1. Supplementary Information

1.1. Supplementary Sections

Section S1 Genome Analysis of the Parent Strain SD96

To reveal the genetic changes that occurred during the ALE experiment, we first de novo sequenced the whole genome of SD96, which is described elsewhere (1). The chromosome of SD96 (2.4 mb) contains 2368 coding sequences (CDS), 19 ribosomal RNAs, and 64 tRNAs. According to NCBI, its closest relative reference genome is IL1403 (92.6% symmetric identity), and the overall closest sequenced strain is Fm03 (92.9% symmetric identity).

Several genes of industrial relevance are located on the ten plasmids of SD96 (**Table S1**). The largest plasmid, pSD96_02 (64.9 kb), contains genes essential for growth in milk, and most importantly, the cell wall-bound protease PrtP (FTN78_RS13140). Genes for lactose utilization (FTN78_RS12660- FTN78_RS12700), which are also essential for growth in milk, were found on plasmid pSD96_05 (49.6 kb). This plasmid also encodes an *opp* operon responsible for oligopeptide transport (FTN78_RS12805 to FTN78_RS12815 & FTN78_RS12525 to FTN78_RS12535). A second *opp* operon is located on the chromosome (FTN78_RS07325 to FTN78_RS07345). As characteristic for *L. lactis* subsp. *lactis* biovar diacetylactis strains, SD96 contains a plasmid, pSD96_04, that carries the citrate permease (CitP, FTN78_RS13490), which enables citrate utilization and diacetyl formation. Besides these crucial and well-described genetic elements, SD96 was found to harbor seven other plasmids, of which three were comparably large: pSD96_01 (11.7 kb), pSD96_03 (37.4 kb), and pSD96_06 (27.4 kb). The remaining four smaller plasmids ranged in size from 2.7 kb to 7.9 kb. pSD96_01 encodes amongst other genes the *cadA* gene (FTN78_RS12845), which is involved in cadmium translocation, and one specificity subunit of a type I restriction-modification system (RMS) (FTN78_RS12835). Among other genes, pSD96_06 encodes a complete type I RMS, including specificity, modification, and restriction

subunits (FTN78_RS00005 to FTN78_RS00015). The function of pSD96_03 was elusive, but many genes were assigned to the Clusters-of-Orthologous-Groups (COG) category "Intracellular Trafficking and Secretion," implying a potential role in mediating transport across the cell envelope. Similarly, the four small plasmids pSD96_07 to pSD96_10 could not be assigned to a specific function, but both encode specificity subunits of type I RMSs (FTN78_RS00140 and FTN78_RS00240, respectively). pSD96_08 encodes genes for bacteriocin production (lactoccocin family), confirmed using BAGEL4 (2). Many of the plasmid-encoded genes are required for mobilizing the plasmid (*mobA* or *mobC*).

Section S2 Determination of Proteolytic Activity

The proteolytic activity of whole cells was determined using azocasein as a substrate with three biological replicates. Azocasein (Sigma Aldrich) was dissolved in sodium phosphate buffer (100 mM, pH 6.5) to obtain a 2.5% (w/v) solution. SD96, RD01, and RD07 were cultivated at 30°C for 48 h on a solid medium containing 1.5% Agar (w/v) and 50% (v/v) UHT milk. Colonies were harvested using cold 0.9% (w/v) NaCl and washed once using the same solution. The number of cells was adjusted to reach OD_{600} of 1.25 in 500 µl. The cell pellets were washed once with MES-Buffer (100 mM, pH 6.0) containing 20 mM $CaCl_2$ and finally suspended in 250 µl of this buffer. For starting the reactions, 250 µl of the azocasein solution was added, and the mixture was incubated at 30°C for 2 h. Samples were withdrawn regularly. Therefore, 50 µl were transferred to a new tube, mixed with 200 µl of a 5% (v/v) trichloroacetic acid solution, and centrifuged at 15000 x g for 2 min. From the supernatant, 50 µl were mixed with 150 µl of a 0.5 M NaOH solution, and absorption was measured at 440 nm using a spectrophotometer. Initial proteolytic activity was obtained by linear regression of three data points. As a blank reaction, a protease-negative strain was used.

