Highlights

1. ALE strategy improved growth performances in several mutants of *Lacticaseibacillus casei* N87

2. Mutants of *Lcb. casei* and *Lpb. plantarum* differed from the parental strains in the ability to use heme and menaquinone

3. ALE generated SNPs in genes involved in carbon metabolism, redox balance, cell wall properties

4. Mutants obtained in this study may be useful for several applications

1   **Title**

2   Growth fitness, heme uptake and genomic variants in mutants of oxygen-tolerant *Lacticaseibacillus*

3   *casei* and *Lactiplantibacillus plantarum* strains

4

5   **Running title**

6   Genomic variants in oxygen-tolerant *Lacticaseibacillus casei* and *Lactiplantibacillus plantarum*

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26

Abstract

Adaptive Laboratory Evolution (ALE) is a powerful tool to improve the fitness of industrially relevant microorganisms, avoiding the constraints related to the use of genetically modified strains.

In this study, we used an ALE strategy (serial batch cultivations in aerobic and respiratory conditions) to generate natural mutants (with improved growth, O_2 and heme consumption, stress survival) from the respiration-competent strain *Lacticaseibacillus casei* N87. Genotypic changes in some selected mutants was investigated through whole genome sequencing (WGS) and identification of untargeted mutations. The O_2-tolerant *Lactiplantibacillus plantarum* C17 and its mutant C17-m58 (obtained from a previous ALE study) was included in heme uptake experiments and in WGS and variant calling analyses.

ALE boosted biomass production, O_2 uptake and oxidative stress tolerance in several mutants of *Lcb. casei* N87 cultivated under aerobic and respiratory conditions. Mutants of *Lcb. casei* and *Lpb. plantarum* differed from the parental strains in the ability to use heme and menaquinone; high concentrations (> 10 mg/L) of the first cofactor were toxic for both wt and mutant strains, while menaquinone relieved heme-associated stress when the latter was supplied at limited doses.

ALE generated single nucleotide modifications (SNPs) in both coding sequences and intergenic regions of mutant genomes. SNPs affected some genes encoding for proteins and transcriptional regulators involved in carbon metabolism, oxidative stress, redox balance, and cell wall properties.

We confirmed that ALE strategy and adaptation to aerobic and respiratory life-style may improve growth fitness and stress robustness in some lactic acid bacteria.

2

Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of microorganisms used as starter, adjunct and/or probiotic cultures for the production of many fermented and functional foods. The industrial potential of LAB, however, is much wider and many strains have been exploited, or are currently being investigated, as microbial cell factories for the production of different value-added compounds, such as food ingredients, nutraceuticals, pharmaceutical precursors, biofuels, building blocks for bioplastics (Brown et al., 2017; Lübeck and Lübeck, 2019; Sauer et al., 2017; Song et al., 2017).

In the last decades, several strategies based on DNA manipulation were used to improve metabolite production, fitness and competitiveness of LAB. Omics approaches and high-throughput sequencing technologies have contributed to the understanding of metabolic features and regulation pathways of LAB, improving their application in food, pharma and biochemical industries (Borner et al., 2019; Gaspar et al., 2013; Hatti-Kaul et al., 2019; Wu et al., 2017), and allowing a more targeted manipulation of the strains. Initial engineering efforts were focused on the central carbon metabolism of LAB (at the pyruvate node) to drive the production of important fermentation end-products (e.g. organic acids, aroma compounds), and were mainly related to dairy applications (de Vos and Hugenholtz, 2004; Kleerebezem and Hugenholtz, 2003; Gaspar et al., 2013). Over time, metabolic engineering strategies have been applied to several LAB species to boost the production of different compounds, to understand the molecular basis of stress defence mechanisms (Bron et al., 2010; Wu et al., 2017) and develop more competitive cultures.

Genetic manipulation, however, suffers from different limitations (Bachmann et al., 2017) mainly related to the in-depth knowledge of target genes and metabolic pathways to be engineered, to the availability of strains with genetically accessible and transformable cells, and to the restrictions on the use of genetically modified microorganisms (GMM). The latter constraint is still debated within the European Union (Borner et al., 2019; Johansen ,2017, 2018) and the reluctance of consumers towards foods containing GMM plays a significant role on the use of GMM in food and biomedical applications.

