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Stress resistant *rpsU* variants of *Listeria monocytogenes* can become underrepresented due to enrichment bias

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ABSTRACT

Population heterogeneity is an important component of the survival mechanism of *Listeria monocytogenes*, leading to cells in a population with diverse stress resistance levels. We previously demonstrated that several ribosomal gene *rpsU* mutations enhanced the stress resistance of *L. monocytogenes* and lowered the growth rate at 30 °C and lower temperatures. This study investigated whether these switches in phenotypes could result in a bias in strain detection when standard enrichment-based procedures are applied to a variety of strains. Detailed growth kinetics analysis of *L. monocytogenes* strains were performed, including the LO28 wild type (WT) and *rpsU* variants V14 and V15, during two commonly used enrichment-based procedures described in the ISO 11290-1:2017 and the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM). WT had a higher growth rate than the variants during the enrichment processes. Co-culture growth kinetics predictions for WT and *rpsU* variants showed that the detection chances of the *rpsU* mutants were reduced from ~52 % to less than ~13 % and ~ 3 % during ISO and BAM enrichment, respectively, which were further validated through subsequent qPCR experiments. Higher heat stress resistance of *rpsU* variants did not lead to faster recovery during enrichment after heat treatment, and different pre-culturing temperatures before heat treatment did not significantly affect the growth kinetics of the WT and *rpsU* variants. Additionally, post-enrichment isolation procedures involving streaking on selective agar plates did not show preferences for isolating WT or *rpsU* variants nor affect the detection chance of *rpsU* variants. The difference in detection chance suggests that the selective enrichment procedures inadequately represent the genotypic diversity present in a sample. Hence, the enrichment bias during the *L. monocytogenes* isolation procedure may contribute to the observed underrepresentation of the *rpsU* mutation among *L. monocytogenes* isolates deposited in publicly available genome databases. The underrepresentation of *rpsU* mutants in our findings suggests that biases introduced by standard isolation and enrichment procedures could inadvertently skew our understanding of genetic diversity when relying on public databases.

1. Introduction

Listeria monocytogenes is a ubiquitous foodborne pathogen that can cause one of the most serious foodborne diseases, listeriosis, with a fatality rate of 13.7 % (EFSA and ECDC, 2022). This bacterium can survive in a wide range of stress conditions, such as low pH, high osmotic pressure, and low temperature (Liu et al., 2019). In addition, *L. monocytogenes* can persist in food processing plants and food-associated environments for years or even decades (Ferreira et al., 2014; Harrand et al., 2020; Vongkamjan et al., 2013). The inherent population heterogeneity is one of the factors that contribute to the robustness and persistence of *L. monocytogenes* in food processing environment (Abee et al., 2016).

Population heterogeneity means that individual cells within the population have genotypic and phenotypic diversity including different stress resistance levels. When exposed to lethal stresses, the stress resistant diversity can lead to tailing of the inactivation curve. Tailing may not only lead to a higher than expected number of surviving cells and inaccurate prediction of inactivation procedures but can also lead to the selection of resistant subpopulations. Previous studies reported the identification of stable stress-resistant variants from *L. monocytogenes* by isolating cells from the tail of inactivation curves upon acid, high hydrostatic pressure (HHP) and heat treatment (Metselaar et al., 2013; Van Boeijen et al., 2011; Van Boeijen et al., 2008).

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Mutations in the ribosomal protein gene *rpsU* were predominantly present in the acid isolated variants, and mutations in this gene were also found in variants isolated after HHP and heat exposure (Metselaar, 2016). These mutations include missense mutation, frameshift mutation, and deletion of the whole *rpsU* gene. Further studies focusing on the amino acid substitution variant V15 and the *rpsU* deletion variant V14 revealed that these variants have increased multi-stress resistance, reduced motility, and reduced growth rates at temperatures below the optimum temperature and the latter was more pronounced at lower temperatures (Koomen et al., 2018; Metselaar et al., 2016). Interestingly, laboratory evolution study of V15 showed that this variant is able to mutate and revert to the wild type like phenotype (Koomen et al., 2021). The mutation happened in the same codon of the *rpsU* gene for two V15 evolved strains. Hence, mutations in ribosomal genes, especially at *rpsU*, enables switching between multiple-stress resistant and high fitness states in *L. monocytogenes* (Koomen et al., 2021). Since mutations in the *rpsU* gene can be a mechanism of *L. monocytogenes* to adapt to different environmental stresses, the *rpsU* gene may be a hot spot of mutation.

Foodborne isolates are often isolated from food using enrichment-based detection procedures. The selective enrichment step of these procedures promotes the growth of the target organism and decreases the growth of background microorganisms, allowing for the isolation of *L. monocytogenes* (Allende et al., 2022). However, the selective enrichment can also lead to an isolation bias of *L. monocytogenes* lineages, serotypes, or strains when growth rate differences exist (Bruhn et al., 2005; Gorski et al., 2006; Zilelidou et al., 2016a; Zilelidou et al., 2016b). Two commonly used *L. monocytogenes* detection standards are the ISO 11290-1:2017 and the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM). The ISO 11290-1:2017 applies a two-step enrichment with two different media, half Fraser broth (HFB) and Fraser broth (FB). The BAM standard uses buffered *Listeria* enrichment broth (BLEB) for the enrichment. To allow the recovery of stressed cells, the ISO standard uses HFB for the first step, which contains less antibiotics than FB, and the BAM standard applies a four hour incubation before adding the antibiotics in the enrichment culture. Both standards require 48 h of enrichment using the required media, and the cultures are streaked onto two different selective agar media for isolation after 24 h and 48 h enrichment.

In this study, genome sequences of strains deposited in the National Center for Biotechnology information (NCBI) database were analyzed to assess the conservation level of the *rpsU* gene. To elucidate whether the detection chance of *rpsU* variants from food may differ from wild type (WT) strains when enrichment-based detection methods are applied, growth kinetics were determined for *L. monocytogenes* LO28 wild type strain and its *rpsU* variants V14 and V15 during the enrichment according to the ISO 11290-1:2017 and BAM methods. This allows to assess whether enrichment-based detection procedures contribute to a bias in the genetic diversity of deposited *L. monocytogenes* isolates.

2. Materials and methods

2.1. Gene variation level analysis and rpsU mutants' isolation origins analysis

A pipeline tool was built (github.com/xchuam/blast_at_local_computer) to construct a genome database at a local computer and run the Basic Local Alignment Search Tool (BLAST). This local analysis was needed because the NCBI online BLAST tool can only display the top 5000 aligned sequences. By using this pipeline, 51,784 genome assemblies (303 complete genomes, 71 chromosomes, 1507 scaffolds and 49,903 contigs) were downloaded from the NCBI ftp site. The consistency of the downloaded genomes was checked by the MD5 checksum tool (GNU coreutils, 8.32). Then, BLAST+ (NCBI, 2.13.0) was used to construct the genome database and run BLAST with all the coding sequences from *L. monocytogenes* EGD-e reference genome sequence (NC_003210.1) as queries. The BLAST hit results that were located at the start or the end of the subject sequence were filtered out. The variation level of each gene was estimated by the following equation:

$$Variation = \frac{N_{type}}{N_{total} \cdot Length} \quad (1)$$

where N_{type} is the number of DNA sequence types of the gene found by BLAST; N_{total} is the total number of DNA sequences of the gene that were found by BLAST; *Length* is the maximum length of the gene DNA sequence. Next to the DNA sequence, also the sample attribute information for each genome assembly was downloaded from NCBI, and the sample isolation origin (animal, clinical, food associated environment, other environment, food, feed and unknown) was manually annotated for each genome assembly according to the sample attribute information.