Section S3 Validation of Enrichment for Protease-Positive Strains

When grown on UHT milk agar (1% UHT milk, 1.5% agar), protease-positive strains formed larger colonies than protease-negative strains, which was exploited for enrichment of protease-positive strains during ALE as described in the main manuscript text. For validating the enrichment, two strains were used: RD07 and RD07c, a derivative of RD07, which was cured for plasmid pSD96_2 and pSD96_4, encoding the cell surface protease (PrtP) and the citrate transporter (CitP), respectively. The plasmid loss was confirmed by whole-genome sequencing (data not shown). Both strains were grown as three independent biological replicates in M17 broth, supplemented with 0.5% lactose. Next, a 1:1 mixture was prepared based on the cultures' optical density at 600 nm. Dilutions of the mixtures were plated on UHT milk agar and UHT milk agar, supplemented with 0.5% casein peptone, to obtain approximately 100-150 colonies per agar plate. Both types of plates were buffered at pH 7 with glycerol phosphate (19 g/L) to avoid the influence of citrate metabolism on cell growth. When colonies with approximately 0.5 mm diameter were visible, all colonies were harvested from the plates, suspended in 0.9% NaCl solution, and plated in serial dilutions on agar plates that indicate citrate utilization as described in the main manuscript text. Since RD07c lacks the plasmid conferring citrate utilization, its colonies remained white on the agar, while RD07 produced blue colonies. The ratio of blue over white colonies thus indicates the ratio of protease-positive strains over protease-negative strains. For colonies grown on UHT milk agar, a ratio of 6.8 ± 0.5 was detected, while UHT agar, supplemented with 0.5% casein peptone, produced a ratio of 0.9 ± 0.5. Accordingly, an approximately 7-fold enrichment was achieved through this method.

1.2. Supplementary Tables

Table S1 Plasmids of SD96.

Name	Size [kb]	Function	Relevant Annotated Genes or Operons
pSD96_01	11.7	Phage defense	cadA (Cd transport), specificity subunit of type I RMS, mobC
pSD96_02	64.9	Protein degradation	PrtP protease, *mobC*, *amaP*, Cu and Cd transport
pSD96_03	37.4	Unknown function	Conjugation protein TraE, thioredoxin, *mobC*
pSD96_04	8.3	Citrate transport	CitP – citrate transporter, specificity subunit of type I RMS
pSD96_05	49.9	Lactose metabolism	opp operon, lac operon, Mn^{2+}-transport, universal stress protein, d-LDH, mobA
pSD96_06	25.4	Phage defense	Complete type I RMS, *mobC*
pSD96_07	7.3	Phage defense	specificity subunit of type I RMS
pSD96_08	4.6	Unknown function	lactococcin family bacteriocin, *mobC*
pSD96_09	2.7	Unknown function	-
pSD96_10	7.9	Phage defense	specificity subunit of type I RMS, *mobC*

Table S2 Differential expression (DE) of mutated genes compared to SD96 DE with low padj (>0.05) and thus high confidence are written in bold font. RD01 2 and RD07 5 were located upstream of codY and FtsH, respectively.

Mutation	Affected gene(s)	DE at 30°C	DE at 39°C
RD01-1	EamA family transporter	-0,7 ± 0.3	-0,8 ± 0.2
RD01-2	Upstream of pleiotropic transcriptional regulator *codY*	**-2,7 ± 0.1**	**-2,9 ± 0.1**
RD01-3	Bifunctional (p)ppGpp synthetase	-0,3 ± 0.1	-0,2 ± 0.2
RD01-4	UDP-N-acetylmuramate-l-alanine ligase	0,1 ± 0.2	0,4 ± 0.1
RD07-1	Phenylalanine-tRNA ligase	0,8 ± 0.2	**-1,2 ± 0.1**
RD07-2	Hypothetical protein	-0,5 ± 0.2	-0,1 ± 0.2
RD07-3	EamA family transporter	-0,4 ± 0.3	0,1 ± 0.2
RD07-4	DNA primase	0,7 ± 0.4	0,04 ± 0.38
RD07-5	Upstream of *hflB*/*ftsH* encoding FtsH	-0,5 ± 0.1	-0,8 ± 0.2
RD07-6	RexA, ATP-dependent exonuclease subunit A	0,2 ± 0.1	-0,6 ± 0.1
RD07-7	Pur operon regulator, *purR*	**1,0 ± 0.2**	0,9 ± 0.2
RD07-8	UDP-N-acetylmuramate- l-alanine ligase	0,2 ± 0.2	0,5 ± 0.1

Table S3 Prophages encoded on the SD96 chromosome as identified using PHASTER (3).