3

79 Because of this, natural strategies based on evolutionary engineering and on the shift of metabolic

80 pathways have been re-evaluated and used to generate industrially relevant microorganisms. Adaptive

81 laboratory evolution (ALE), generally, entails the prolonged cultivation (e.g. serial batch

82 propagations, chemostat cultures) of a microorganism in defined conditions for hundreds to thousands

83 of generations in attempt to develop an "improved phenotype" with desired features (Bachmann et

84 al., 2015, 2017). Although ALE does not require specific knowledge of genomic traits of strain, the

85 propagation system (i.e. selective forces, population size, number of propagations) may affect the rate

86 and type of mutations (Bachmann et al., 2015, 2017; Dragosits and Mattanovich, 2013). The

87 correlations among phenotype and genotype (i.e. untargeted mutations) of evolved strains can be

88 easily obtained by whole genome re-sequencing and identification of genomic variants. To date,

89 ALE has been applied to different LAB to modulate biomass yield, stress tolerance and survival,

90 production of organic acids and bacteriocins, antibiotic adaptation (Bachmann et al., 2017; Cubas-

91 Cano et al., 2019; Kwon et al., 2018; Liang et al., 2020; Papiran et al., 2021; Wang et al., 2017).

92 Regarding the boosting strategies based on natural metabolic shift, several authors demonstrated that

93 in some LAB the shift from anaerobic fermentative metabolism towards aerobic respiration may

94 result in different physiological advantages, including increased production of biomass and

95 metabolites, synthesis of ROS-scavenging enzymes, and robustness to several stresses (i.e. oxidative,

96 freezing, freeze-drying; Zotta et al., 2017).

97 In this work we combined the ALE strategy and the natural metabolic shift (serial batch cultivations

98 under aerobic and respiratory pressures) to generate naturally evolved mutants from the respiration-

99 competent strain *Lacticaseibacillus casei* N87 (Ianniello et al., 2016). Growth fitness, tolerance of

100 oxygen, heme and menaquinone (cofactors for the synthesis of a respiratory chain), as well as the

101 genomic variants that may possibly have affected the evolved phenotypes were evaluated. The

102 evolutive mutations generated in a natural mutant of the O_2-tolerant *Lactiplantibacillus plantarum*

103 C17 (obtained from a previous ALE study; Zotta et al., 2014a) were identified and analysed in this

104 study, and correlated to the phenotypic performances of the strain.

105

2. Materials and methods

107

2.1. Strains and culture conditions

Lacticaseibacillus casei N87 (from infant faeces; Ianniello et al., 2016), *Lactiplantibacillus plantarum* C17 (from Caciocavallo cheese) and its mutant *Lpb. plantarum* C17-m58 (Ricciardi et al., 2015a; Zotta et al., 2014a) were used in this study. The strains were maintained as freeze-dried stocks in reconstituted 11% (w/v) Skim Milk containing 0.1% (w/v) ascorbic acid, in the culture collection of the Laboratory of Industrial Microbiology, Università degli Studi della Basilicata, and were routinely propagated in Weissella Medium Broth, pH 6.8 (WMB; Zotta et al., 2012), for 16 h at 37°C (*Lcb. casei*) or 35°C (*Lpb. plantarum*).

Lcb. casei N87 was used to generate random mutants through serial batch propagations (this study, section 2.2), while a menadione and H_2O_2 tolerant mutant *Lpb. plantarum* C17-m58 was obtained through a colony-selection experiment in a previous study (Zotta et al., 2014a).

119

2.2. Adaptive evolution of Lcb. casei N87 through serial batch propagations

The wild-type (wt) *Lcb. casei* N87 (hereafter wt-N87) was propagated at 37°C in buffered (with 0.1 mol/L morpholin propanesulphonic acid, MOPS) modified WMB (mWMB; Ianniello et al., 2015), initial pH 6.8, in aerobic (AE; shaken flasks on a rotary shaker at 150 rpm) and respiratory (RS; AE + supplementation with 2.5 µg/uL hemin, 1 µg/uL menaquinone) conditions. Sixteen serial batch AE and RS propagations were performed using different levels of inoculum and time of incubation, as described in **Table 1**. The first propagation (P0, control) of both AE and RS conditions was inoculated (1% v/v) with an overnight wt-N87 culture, grown anaerobically (AN, static bottle) in WMB, and washed twice in 20 mmol/L phosphate buffer pH 7.0 (PB7). The subsequent (P1-P16, adaptive growth) AE and RS propagations were inoculated, respectively, with the previous propagated AE or RS cultures, washed twice in PB7. The AN culture of wt-N87, grown in mWMB for 24 h at 37°C,

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131   was used as control to evaluate the growth fitness of wt and mutant strains.

