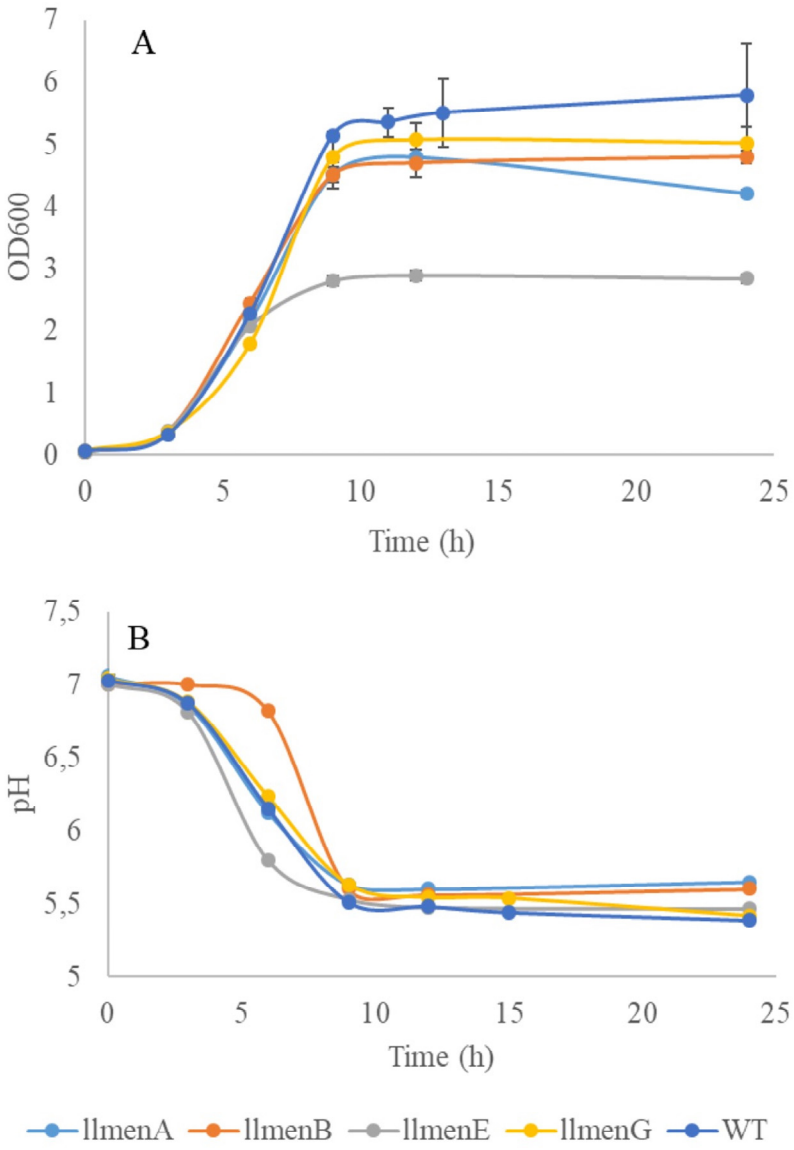

Figure S5. Growth profile (A) and pH analysis (B) of *L. lactis* NZ9000 (WT) and engineered deficient strains complemented by homologous genes (*llmenA*, *llmenB*, *llmenE*, *llmenG)*. 300 mL of GM17 medium supplemented with heme (2µg/mL) were used, 1 ng/mL of nisin was added as an inducer at OD_{600} = 0.4.