Prophage number	Region length [kb]	Completeness	Total proteins	Region position	Most common Phage (Accession no.)
1	47.1	Intact	55	53121-100233	Lactococcus bIL286 (NC_002667)
2	21.8	Intact	36	106834-128670	Lactococcus bIL312 (NC_002671)
3	43.1	Intact	65	1487673-1530792	Lactococcus bIL309 (NC_002668)
4	27.8	Incomplete	24	1837787-1865594	Lactococcus bIL312 (NC_002671)
5	27.5	Incomplete	15	1879571-1907158	Staphylococcus SPbeta like (NC_029119)
6	18.9	Intact	32	1997320-2016230	Lactococcus bIL310 (NC_002669)
7	15.5	Intact	23	2400586-2416120	Lactococcus bIL311 (NC_002670)

1.3. Supplementary Figures

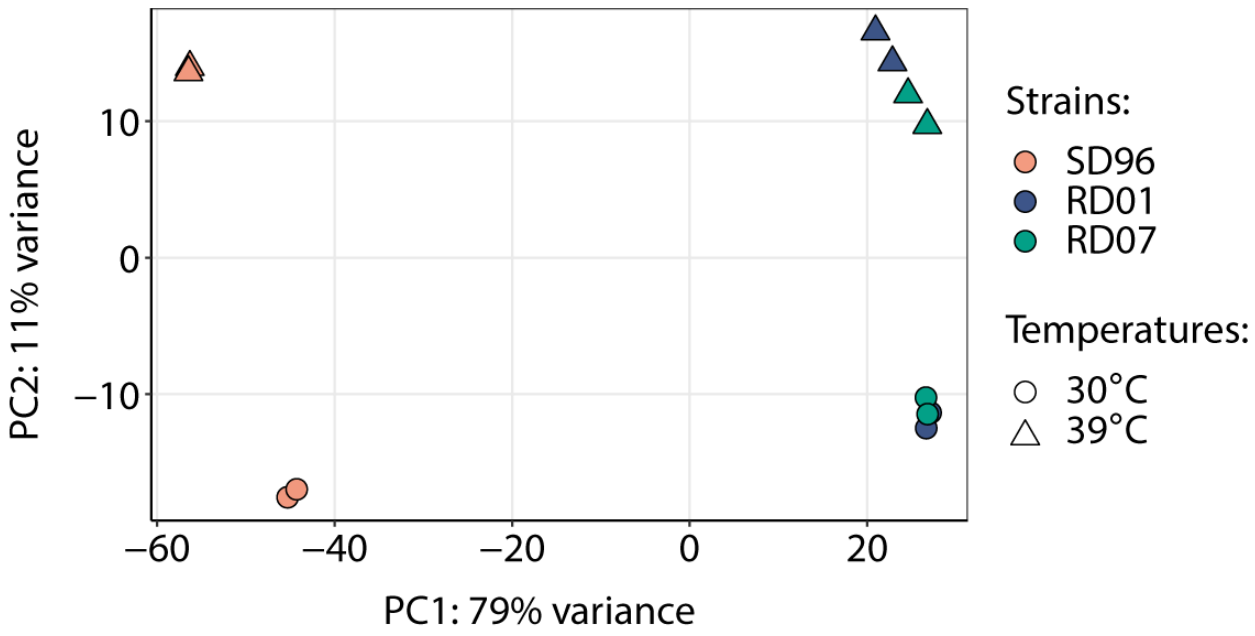

Figure S1 Principal component analysis of the sample groups. Biological replicates are shown as separate dots in the same color. Strains are indicated with different colors (SD96: rose, RD01: blue, RD07: green). Samples from different temperatures are shown with different shapes (30°C: circles, 39°C: triangles).