132

2.3. Analyses during serial batch propagations

134   Optical density at 650 nm (OD_{650}; SmartSpec Plus Spectrophotometer, Bio-Rad Laboratories), pH

135   (Double Pore Slim electrode, Hamilton Company, Reno, Nevada, USA), biomass production (cell

136   dry weight, CDW, g/L), colony count on WMB agar (WASP Spiral Plater, bioMérieux Italia SpA,

137   Bagno a Ripoli, Firenze, Italy; incubation of plates at 37°C for 48 h) and O_2 uptake (time of resazurin

138   discoloration; Ricciardi et al., 2014) were measured at the end of each propagation (P0-P16). Cell

139   morphology was checked (Axioskop microscope, Carl Zeiss, Germany) to verify possible

140   contaminations during propagation. The AN culture of wt-N87 (24 h, 37°C) was used as control. Two

141   technical replicates were performed for each analysis.

142

2.4. Isolation and selection of evolved mutants

144   A total of 710 colonies (**Table 1**) were randomly picked from the WMA plates of P1-P16

145   propagations (see section 2.3) and frozen (-24°C) in 25% (v/v) glycerol. Colonies of wt-N87 grown

146   in AN, AE and RS conditions were collected and used as controls.

147   For the selection of potential mutants, all colonies were cultivated overnight in WMB, washed twice

148   with PB7, inoculated (10% v/v) in mWMB (96-well/microplate experiments; 180 µL) in AN, AE and

149   RS conditions, and screened for their tolerance to one or more tested conditions. At the end of

150   incubation (24 h, 37°C), the growth of all isolates was evaluated (OD_{650} measurement with a

151   Multiskan microplate reader; Thermo Fisher Scientific Inc., Waltham, MA, USA) and compared with

152   that of wt-N87 cultivated in the same conditions.

153   Sixty mutants showing differences in   growth performances compared to wt-N87 (< -1.5 or > +1.5

154   fold-change in at least one of the tested conditions) were selected, and their identity   was confirmed

155   by RAPD-PCR (M13 primer; Parente et al., 2010) and Multiplex-PCR (Ventura et al., 2003). Purity

156   of evolved mutants was confirmed by colony morphology on mMRS (Ricciardi et al., 2015b). The

6

157 capability of the 60 selected mutants to grow (OD_{650} values) in AN, AE and RS conditions was

158 confirmed in a 24-well/microplate (1 mL) experiment. All growths experiments were run in

159 duplicates.

160

161 ### 2.5. Kinetics of growth and oxidative stress tolerance

162 Ten mutants selected for the stability in growth behaviour (see results) and for the growth differences

163 in AN, AE and/or RS (section 2.4; 24-well/microplate experiment) compared to wt-N87 (< -1.5 or >

164 +1.5 fold-change) were cultivated in mWMB (2% v/v inoculum) in static bottles (AN) or shaken

165 flasks (AE and RS), for 24 h at 37°C; wt-N87 was used as control. OD_{650} and pH values were

166 measured every 60 min up to 24 h of incubation; for each condition, a standard curve correlating

167 OD_{650} and biomass production (CDW, g/L) was generated. The kinetics of growth of wt and mutant

168 strains were estimated with the dynamic model of Baranyi and Roberts (1994) using the DMFit v.3.5

169 program (Baranyi, 2015). Two biological replicates were carried for each condition.

170 O_2 consumption was measure with resazurin assay, while the tolerance of different H_2O_2

171 concentrations (2-fold dilutions from 220 to 6.87 mmol/L; 96-well/microplate experiment) was

172 measured as describe in Ianniello et al. (2016). Two technical replicates were performed for each

173 cultivation.

174

175 ### 2.6. Effect of hemin and menaquinone on the growth of wt and mutant strains

176 The effect of hemin and/or menaquinone on the growth of *Lcb. casei* wt-N87, *Lpb. plantarum* wt-

177 C17 and their mutants *Lcb. casei* N87-AE51, N87-AE284, N87-RS337 (this study, section 2.5; bold

178 in **Table 2**) and *Lpb. plantarum* C17-m58 (Zotta et al., 2014a) was evaluated by using a concentration

179 gradient experiment carried out in 96-well microplates. Pre-cultures, grown anaerobically in WMB,

180 were standardised (OD_{650}=1) and used to inoculate (10% v/v) mWMB supplemented with different

181 concentrations of hemin (20, 15, 10, 7.5, 5.0, 2.5, 1.25, 0.65, 0.32, 0.16, 0.08, 0 μg/mL; 1-12

182 microplates columns) and/or menaquinone (8, 4, 2, 1, 0.5, 0.25, 0.125, 0 μg/mL; A-H microplates

rows). The microplates were incubated in AE conditions (150 rpm on a rotary shaker) for 24 h at 37°C (*Lcb. casei*) or 35°C (*Lpb. plantarum*). Wild-type and mutant strains cultivated in unsupplemented mWMB under static AN condition (AnaeroGen bags, 24 h at 37°C or 35°C) were used as controls. At the end of incubation (24 h), cell suspensions were 10-fold diluted in PB7 and the OD_{650} was measured using a Multiskan microplate reader. Three biological replicates were carried out for each strain and treatment.