2.2. Bacterial strains and mono-culture enrichment conditions

L. monocytogenes strain LO28 WT, *rpsU* deletion variant V14 and *rpsU* point mutation variant V15 were used in this study (Metselaar et al., 2013). Enrichment procedures were followed using the ISO 11290-1:2017 standard and the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM). Cultures were made by inoculating 10 mL of Brain Heart Infusion (BHI, Oxoid, Ltd., Basingstoke, England) broth with a single colony from a BHI agar plate (1.5 % (w/w), bacteriological agar no. 1 Oxoid) obtained from −80 °C freezer stocks. Cultures were grown at 30 °C under shaking at 160 rpm for 17 to 30 h to obtain a working culture. Afterwards, two parallel time-shifted overnight (ON) cultures were made by inoculating 10 μL of the working culture in 10 mL BHI broth in the morning and in the afternoon, respectively. Both parallel ON cultures were grown at 30 °C under shaking at 160 rpm for 22 to 24 h and subsequently diluted 1,000,000 times in the enrichment media or exposed to heat treatment as described below (see Section 2.6). For the enrichment according to the ISO 11290-1:2017, 5 mL diluted culture or heat-treated culture was added to 45 mL HFB, which was made by supplementing Fraser broth base (Oxoid) with half Fraser supplement (Oxoid). The parallel time-shifted HFB cultures were incubated at 30 °C for 24 h and sampled at time points 0, 2, 4, 6, 8, 10, 14, 18, 22, and 24 h. After 24 h enrichment, 0.1 mL of HFB enrichment was transfered into 10 mL FB, which was made by supplementing Fraser broth base with Fraser supplement (Oxoid). The FB cultures were incubated at 37 °C for 24 h and sampled at time points 0, 2, 4, 6, 8, 10, 14, 18, 22, and 24 h. For the enrichment according to the BAM standard, a 5 mL diluted culture or heat-treated culture was added to 45 mL BLEB (Oxoid) and cultured at 30 °C for 48 h. Listeria Selective Enrichment Supplement (Oxoid) was added to the enrichment culture after 4 h incubation at 30 °C. The BLEB enrichment cultures were sampled at time points 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 40, and 48 h. All the samples were spread-plated on BHI agar plates after appropriately diluting and plates were incubated at 30 °C for 24 h before counting. Three independent biological reproductions were carried out.

2.3. Growth model fitting

Growth of *L. monocytogenes* LO28 WT, V14 and V15 during mono-culture enrichment was modeled with the three-phase model (Buchanan et al., 1997):

$$y = \begin{cases} \log_{10}N_0 & t \le \lambda \\ \log_{10}N_0 + \mu(t-\lambda) & \lambda < t < t_s \\ \log_{10}N_{max} & t \ge t_s \end{cases} \quad (2)$$

where y is the $\log_{10}$ concentration ($\log_{10}$CFU/mL) at time t (h); $\log_{10}N_0$ is the initial concentration ($\log_{10}$CFU/mL); $\log_{10}N_{max}$ is the concentration at stationary phase ($\log_{10}$CFU/mL); μ is the maximum growth rate ($\log_{10}$/h); λ is the lag time (h); t_s is the time to reach stationary growth

phase (h). In some cases, the growth data did not show a clear stationary phase, so a two-phase model, which was a three-phase model without stationary phase, was used for model fitting in those cases:

$$y = \begin{cases} \log_{10}N_0 & t \le \lambda \\ \log_{10}N_0 + \mu(t-\lambda) & t > \lambda \end{cases} \quad (3)$$

The model was fitted using an adapted version of the R package biogrowth (0.2.3) (Garre et al., 2023), accessed from https://github.com/xchuam/biogrowth/tree/two_phase_model.

The model was fitted to the biological replicates data together. The fitting results were evaluated to check whether λ was significantly ($\alpha = 0.05$) different from zero. If the λ was not significantly different from zero, the F-test was applied to verify if fixing the λ to zero was statistically acceptable. The f value was calculated by the following equation:

$$f = \frac{(RSS_2 - RSS_1)/(DF_2 - DF_1)}{RSS_1/DF_1} \quad (4)$$

where RSS_1 is the residual sum of squares of the full model (i.e., model with λ); RSS_2 is the residual sum of squares of the reduced model (i.e., model without λ); DF_1 and DF_2 are the degrees of freedom for the full and reduced models, respectively. The f value was tested against the F table value (95 % confidence, $F_{DF_2}^{DF_2-DF_1}$). If the f value was smaller than the F table value, the F-test was accepted and the λ was fixed at zero.

To decide the inclusion of the stationary phase in the three-phase model, the adequacy and the fitting performance of the models with determined λ setting was further checked according to Den Besten et al. (2006). The mean square error (MSE_{model}) was used to measure the adequacy of the model to describe the data.

$$MSE_{model} = \frac{RSS}{DF} = \frac{\sum_{i=1}^{n}\left(\log_{10}N_i^{observed} - \log_{10}N_i^{fitted}\right)^2}{n-p} \quad (5)$$

RSS is the residual sum of squares; DF is the degree of freedom; n is the number of data points; p is the number of parameters of the model; $\log_{10}N_i^{observed}$ is the observed population level ($\log_{10}$CFU/mL); $\log_{10}N_i^{fitted}$ is the fitted population level ($\log_{10}$CFU/mL).

The F-test was used to decide if the fitting performance of the model was statistically accepted. The f value was calculated by the following equation:

$$f = \frac{MSE_{model}}{MSE_{data}} \quad (6)$$

where MSE_{model} is the mean square error of the model and MSE_{data} is the mean square error of the data for replicate values, which indicates the measuring error. MSE_{data} was calculated by the following equation:

$$MSE_{data} = \frac{RSS}{DF} = \frac{\sum_{i=1}^{m}\sum_{j=1}^{k}\left(\log_{10}N_i - \log_{10}N_{ij}\right)^2}{n-m} \quad (7)$$

where $n = m{\cdot}k$ is the number of data points; m is the number of time points (sampling times); k is the number of replicates at each time point i; $\log_{10}N_{ij}$ ($\log_{10}$CFU/mL) is the population at time point i for specific replicate j; $\log_{10}N_i$ ($\log_{10}$CFU/mL) is the mean value of the population at time point i.

For the F-test, the f value was tested against F table value (95 % confidence, $F_{DF_{data}}^{DF_{model}}$). If the f value was smaller, the F-test was accepted, and the model describes the observed data well.