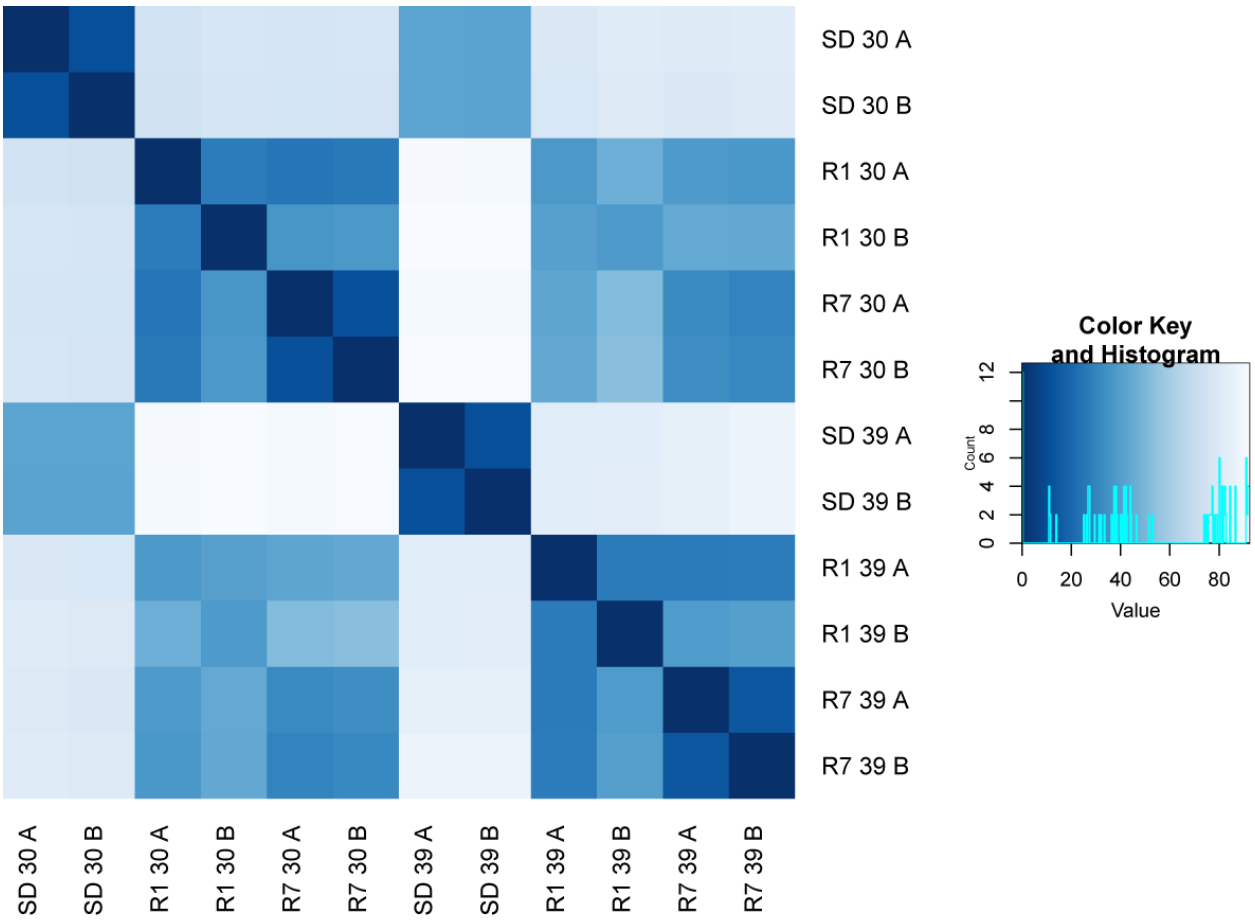

Figure S2 Sample distance between biological replicates (A and B) and conditions (30°C and 39°C). Abbreviations are as follows: SD: SD96, R1: RD01, R7: RD07. Labels should be read as follows: strain - temperature - replicate. The darker the shape, the closer are the samples to each other.