To confirm the toxic effect of hemin on the growth kinetics of wt and mutants, all strains were cultivated (24 h at 37°C or 35°C, respectively for *Lcb. casei* and *Lpb. plantarum*; two biological replicates for each condition) in AE conditions (shaken flasks, 150 rpm) in mWMB supplemented with 10 µg/mL hemin and 1 µg/mL menaquinone. Cultivations in unsupplemented AE (without hemin and menaquinone) and menaquinone-supplemented AE (only 1 µg/mL menaquinone) were included for comparison. OD_{650} were measured every 60 min up to the end of incubation, and the parameters of growth kinetics were estimated as described in *section 2.5*.

2.7. Genome sequencing and identification of genomic variants

The genomic DNA of *Lpb. plantarum* wt-C17 and its mutant C17-m58, and *Lcb. casei* N87-AE51, N87-AE284 and N87-RS337 (mutants of *Lcb. casei* wt-N87; bold in **Table 2**), was isolated by using the GeneElute Bacterial Genomic DNA Kit (Sigma-Aldrich) and quantified with a NanoDrop 1000c spectrophotometer (Thermo Scientific, Wilmington, DE). The whole-genome sequencing was performed by IGA Technology Service (Udine, Italy). Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used for library construction. Libraries were processed with the Illumina cBot and sequenced on the HiSeq 2500 (Illumina, San Diego, USA, CA) pair-end with 100 cycles/read. Reads were analysed with Illumina pipeline to perform base calling, while trimming and assembly were carried out with CLC Genomics WorkBench v7.0 (CLC bio, Cambridge, MA) using the default parameters. RAST (Aziz et al., 2008) was used for annotation, distribution and classification of all sequenced genes.

209 The genome of parental strain *Lcb. casei* wt-N87 was already sequenced (Zotta et al., 2016).

210 The genome variant calling and the detection of untargeted mutations in *Lpb. plantarum* C17-m58,

211 and *Lcb. casei* N87-AE51, N87-AE284 and N87-RS337 mutants were carried-out against the relative

212 wt genomes. Briefly, the trimmed reads were aligned to the reference genome with Burrows-Wheeler

213 Aligner (BWA; Li and Durbin, 2009). PCR and optical duplicates were removed from all alignments

214 with Picard (http://broadinstitute.github.io/picard/). Single Nucleotide Polymorphism (SNP) calling

215 and genotyping was performed with GATK (McKenna et al., 2010) tools (i.e. local realignment,

216 UnifiedGenotyper, Variant Filtration). VCF (Variant Call Format) files were exported and used for

217 statistical analyses.

218

219 *2.8. Statistical analyses*

220 Statistical analyses and graphs were performed using Systat 13.0 for Windows (Systat Software Inc.,

221 San Jose, CA, USA). Dose-response curves to heme and menaquinone were modelled using the Hill-

222 five equation of R-package mixtox (Zhu, 2017).

223

224 **Results**

225 *Adaptive evolution of Lcb. casei wt-N87 and mutant selection*

226 In this study, sixteen batch propagations (~ 80 generations; **Table 1**) were carried out in both AE and

227 RS conditions to investigate the effect of O_2 (AE) or O_2 and heme and menaquinone supplementation

228 (RS) on the growth performances, oxidative stress tolerance and genome dynamism (generation of

229 untargeted mutations) of *Lcb. casei* wt-N87.

230 The growth behaviour and O_2 uptake in both AE and RS propagations were similar (**Supplementary**

231 **Fig. 1**), and the selective pressures promoted biomass production and enhanced the rate of O_2

232 consumption in both AE and RS propagations, indicating that prolonged cultivation in AE or RS

233 conditions increased the frequency of O_2-tolerant phenotypes in the population of *Lcb. casei* wt-N87.

A total of 710 colonies were randomly isolated from AE and RS propagations (**Table 1**) and the potential mutants were successively cultivated in anaerobiosis (AN, static growth), AE and RS to evaluate their tolerance or sensitivity to one or more conditions, compared to parental strain. A large number of colonies were collected from both P16-AE and P16-RS (growth parameters and O_2 consumption indicated a clear improvement of wt-N87 culture) to ensure the presence of putative mutants with evolved phenotype. The ability to grow in a given condition was measured as ratio between OD_{650} of mutant and parental strain (OD ratio mutant/wt; **Fig. 1**; **Supplementary Figs. 2, 3, 4**). Putative mutants with an OD ratio < -1.5 were considered more sensitive than *Lcb. casei* wt-N87 to the growth conditions, while an OD ratio > +1.5 indicated the expression of tolerant phenotypes. Most mutants exhibited a growth behaviour comparable to wt-N87 (OD ratio -1.5 $\leq$ OD $\leq$ +1.5) in all conditions.