To compare the differences between each strain, the λ and the μ were estimated by fitting the selected model to each biological replicate, and significant differences between strains were tested and plotted in R with the ggsignif package (Ahlmann-Eltze and Patil, 2021).

2.4. Co-culture kinetics prediction

The three-phase model was used to predict the growth kinetics of WT and V14 or V15 during co-culture. In the prediction, the initial concentration $\log_{10}N_0$ was assumed the same as the concentration in the quantitative PCR (qPCR) experiment (see Section 2.5). The λ and μ were estimated by fitting the selected model to the biological replicates data together of the mono-cultures, and these parameter estimates were used for the prediction of the co-culture growth kinetics. The $\log_{10}N_{max}$ was assumed to be the mean value of the highest concentration from each replicate of mono-culture. Also, the prediction model took into account the Jameson effect, assuming that when the concentration of one strain reaches $\log_{10}N_{max}$, both strains reach the stationary phase (Jameson, 1962). The lowest and highest confidence interval (95 %) value of $\log_{10}N_0$, λ, μ, and $\log_{10}N_{max}$ were used to determine the confidence intervals of each of the strains. The predicted variants detection chances after enrichment were computed by dividing the predicted variants concentrations by the sum of the predicted variants and WT concentrations.

2.5. Co-culture kinetics identification by qPCR

Previously reported WT-specific and V14-specific primers (Metselaar et al., 2016) were used for qPCR by targeting the DNA deletion region in V14 (Table S1). The ON cultures of WT and V14 were made and plated on BHI agar plates after appropriately diluting to determine the initial concentration. Then, WT and V14 ON cultures were diluted 100,000 times in the enrichment media, and 500 μL diluted culture of each strain was added together to the same flask with 49 mL enrichment media and enriched as described previously (see Section 2.2). For heat treatment effect investigation, equal amount of WT and V14 ON culture were mixed and exposed to heat treatment as describe below (see Section 2.6). Then, a 5 mL heat-treated sample was added to 45 mL enrichment media and enriched as described previously. At time points 24, 36 and 48 h, the co-culture enrichment culture was plated on BHI agar after appropriately diluting, and 2 mL culture was sampled for DNA isolation by the DNeasy blood and tissue kit (Qiagen), using the protocol with pretreatment for Gram-positive bacteria with lysozyme and proteinase K incubations for 1 h. DNA was stored at −20 °C with maximum three times freeze-thaw cycle until qPCR analysis. The BHI agar plates were incubated at 30 °C for 24 h before counting. Based on the counting results, the total concentration of WT and V14 during co-culture enrichment could be determined and used for qPCR results verification. To make the qPCR standard curve suitable for each time point, WT and V14 were also enriched as mono-culture. At time points 24, 36 and 48 h, the mono-culture enrichment cultures of WT and V14 were plated on BHI agar plates after appropriately diluting, and 2 mL culture of each strain were mixed for DNA isolation, using the same protocol as co-culture enrichment. The DNA samples of the co-culture enrichment and standard curve were serially diluted by Milli-Q water, mixed with Power SYBRgreen mastermix (Applied Biosystems), and added to a Hard-Shell 96-well PCR plate (Bio-Rad). The qPCR was done using the qPCR machine CFX96 (Bio-Rad) at an annealing temperature of 60 °C. Threshold cycle (C_T) values were determined with automatic baseline settings. The concentration of WT and V14 were calculated based on the standard curve and verified by comparing with the plate counting results of the total concentration. The qPCR measured V14 detection chance was computed by dividing the V14 concentration by the total concentration of both V14 and WT. To validate the V14 detection chance in co-culture enrichments, colonies from BHI plates at time points 24 h and 48 h were randomly selected for DNA isolation using InstaGene matrix (Bio-Rad). The isolated DNA was mixed with Power SYBRgreen mastermix, added to a Hard-Shell 96-well PCR plate, followed with qPCR analysis with V14-specific primers, using the DNA sample of WT and V14 as negative and positive control, respectively. Three independent biological reproductions were carried out for each co-culture experiment.

2.6. Heat treatment conditions

Heat treatments were carried out in BHI at 60 °C for 8.5 min. For mono-culture enrichment, 0.1 mL WT, V14 or V15 ON culture were added to 9.9 mL 60 °C pre-heated BHI (i.e., 1 % [v/v]). For co-culture enrichment, equal amount of WT and V14 ON culture were mixed, and 0.4 mL mixed ON culture were added to 19.6 mL 60 °C pre-heated BHI (i.e., 1 % [v/v] of both WT and V14). After 8.5 min, a 5 mL heat-treated culture was transferred to 45 mL enrichment media immediately and cultured as described previously. At least three independent biological reproductions were carried out for each strain.

2.7. Mono-culture enrichment after low pre-culturing temperature and heat treatment

ON cultures of WT, V14 and V15 were inoculated into fresh BHI (0.1 % [v/v]) and incubated at 20 °C, 10 °C or 7 °C. The culture was grown under shaking at 160 rpm until the stationary phase (~9 $\log_{10}$CFU/mL). The stationary phase culture was exposed to heat treatment as described previously (see Section 2.6). The heat-treated cultures were added to enrichment media and cultured as described in Section 2.2 with sampling at time points 0, 24 and 48 h. All the samples were spread plated on BHI agar plates after appropriately diluting and plates were incubated at 30 °C for 24 h before counting. Three independent biological reproductions were carried out.

2.8. Colony difference identification for L. monocytogenes isolation procedure

WT, V14 and V15 were enriched as described in Section 2.2. After 24 h of enrichment in HFB, FB, and BLEB and 48 h enrichment in BLEB, the culture was streaked on an ALOA agar plate (BioMérieux), a Rapid' *L. mono* agar plate (Bio-Rad), a PALCAM medium agar plate (Oxoid) and on a OXA plate (Listeria Selective Agar [Oxford], Oxoid). The ALOA plates and Rapid' *L. mono* plates were cultured at 37 °C, and the PALCAM plates and OXA plates were cultured at 35 °C following the recommendations of the suppliers. All the plates were checked after 24 h and 48 h of incubation.

3. Results and discussion

3.1. rpsU gene is conserved in the L. monocytogenes genome database

To analyze the genotype variation of the *L. monocytogenes* genes, a *L. monocytogenes* genome database was constructed, which includes 51,784 genome assemblies for strains isolated from seven main categories: food, feed, clinical, animal, food associated environment, other environment, and unknown (Fig. 1). For each of the genomes, 2867 *L. monocytogenes* genes were analyzed, and the gene variation levels are shown in Fig. 2. Among all the analyzed genes, *rpsU* exhibited the lowest variation level, with a notable distinction from the other genes. The *rpsU* gene has been found in 51,768 genomes, but there were only 49 genomes that showed a mutation in the *rpsU* gene, which was around 0.1 % of all the genomes available in the genome database. These 49 genomes with *rpsU* mutations were from clinical isolates (28), food isolates (8), other environment isolates (8), and unknown resource (5). Notably, in the rest 16 genomes without identified *rpsU* sequence, 8 genomes exhibited partial *rpsU* sequences due to their location at the contig edges, while the remaining 8 genomes raised concerns due to low data quality warnings on the NCBI website or their high contig count, exceeding 370. Collectively, these findings suggest that the *rpsU* gene exhibits a remarkable level of conservation within the *L. monocytogenes* genome database.