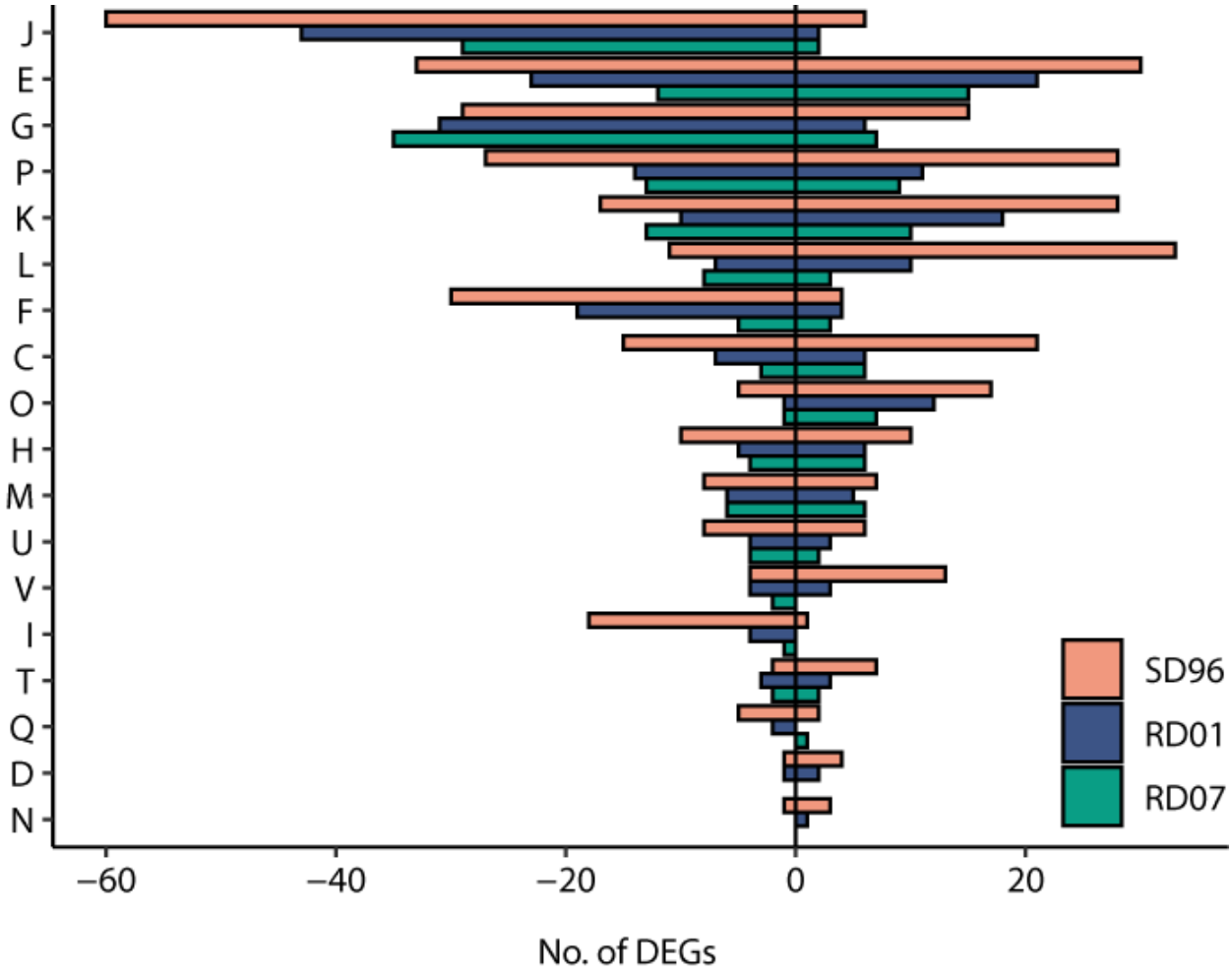

Figure S3 COG analysis comparing expression at 30°C with expression at 39°C for SD96 (rose), RD01 (blue), and RD07 (green). Categories are as follows: C: Energy production and conversion, D: Cell cycle control and mitosis, E: Amino Acid Metabolism and Transport, F: Nucleotide Metabolism and Transport, G: Carbohydrate Metabolism and Transport, H: Coenzyme Metabolism, I: Lipid metabolism, J: Translation, K: Transcription, L: Replication and Repair, M: Cell Wall/Membrane/Envelope Biogenesis, N: Cell motility, O: Post-translational Modification, Protein Turnover, Chaperone Functions, P: Inorganic Ion Transport and Metabolism, Q: Secondary Structure, T: Signal Transduction, U: Intracellular Trafficking and Secretion.