The early stages of AE and RS propagations (P1-P6) slightly impaired the growth performances of wt-N87 and several sensitive mutants occurred when cultivated in AN, AE and RS conditions (**Fig.1, panels A, B, C**). The prolonged cultivation in AE and RS (P10-P16 in **Fig. 1**) increased the frequency of O_2-tolerant phenotypes (**Fig. 1B**), while some mutants sensitive to AN and RS conditions, were still isolated in the latest propagations (**Figs. 1A**, **1C**). At the end of adaptation (P16) several mutants acquired a pronounced AE and/or RS phenotype (+1.5 fold-change), but suffered when cultivated AN condition (up-left scatter in **Supplementary Figs. 2** and **3**). Many isolates, however, grew slightly better in unsupplemented AE (**Supplementary Fig. 4**).

Sixty isolates showing growth levels <-1.5 or > +1.5-fold compared to wt-N87 were selected to confirm the adequacy of serial propagations (in terms of culture purity) and the growth fitness. All isolated had RAPD-PCR and Multiplex-PCR profiles as well as colony morphology (glossy white colony on mMRS) like those of parental strains, indicating that no contamination occurred during propagations. The capability of selected mutants to grow in AN, AE and RS conditions were reported in **Supplementary Fig. 5**. Although the growth experiments were slightly different (96-well microplates, first assay; 24-well microplates, second test), most of selected mutants exhibited a

growth behaviour similar to that of first screening phase (AN, Pearson correlation coefficient $r=0.957$; AE, $r=0.962$; RS, $r=0.928$; **Supplementary Figs. 5A**, **B**, **C**), indicating some phenotypic stability.

Kinetics of growth and oxidative stress tolerance of ten selected mutants

Ten putative mutants (see *section 2.5*) were selected and propagated in AN, AE and RS conditions to evaluate possible differences in growth kinetics, biomass production, O_2 consumption, and robustness to oxidative stress (**Table 2**). The specific growth rate (μ_{max}) and biomass production of wt-N87 (P0, control) confirmed the respiratory-phenotype of the strain.

Mutants N87-AE51 and N87-RS37, recovered in the early stages of propagations, had impaired growth (both biomass and μ_{max}), O_2 uptake and H_2O_2 tolerance. Prolonged cultivation (P16) led the development   of robust phenotypes, and the ability to consume O_2 and cope with oxidative stress was clearly increased in some tolerant mutants (e.g. N87-AE284, N87-RS337).

As the adaptive evolution in AE and RS conditions affected the evolved phenotypes in different way, the mutants N87-AE51 (sensitive to AE and RS growth) and N87-AE284 and N87-RS337 (highest μ_{max}, biomass production, oxidative stress tolerance, O_2 consumption) were selected to verify the effect (stimulatory or inhibitory) of several doses of hemin and menaquinone on growth performances. The above mutants were also subjected to whole genome re-sequencing and analysis of genomic variants.

Effect of hemin and menaquinone on the growth of wt and mutant strains

In this study, we investigated the effect of different hemin (H; 0-20 mg/L) and menaquinone (M; 0-8 mg/L) concentrations on the growth of *Lcb. casei* wt-N87, N87-AE51, N87-AE284 and N87-RS337 to verify whether the evolved phenotypes developed a greater capability to use these cofactors, or if the adaptation to AE and RS conditions induced a sensitivity to these compounds. *Lpb. plantarum* wt-C17 and its mutant C17-m58 were included in the dose-response experiment.

In unsupplemented medium, *Lcb. casei* N87-AE284, N87-RS337 and *Lpb. plantarum* C17-m58 grew better than respective parental strains (**Fig. 2**); on the contrary, the growth of N87-AE51 was significantly lower, indicating that prolonged cultivation in AE conditions impaired its functionality. The effect of respiratory cofactors was reported as dose-response curves in **Fig.3**. Strain behaviour to hemin supplementation was fitted for each level of menaquinone (different colour in **Fig. 3**) and was calculated as inhibition rate (I0-I)/I0, where I0 was the response of control (OD_{650nm} at 0 mg/L hemin) and I the response at any given concentration of hemin. Dose-response relationships were non-monotonic (**Fig. 3**), and the stimulatory effect of hemin was strain-dependent. Regarding *Lcb. casei* strains, the growth of wt-N87 was stimulated up to 5 mg/L hemin, but higher concentrations reduced the OD_{650} levels. Mutants N87-AE51, N87-AE284 and N87-RS337 were more sensitive to heme toxicity than parental strain, and the non-growth-damaging concentrations were significantly lower (up to 2.5 mg/L hemin); in the evolved strains the stimulatory effect of hemin was much milder than wt-N87. Menaquinone did not relieved heme toxicity in N87-AE51, but reduced the detrimental effect in wt-N87, N87-AE284 and N87-RS337, albeit to different extent.