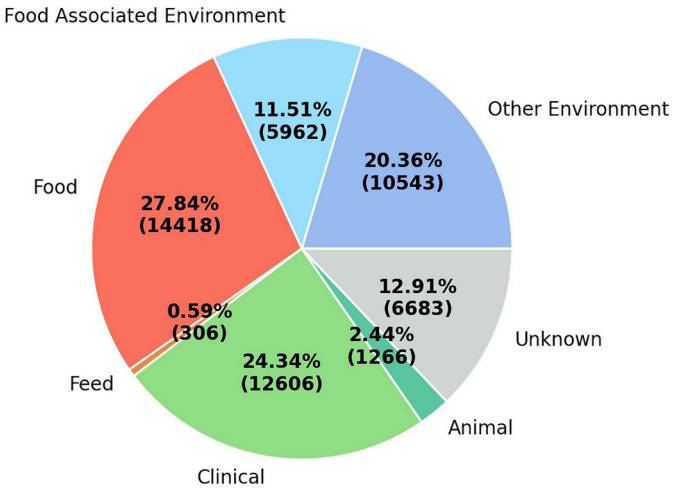

Fig. 1. Isolation origins of sequenced *L. monocytogenes* isolates deposited in the NCBI genome database. The prevalence is shown in percentage with the sample number between brackets.

3.2. rpsU mutant detection chance reduced during enrichment

Our previous research showed that *rpsU* variants have a stress resistance advantage over the WT strain, and mutations in *rpsU* enables switching between multiple-stress resistant and high fitness states in *L. monocytogenes* (Koomen et al., 2021; Koomen et al., 2018; Metselaar et al., 2015; Metselaar et al., 2013). Therefore, it was expected that *rpsU* mutations were widely spread in *L. monocytogenes*. However, the genome analyses demonstrated that the *rpsU* gene had a high conservation level in the *L. monocytogenes* genome database. A possible explanation may be that the *L. monocytogenes* detection methods may introduce an isolation bias and a reduced detection chance of *rpsU* variants. To further investigate this, the *L. monocytogenes* LO28 WT and *rpsU* variants V14 and V15 were cultured and plated according to two commonly used *L. monocytogenes* detection methods, the ISO 11290-1:2017 standard and the FDA BAM standard.

The *L. monocytogenes* strain LO28 WT and *rpsU* variants V14 and V15 were individually cultured according to the ISO 11290-1:2017 and the BAM methods (Fig. 3). The three-phase model was used to fit the growth data and the inclusion of lag phase λ and stationary phase $\log_{10}N_{max}$ was tested. For each strain, the lag phase was not included in the model for FB and the stationary phase was not included for HFB. In HFB and BLEB, there was no significant difference in the λ between the WT and the *rpsU* variants (Fig. 4, A), while the WT had a significantly higher growth rate than the *rpsU* variants in HFB and BLEB but not in FB (Fig. 4, B). Specifically, the average growth rates of WT, V14, and V15 in HFB were 0.25, 0.20, and 0.20 $\log_{10}$/h, respectively, and in the BLEB medium, the average growth rates of WT, V14, and V15 were 0.30, 0.24, and 0.24 $\log_{10}$/h, respectively. However, in the FB medium, the average growth rate of WT was even slightly but not significantly lower than that of V14 and V15, with values of 0.29, 0.30 and 0.32 $\log_{10}$/h, respectively. Notably, the culture temperature is 30 °C in HFB and BLEB but 37 °C in FB. Previous research based on nutrient-rich medium BHI showed that the growth rate of *rpsU* variants, relative to the WT, is more significantly reduced at lower temperatures (Fig. S7) (Metselaar et al., 2016). Therefore, the differences in growth rates between the WT and the *rpsU* variants in HFB and BLEB may be attributed to the culture temperature of 30 °C rather than the culture media.

3.2.1. rpsU variants detection chance reduced during co-culture enrichment

To investigate how the growth rate differences affected the detection chance of *rpsU* variants after the enrichment, the growth behavior of WT and *rpsU* variant V14 during co-culture were predicted by using the fitted parameters of mono-culture growth data (Fig. 5). Since the

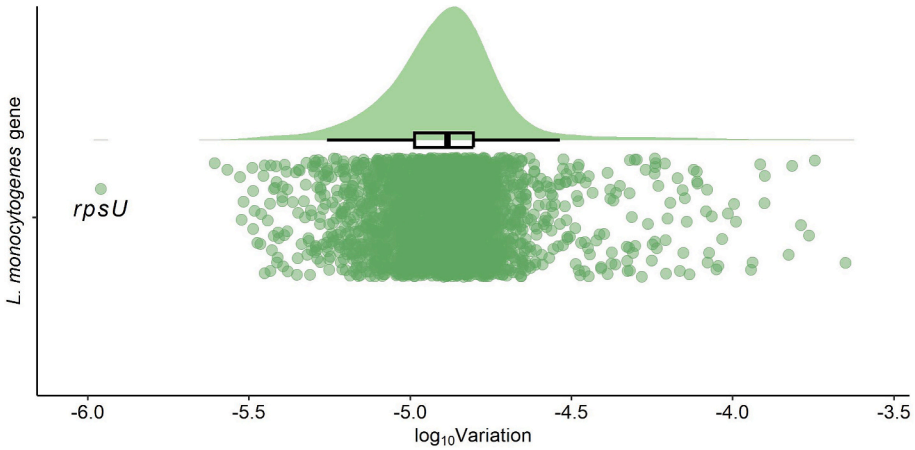

Fig. 2. Raincloud plot of *L. monocytogenes* gene variation levels in the genome database. The point that represents the *rpsU* gene variation level has been labeled.