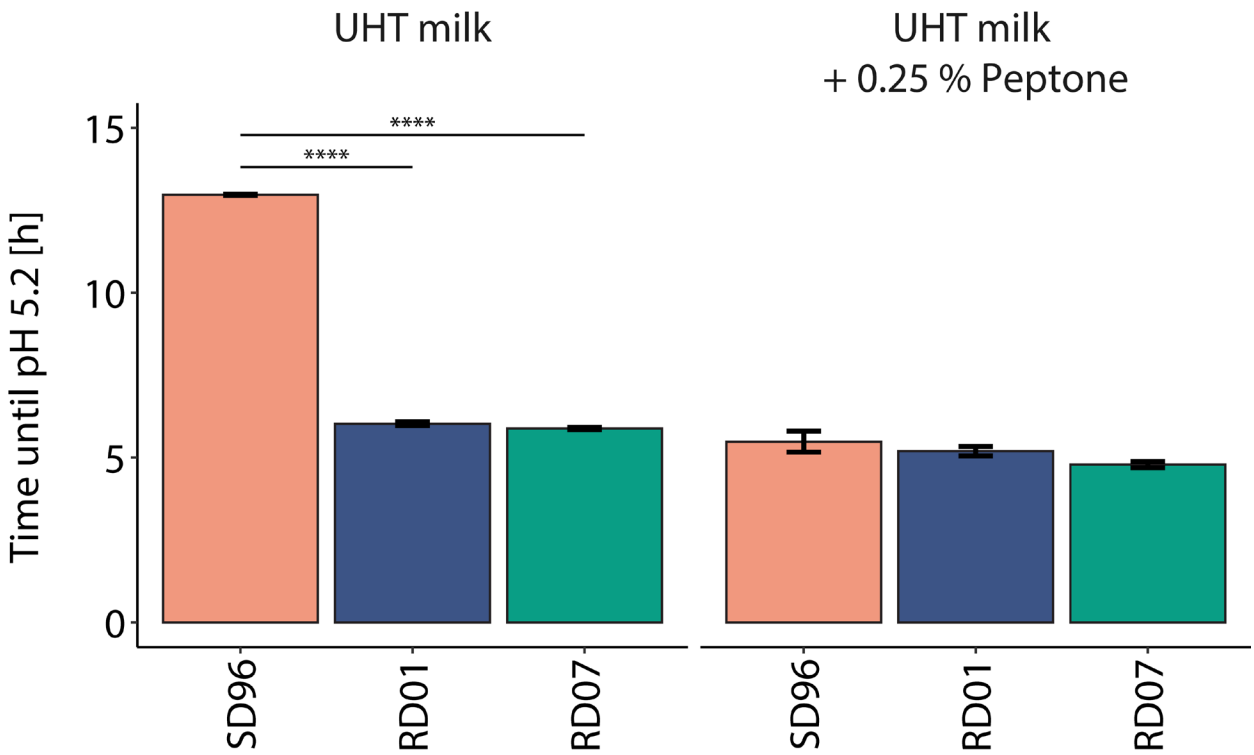

Figure S4 Acidification of SD96, RD01, and RD07 (rose, blue, and green, respectively) in UHT milk and UHT milk supplemented with 0.25% (w/v) casein peptone. The time until pH 5.2 is shown with standard deviations from two independent fermentations at 30°C.