The dose-response curves of *Lpb. plantarum* wt-C17 and C17-m58 were similar, with a stimulatory effect of hemin in the range 0-2.5 mg/L; a clear breakpoint was evident in both wt-C17 and C17-m58 curves when >1 mg/L menaquinone was added, indicating a protective effect of this cofactor. For all strains, however, the beneficial effect of menaquinone, regardless of its concentration, faded at hemin levels >10 mg/L.

The dose-response curves were modelled using Hill-five equation of mixtox package for R (Zhu, 2017). Curves parameters and the predicted concentrations of hemin causing the maximum growth stimulation (minx) and 10, 25 and 50% inhibition (EC10, EC25, EC50) are reported in **Supplementary Table 1**. The Hill-five model provided an excellent fit for almost all curves (adjusted R^2 from 0.96 to 1.00; data not shown), except for some wt-N87 curves (adjusted R^2 from 0.83 to 0.97). *Lcb. casei* wt-N87 and the mutant N87-RS337 were most stimulated by heme addition (**Supplementary Table 1**) and the menaquinone had a clear boosting effect in the range 4-5 mg/L

312 (**Fig. 4**). For the other strains (both *Lcb. casei* and *Lpb. plantarum*), the concentrations of hemin

313 causing the maximum growth stimulation (minx) were lower (especially for N87-AE51) and

314 menaquinone had not a significant effect on minx parameter (**Fig. 4**).

315 High concentrations of hemin (i.e. 10 mg/L) significantly reduced the growth parameters (both μ_{max}

316 and biomass production) of *Lcb. casei* and *Lpb. plantarum* strains, and this was particularly evident

317 for the weakened phenotype N87-AE51 (**Table 3**). On the other hand, menaquinone alone did not

318 provide a growth improvement, confirming the inability of these strains to synthesize heme and the

319 need for an exogenous supplementation.

320

321 **Genome sequencing and identification of genomic variants**

322 The genomic features of *Lcb. casei* N87 were reported in Zotta et al. (2016; NCBI database, WGS

323 GenBank accession number LCUN00000000). The draft genome of *Lpb. plantarum* C17 (this study)

324 contained 78 contigs, a circular chromosome of 3,192,244 bp, an overall GC content of 44.2%, a total

325 of 3,034 protein-coding sequences, 63 tRNA genes, 5 rRNA genes. Contrarily to *Lcb. casei* N87, no

326 CRISPR system was found in *Lpb. plantarum* C17.

327 The analysis of genomic variants of *Lcb. casei* N87-AE51, N87-AE287, N87-RS337 and *Lpb.*

328 *plantarum* C17-m58 was carried-out on both coding sequences (CDS) and intergenic-regions, and

329 the results were reported, respectively, in **Table 4** and **Table 5**. The adaptive evolution resulted

330 exclusively in point mutations (Single Nucleotide Polymorphism, SNP), while no large InDels

331 (insertions/deletions) were found.

332 Some SNPs retrieved in CDS were common to all *Lcb. casei* mutants, others instead were associated

333 to a single phenotype (**Table 5**). *Lcb. casei* N87-AE51, N87-AE287 and N87-RS337 shared SNPs in

334 sequences encoding for an α-galactosidase and a hypothetical protein, while the robust mutants N87-

335 AE287 and N87-RS337 shared several variants in a cell wall hydrolase belonging to MltA (membrane

336 bound lytic transglycosylase A)/3D domain family (stop codons were generated). A missense SNP

337 affected a LPXTG-motif cell wall anchor domain-containing protein only in N87-AE284, while

13

338 variants within serine/threonine protein kinase PrkC, magnesium and cobalt efflux protein CorC and

339 LacI family transcriptional regulator were identified only in N87-RS337. Furthermore, in the latter

340 genome, a single nucleotide deletion was found in the intergenic-region between redox-sensitive

341 transcriptional repressor Rex (68 bp downstream) and valyl-tRNA synthetase (450 bp upstream),

342 while a transversion (T - G) was identified between an oligo-1,6-glucosidase (68 bp downstream) and

343 a Mn-dependent catalase (481 bp upstream) (**Table 5**).