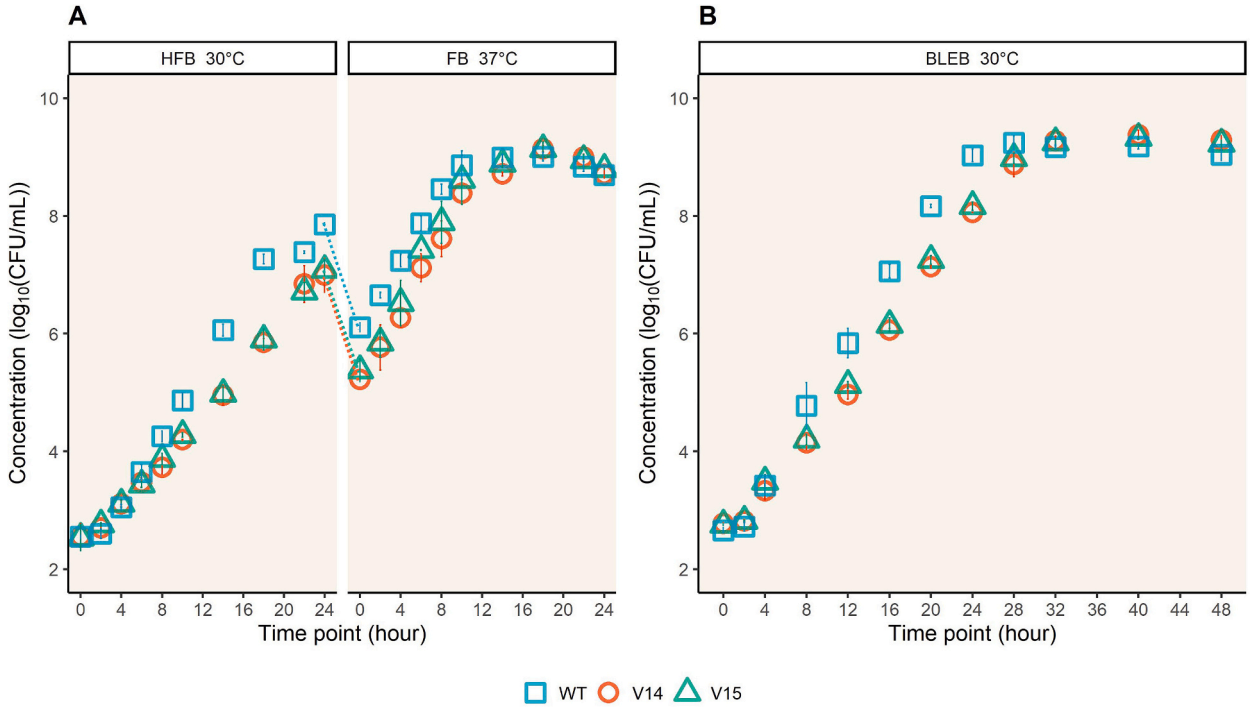

Fig. 3. Mono-culture growth kinetics of LO28 WT, V14 and V15 during enrichment by following the ISO standard (A) and the BAM standard (B). The dotted lines indicate a 1:100 (v/v) inoculation from HFB to FB.

enrichment culture should be sampled after incubation for 24 and 48 h according to the ISO and the BAM detection procedure, the variant detection chances at 24 and 48 h were calculated. The predicted results show that the detection chance of *rpsU* variants reduced from ~52 % to ~7 % in HFB after 24 h (Fig. S2, A). In BLEB, the detection chance reduced from ~52 % to ~3 % after 24 and 48 h co-culture (Fig. S2, A). The prediction of WT and V15 co-culture behavior shows similar results as expected, since the growth parameters were similar between V14 and V15 (Fig. S1 and Fig. S2, A). To verify the prediction results, WT and V14 were co-cultured according to these isolation standards and measured by qPCR (Fig. 5). Comparing the qPCR results and the prediction results, the qPCR results were mostly found in the confidence interval of the predicted results. The prediction model might however overestimate the growth of V14 in FB and BLEB, since in both media the V14 qPCR results were at the lower end of the confidence interval. Nevertheless, the qPCR results confirmed that the detection chances of V14 were reduced after co-culture enrichment.

In FB with 37 °C as culture temperature, the WT strain did not exhibit a significantly higher growth rate than the V14 strain during mono-culture (Fig. 4, B). However, the detection chance of V14 reduced during co-culture enrichment in FB (Fig. S2, B), suggesting that factors beyond mere differences in mono-culture growth rates contribute to the competitive advantage of the WT over *rpsU* variants in co-culture conditions. Indeed, previous studies have reported that the outgrowth of a strain in co-culture cannot only be explained by growth rate differences during mono-culture (Gorski et al., 2006; Mellefont et al., 2008; Zilelidou et al., 2016b; Zilelidou et al., 2015). This evidence underscores the complexity of competitive dynamics between WT and the *rpsU* variants in co-culture environments, which cannot be fully exampled by a Jameson-effect model.

3.2.2. Detection chance of heat-treated rpsU variants also reduced during co-culture enrichment

L. monocytogenes rpsU variants have higher stress resistance than the

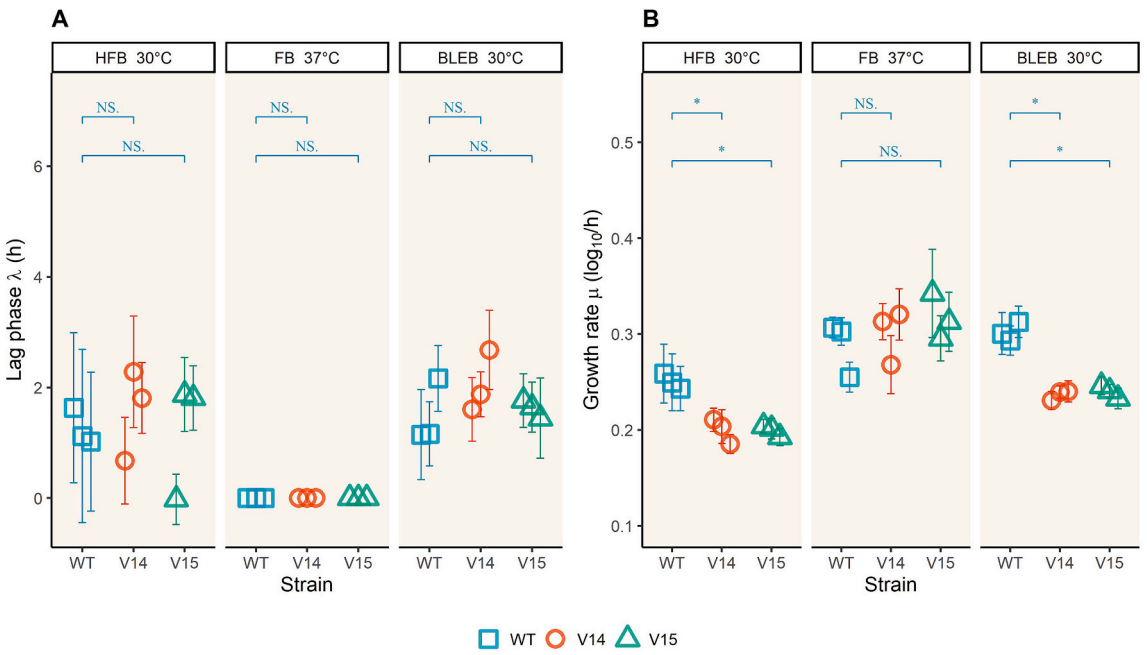

Fig. 4. Mono-culture growth parameter estimates of LO28 WT, V14 and V15 in enrichment broth. Panel (A) and Panel (B) show the fitting results of lag phase (h) and growth rate (log_{10}/h), respectively. The points represent the best estimated values, and the error bars represent the 95 % confidence interval. Significant differences are indicated by an asterisk, and no significant differences are indicated by NS.