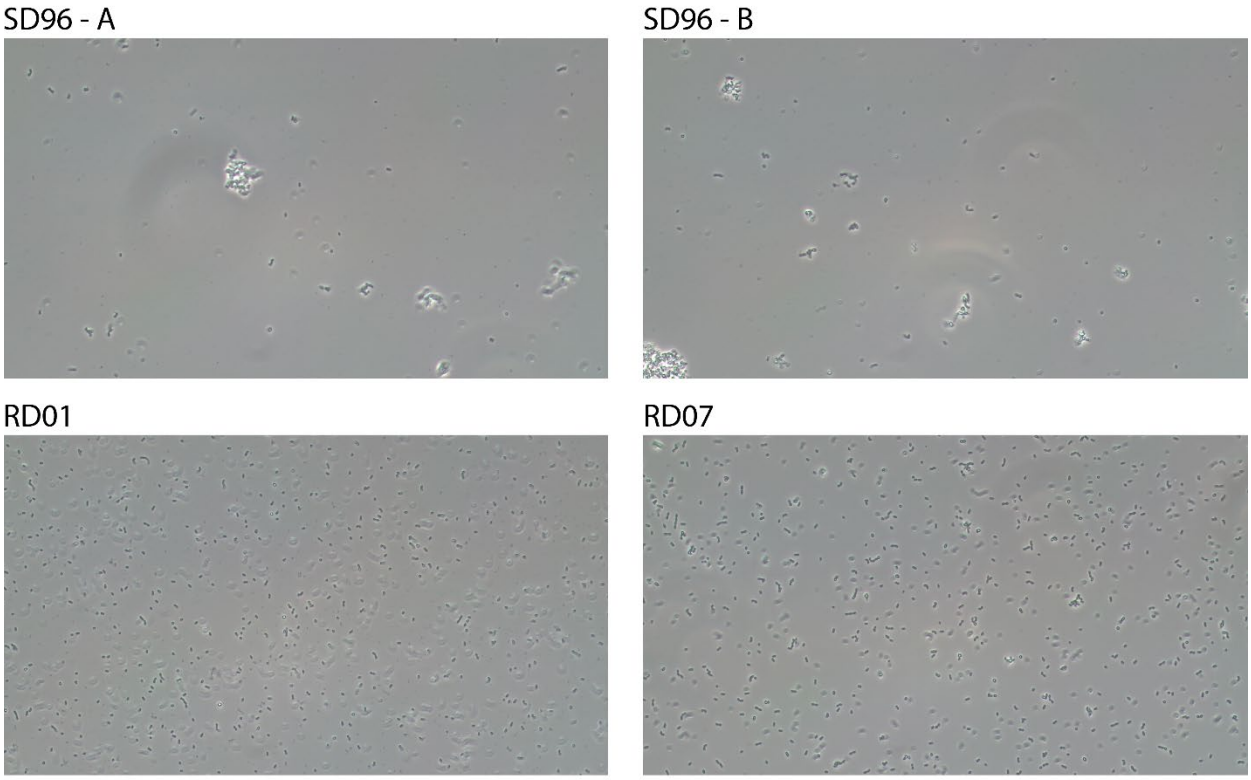

Figure S5 Microscopic pictures of SD96, RD01 and RD07. SD96 shows a higher tendency to form globular structures than RD01 and RD07. The former two strains appear identical under the microscope.

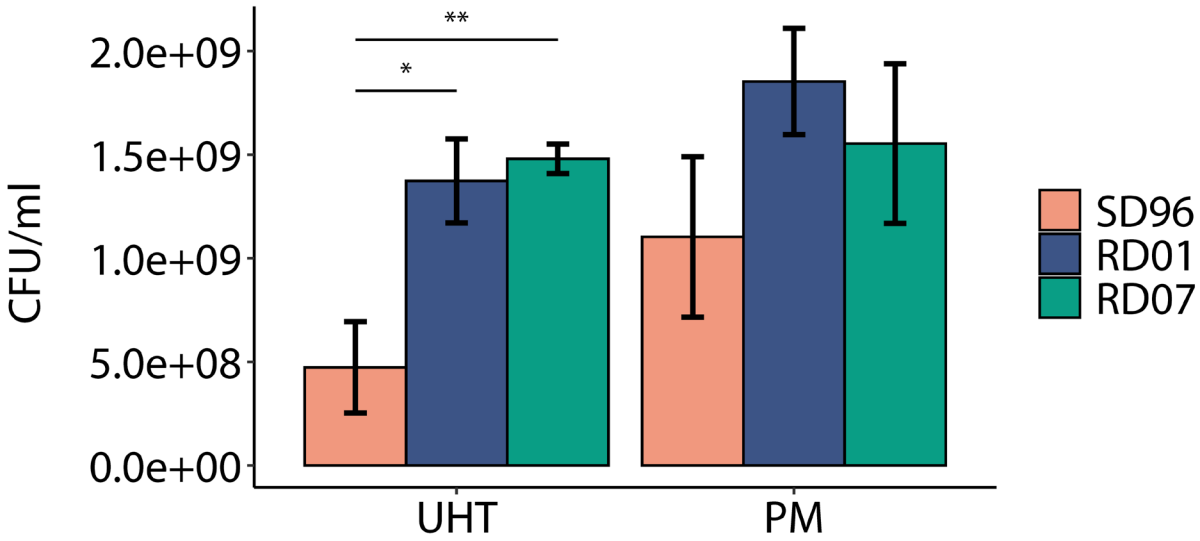

Figure S6 Colony forming units (CFU) per ml overnight culture. Values were determined from three biological replicates and statistically significant differences are indicated with asterisks after performing a T-test ($p < 0.05$: *, $p < 0.005$: **).

References

1. Dorau R, Chen J, Jensen PR, Solem C. Complete Genome Sequence of Lactococcus lactis subsp. lactis bv. diacetylactis SD96. *Microb Resour Announc* **2020**, 9(3):1–2.

2. Van Heel AJ, De Jong A, Song C, Viel JH, Kok J, Kuipers OP. BAGEL4: A user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Res* **2018**, 46:278–81.

3. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, Wishart DS. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* **2016**, 44:16–21.