344 The sensitive mutant N87-AE51 harbours a missense and a nonsense mutation, respectively, within

345 a nicotinate phosphoribosyl transferase and a membrane protein belonging to the YdcF-like family.

346 A further SNP was also found in the intergenic region between an aminoacid permease (192

347 downstream) and the DNA mismatch repair protein MutS (99 upstream).

348 The number of SNPs in *Lpb. plantarum* C17-m58 was significantly lower, probably due to the smaller

349 impact of the evolutionary strategy used for mutant generation (i.e. chemostat cultivation instead of

350 serial batch propagations). No mutations within CDS were identified (**Table 4**). On the contrary, a

351 transition (T - C) was found in the intergenic-region between Rrf2 family transcriptional regulator

352 (51 bp downstream) and pyruvate oxidase (31 bp upstream; *pox4* in *Lpb. plantarum* WCFS1), while

353 a transversion (C - A) was identified in the intergenic-region between sequences annotated as negative

354 regulator of proteolysis (87 bp downstream; transcriptional regulator Spx in other *Lpb. plantarum*

355 genomes) and predicted transcriptional regulator (84 bp upstream; transcriptional regulator MarR

356 family in *Lpb. plantarum* WCFS1; **Table 5**).

357

358 **Discussion**

359 ALE provided a powerful tool for generation of boosted phenotypes, avoiding the constraints related

360 to the genetically manipulated strains. The success of an ALE strategy depends on several factors,

361 including the choice of selection criteria (often related to the type of the applied pressure) for potential

362 mutants. In this study, the growth behaviour in AN, AE and RS conditions was the only selection

363 criterion used to identify the potential *Lcb. casei* mutants recovered from ALE propagations

364   (screening phase). Successively, other features, such as growth rate, biomass production, oxygen

365   consumption and oxidative stress survival were used as fitness criteria for the selection of evolved

366   phenotypes to send to whole genome re-sequencing and identification of genomic variants.

367   Although   generation of   mutants and the presence of genomic variants has been demonstrated

368   in several LAB undergoing an ALE system with a large number of generations (from 100-500;

369   Dragosits and Mattanovuich, 2013), in this study we obtained phenotypes with significant differences

370   in growth performances, heme uptake and stress robustness, even applying a lower number of

371   generations; this suggests that the selective pressures that we used (i.e. aerobic and respiratory state,

372   oxidative stress) strongly affected the genome and phenotype of *Lcb. casei* wt-N87. On the other

373   hand, the mutant *Lpb. plantarum* C17-m58 was previously obtained through the combination of

374   chemostat cultivation (i.e. respiratory condition under controlled growth rate, pH, T°C, oxygen level)

375   and agar-plate selection experiment (i.e. recovery on medium supplemented of pyrogallol or

376   menadione as superoxide generators; Zotta et al., 2014a); chemostat cultivation provides several

377   advantages, including constant growth rate, incubation parameters and population density, although

378   the higher operating costs and the shorter time scale are limiting factors compared to serial batch

379   propagations.

380   The adaptation to AE and RS conditions affected in different ways the growth performances and

381   stress tolerance of selected *Lcb. casei* mutants, and the greatest differences with parental strain were

382   found for the ability to consume O_2 and tolerate H_2O_2 rather than growth features (both biomass and

383   μ_{max}). Previous studies on *Lcb. casei* N87 (Ianniello et al., 2015, 2016) and *Lpb. plantarum* C17 (Zotta

384   et al., 2013, 2014b) had already demonstrated that the shift towards aerobic respiration conferred

385   advantages in growth performances (i.e. biomass yield, μ_{max}), but to a greater extent   increased the

386   synthesis of O_2-consuming and ROS-degrading enzymes, and strongly boosted the survival to

387   oxidative, freezing and freeze-drying stresses.

388   Adaptive evolution also affected the ability to use heme and menaquinone in both *Lcb. casei* and *Lpb.*

389   *plantarum* mutants. *Lcb. casei* wt-N87 and *Lpb. plantarum* wt-C17 were already characterized and

recognized as respiration-competent strains (Ianniello et al., 2016; Ricciardi et al., 2015a; Zotta et al., 2014a), and were able to use heme also for the synthesis of a heme-dependent catalase (Ricciardi et al., 2018; Zotta et al., 2014a). In many LAB the presence of heme may be harmful to cells; when the cofactor is not used for the assembly of catalase and/or cytochrome oxidase (component of respiratory chain) its accumulation may result in a heme-induced toxicity. Zotta et al. (2014b, 2018) already verified the detrimental effect of heme (i.e. reduced growth) in several *Lcb. paracasei* and heterofermentative (i.e. *Limosilactobacillus fermentum*, *Lmb. reuteri*) strains when cultivated in aerobic conditions.