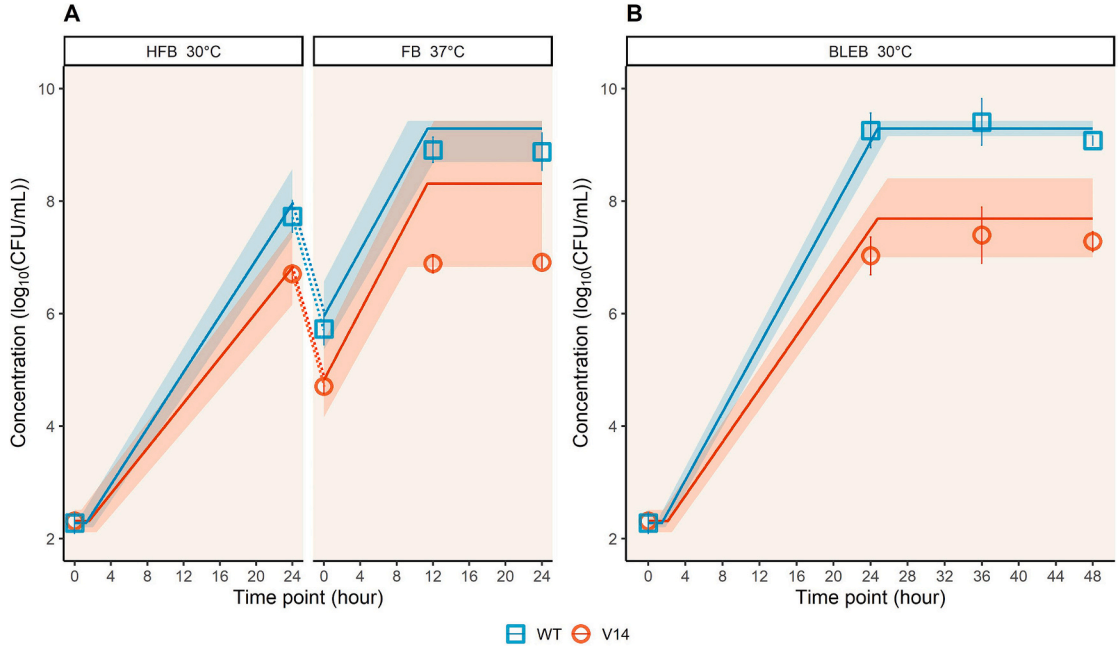

Fig. 5. Co-culture growth predictions and validations of LO28 WT and V14 during enrichment by following the ISO standard (A) and the BAM standard (B). Co-culture growth predictions, which are shown as solid lines with confidence interval as shadow, according to the three-phase model were based on estimated parameters from mono-culture. Validations were done by qPCR (blue square for WT and red circle for V14) of co-culture. The error bars indicate standard deviations. The dotted lines indicate a 1:100 (v/v) inoculation from HFB to FB.

LO28 WT (Metselaar et al., 2015). To investigate if the higher stress resistance of *rpsU* variants V14 and V15 results in a faster recovery after heat treatment during enrichment, the *L. monocytogenes* strain LO28 WT and *rpsU* variants V14 and V15 were exposed to 60 °C for 8.5 min and then individually cultured according to the ISO 11290-1:2017 and the BAM methods (Fig. S3). The three-phase model was used to fit these growth data. For each strain, the lag phase was not included in the model for FB and the stationary phase was not included for HFB. After heat treatment, there were again no significant differences between the lag phase of the WT and the *rpsU* mutants V14 and V15 in HFB, with average values of 2.8, 2.7, and 2.3 h, respectively. V15 had even a significantly higher lag phase than WT in BLEB, with average values of 5.6 and 2.7 h, respectively (Fig. 6, A). The growth rate of the WT was again significantly higher compared to the *rpsU* variants in HFB and BLEB but not in FB (Fig. 6, B). Therefore, high resistance *rpsU* mutants did not have a faster recovery but again had a growth disadvantage

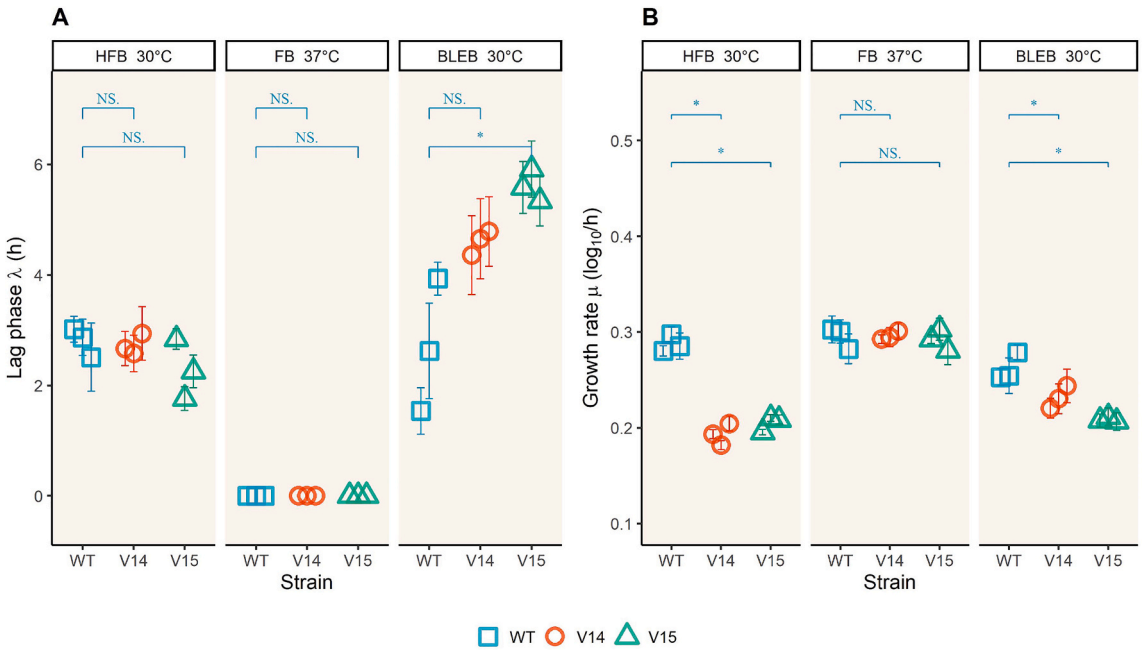

Fig. 6. Mono-culture growth model fitting results of heat-treated LO28 WT, V14 and V15. Panel (A) and Panel (B) show the fitting results of lag phase (h) and growth rate (log_{10}/h), respectively. The points represent the best estimated values, and the error bars represent the 95 % confidence interval. Significant differences are indicated by an asterisk, and no significant differences are indicated by NS.

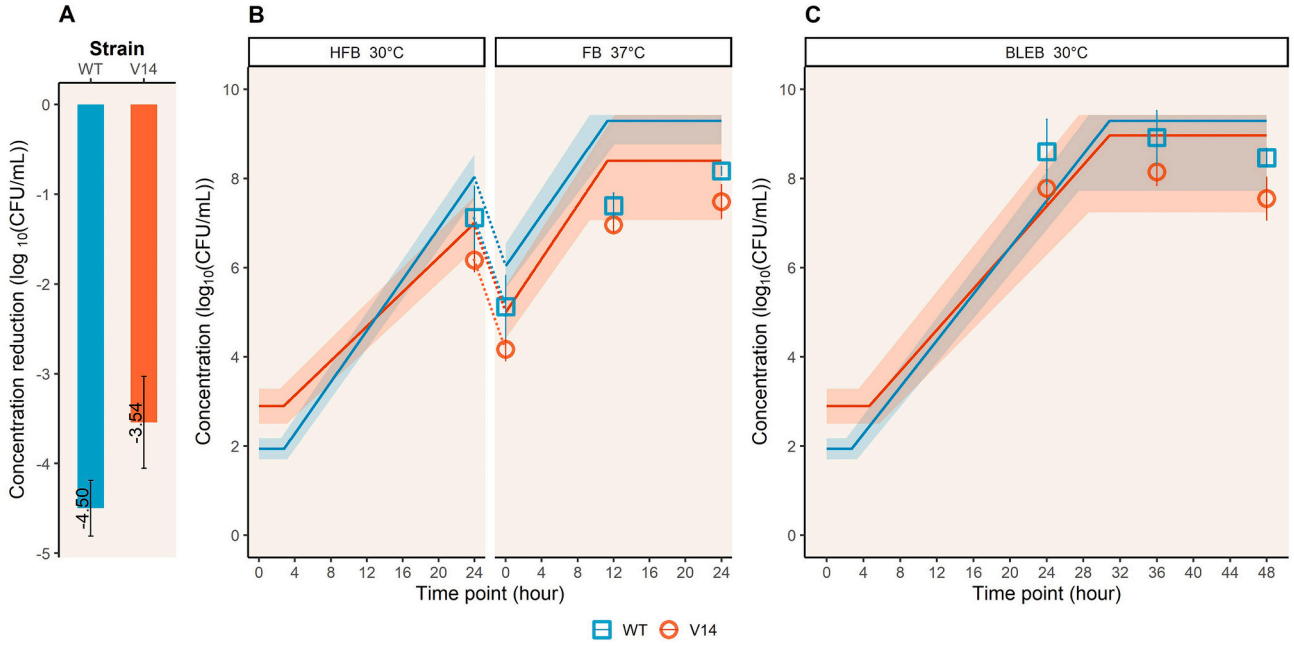

Fig. 7. Heat reduction of LO28 WT and V14 (A) and co-culture growth predictions and validations of heat-treated LO28 WT and V14 during enrichment by following the ISO standard (B) and the BAM standard (C). Heat reductions were measured by exposing stationary phase culture at 60 °C in BHI for 8.5 min and shown as bar plot with reduction level and standard deviation. Co-culture growth predictions, which are shown as solid lines with confidence interval as shadow, according to the three-phase model were based on estimated parameters from mono-culture after heat treatment. Validations were done by qPCR results (blue square for WT and red circle for V14) in co-culture. The error bars indicate standard deviations. The dotted lines indicate a 1:100 (v/v) inoculation from HFB to FB.

during enrichment after heat treatment.

To investigate how the stress resistance difference affected the growth behavior and detection chance of WT and *rpsU* variants during co-culture, the co-culture growth behavior of WT and V14 were predicted using the fitted parameters of the mono-culture growth data after heat treatment (Fig. 7, B and C). For prediction, the initial concentration of WT and the *rpsU* variants was assumed to be the same before heat treatment. Since the WT had around 1 log_{10}(CFU/mL) more reduction than V14 after heat treatment (Fig. 7, A), WT was around 1 log_{10}(CFU/mL) less than V14 at t0 of the enrichment. The predicted results show that the detection chances were still reduced for V14 in both enrichment methods (Fig. S5, A). The prediction of WT and V15 co-culture behavior shows similar results (Fig. S4 and Fig. S5, A). To verify the prediction results, WT and V14 ON culture were mixed, exposed to heat treatment, co-cultured according to these isolation standards and measured by qPCR (Fig. 7, B and C). This confirmed that the detection chance of heat-

stressed resistance variants was reduced after the enrichment (Fig. 7 and Fig. S5, B).

It has been reported that the growth defect of *rpsU* variants was more pronounced at lower temperature (Metselaar et al., 2016). To further investigate the effect of pre-culturing temperature on the growth of *L. monocytogenes* WT and the *rpsU* variants during enrichment, LO28 WT, V14 and V15 were pre-cultured at 7 °C, 10 °C or 20 °C, exposed to heat treatment and then enriched in HFB, FB, or BLEB. The growth kinetic of these cultures during enrichment were rather similar to 30 °C pre-cultured cells (Fig. 8), suggesting that the detection chance reduction of the heat treated *rpsU* mutants when applying the ISO or BAM procedure will not be altered by the pre-culturing temperature followed

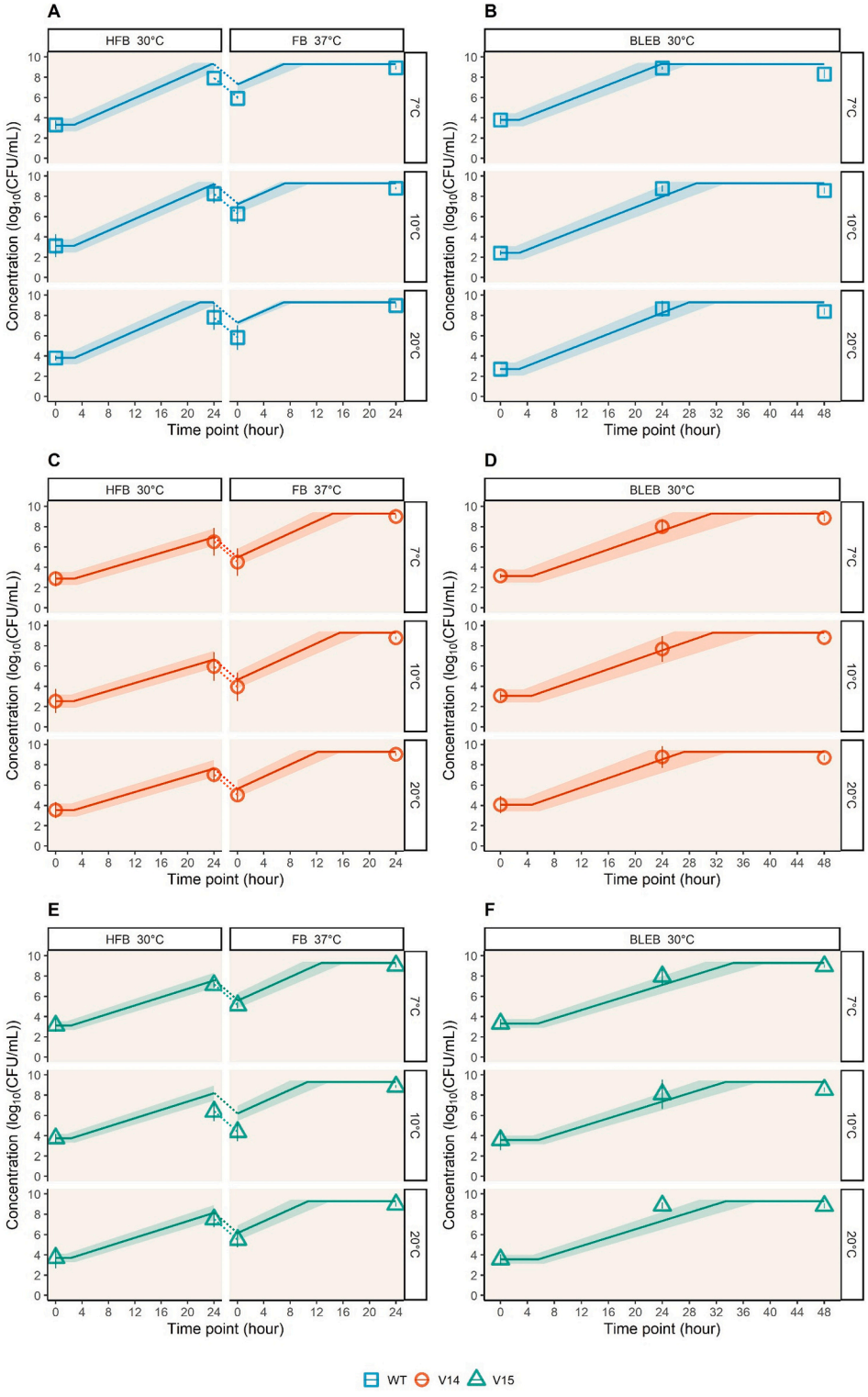

Fig. 8. After pre-culturing at 7 °C, 10 °C and 20 °C and heat treatment at 60 °C for 8.5 min, mono-culture growth kinetics of LO28 WT (green square), V14 (red circle) and V15 (green triangle) during enrichment by following the ISO standard (A, C and E) and the BAM standard (B, D and F). The solid lines indicate the growth prediction according to the three-phase model based on estimated parameters from mono-culture after 30 °C ON culture and heat treatment. The shadow indicates the prediction confidence interval. The dotted lines indicate a 1:100 (v/v) inoculation from HFB to FB. The error bars indicate standard deviations.

by heat treatment. Notably, the predicted concentrations in HFB after 24 h were consistently slightly higher than the plate-counting results. This may be due to a longer lag phase for adaptation from lower pre-culture temperatures to the enrichment temperature. Further studies are needed to elucidate the detailed growth kinetics of these strains after pre-culturing at lower temperatures and exposing to heat treatment.

3.2.3. Isolation procedure does not affect the detection chance of rpsU variants

After 24 and/or 48 h of enrichment in HFB, FB or BLEB, the enrichment culture should be streaked on two different types of selective agar plates following the isolation procedure according to the ISO 11290-1:2017 and the BAM methods. Colony differences between WT and the *rpsU* variants on selective agar plate might lead to the selection preference of a certain strain. To investigate the colony differences between WT and the *rpsU* variants on the selective agar plates, *L. monocytogenes* LO28 WT, V14 and V15 were enriched and streaked on four different types of selective agar plates including ALOA, Rapid' *L. mono*, PALCAM, and OXA plates. As shown in the Fig. S6, WT and *rpsU* mutants had similar colony shape, size, and colour, so it was difficult to distinguish WT and *rpsU* mutants based on the colony characterization on these selective agar plates. Therefore, the isolation procedure seems not to affect the detection chance of *rpsU* variants.

Here, we demonstrated that the lower fitness of the *rpsU* mutants resulted in a lower detection chance compared to the WT strain when enrichment-based detection procedures are applied to isolate *L. monocytogenes*. Consequently, this approach may underestimate the genotypic diversity of *L. monocytogenes* in a sample for enrichment. Therefore, this phenomenon could contribute to the underrepresentation of *rpsU* mutants in the *L. monocytogenes* genome database. Previous studies reported a bias in the *L. monocytogenes* enrichment procedure at the lineage and strain levels (Bannenberg et al., 2021; Bruhn et al., 2005; Zilelidou et al., 2016a; Zilelidou et al., 2016b), and the current study underlines that such a bias can be extended to the sub-strain level. In line with this, *rpsU* mutations were not identified in studies employing whole-genome sequencing to investigate persistent *L. monocytogenes* strains isolated using enrichment-based methods, including the ISO 11290-1:2017 and the BAM methods (Castro et al., 2021; Cherifi et al., 2018; Lucchini et al., 2023; Palma et al., 2020; Simmons et al., 2014; Stasiewicz et al., 2015). Although stress resistance variants, such as *rpsU* variants, may significantly contribute to the overall stress resistance of the *L. monocytogenes* population, the fraction of these stress resistance variants is generally low in non-stressed populations (Metselaar, 2016). The detection bias induced during enrichment, resulting in infrequent isolation, and their rareness in non-stressed population may have contributed to the observed low prevalence of *rpsU* variants in the genome and phenotype databases.

Also in other *Bacillales* bacteria, *rpsU* mutations have been reported to impact phenotype significantly. In *Bacillus subtilis*, a nonsense mutation in the second codon of *rpsU* led to impaired cell separation, defective motility, and robust biofilm formation (Takada et al., 2014). Furthermore, a study on clinical strains of *Staphylococcus aureus* identified *rpsU* mutants after five days of vancomycin treatment, exhibiting increased resistance to vancomycin and lysostaphin, thicker cell walls, and a reduced growth rate (Basco et al., 2019). These findings highlight the phenotypic alterations associated with *rpsU* mutations across various bacterial species beyond *L. monocytogenes*, underscoring the critical role of *rpsU* mutations in bacterial physiology and adaptation. Notably, the level of *rpsU* variation in the genome databases of *B. subtilis* and *S. aureus* is markedly higher than in *L. monocytogenes*, with $\log_{10}$*Variation* values of −4.1, −4.2, and − 6.0, respectively, which suggests a higher *rpsU* mutation detection chance for *B. subtilis* and *S. aureus*. The potential link between the higher detection rates of *rpsU* mutations in these two species and the growth behavior of wild type strains and *rpsU* mutants during enrichment requires further investigation.

Interestingly, despite *rpsU* mutants being infrequently isolated, a significant proportion of the *rpsU* variants documented in the NCBI genome database were clinical isolates (Fig. S8). This association underscores the potential relevance of *rpsU* variants in epidemiological contexts. A previous study showed that the *rpsU* variants V14 and V15 exhibit elevated expression of internalins, coupled with enhanced adhesion and invasion capabilities in Caco-2 cells, pointing towards a potentially increased virulence of these variants (Koomen et al., 2018). Consequently, the virulence potential of *rpsU* variants, in conjunction with their enhanced stress resistance, underscores the need to closely monitor such genotypes in epidemiological studies, as they may be underrepresented in genomic databases yet play a role in disease incidences.

In conclusion, selective enrichment procedures at 30 °C may inadequately represent the genotypic diversity present in a sample. Hence, this enrichment bias contributes to the underrepresentation of natural mutants in the *L. monocytogenes* genome database.

CRediT authorship contribution statement

Xuchuan Ma: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Jingjie Chen:** Writing – review & editing, Visualization, Software, Methodology, Data curation. **Marcel H. Zwietering:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition. **Tjakko Abee:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Heidy M.W. Den Besten:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110680.

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