Although LAB do not possess the genetic pathway for the synthesis of heme, they can use it as an exogenous cofactor and may activate several mechanisms to prevent its accumulation; some LAB, for example, harbour heme transport systems (e.g. *Streptococcus agalactiae*, HssR and HssS transcriptional regulators and HrtB and HrtA heme-regulated transporters, Fernandez et al., 2010; *Lactococcus lactis*, HrtR regulator and HrtBA, Joubert et al., 2014; Lechardeur et al., 2012; *Enterococcus faecalis*, FhtR regulator and HrtBA, Saillant et al., 2021; *Latilactobacillus sakei*, ABC transporter homologous of HrtBA, ECF energy-coupling factor transporter system, Fhu-like ferrous iron transporters, Verplaetse et al., 2020) that regulate and drive heme efflux across the membrane, ensuring its intracellular homeostasis, and avoiding heme overload and toxicity.

The genome of *Lcb. casei* N87 harbours a putative hemin ABC transporter ATP-binding protein, two ECF-type transporter system and two ferrous iron transporters (Feo system) (**Fig. 5**), already found in *Ltb. sakei* 23K (Verplaetse et al., 2020). Additionally, an iron-dependent peroxidase (annotated as Dyp-type peroxidase in *Ltb. sakei* 23K, putatively involved in heme modification) was also present in *Lcb. casei* N87; the DyP-type peroxidases (lignin-degrading enzymes) are a novel family of microbial heme-dependent peroxidases (RedoxiBase database; https://peroxibase.toulouse.inra.fr/) that seem to be involved in oxidative stress defence (H_2O_2 removal) in several microorganisms, including the lactic acid bacterium *Streptococcus thermophilus* (Zhang et al., 2015).

16

The genome of *Lpb. plantarum* C17 includes some of the above-mentioned proteins and the % identity with sequences of *Lcb. casei* N87 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were reported in **Fig. 5**. However, the role of these proteins in heme uptake and detoxification should be experimentally confirmed in both *Lcb. casei* N87 and *Lpb. plantarum* C17.

The role of menaquinone in heme-induced toxicity is controversial. Wakenam et al. (2012) found that menaquinone exacerbated the heme-associated oxidative damage in *Staphylococcus aureus*; in *Lactococcus lactis* menaquinone promoted the reduction and accumulation of heme in cell membrane, but aerobic cultivation seems to prevent its reducing activity and heme-associated stress (Joubert et al., 2014). More recently, Zotta et al. (2018) found that menaquinone supplementation (>0.25 mg/L) stimulated the aerobic growth of some *Leuconostoc mesenteroides* strains in presence of limited doses of hemin (0-2.5 mg/L).

In this study, we confirmed the beneficial effect of menaquinone for both *Lcb. casei* and *Lpb. plantarum* strains at low heme concentrations (0-2.5 mg/L), confirming that elevated levels of heme are toxic for LAB. High dose of hemin (10 mg/L) significantly reduced the growth performances of wt and mutants, while menaquinone alone did not provide further advantages compared to unsupplemented AE. In previous studies (Ianniello et al., 2016; Ricciardi et al., 2018; Zotta et al., 2013), the amounts of cofactors used for respiratory growth of *Lcb. casei* N87 and *Lpb. plantarum* C17 were 2.5 mg/L hemin and 1 mg/L menaquinone, which were appropriate for the synthesis and activation of catalase and cytochrome oxidase, and for generation of oxidative-tolerant phenotypes. The prolonged adaptation to unsupplemented AE reduced the stimulatory effect of heme and menaquinone in the robust mutant N87-AE284, probably because of lower requirements of these cofactors for the activation of a minimal respiratory chain. As the availability of heme and menaquinone, as well as O_2, is possible in several environments and circumstances (e.g. may be present in some foods and in human body, or may be deliberately added), the interactions between heme and menaquinone, and their effect on strain robustness deserve further investigations.

To understand the relationship between phenotypic (growth fitness, heme uptake, stress robustness) and genotypic changes, a whole genome re-sequencing was carried-out and the random mutations in the genomes of evolved *Lcb. casei* and *Lpb. plantarum* mutants were identified. Adaptive evolution resulted in nucleotide modifications (mainly nucleotide substitution, more rarely frameshift deletions) of some proteins involved in carbon metabolism, redox balance and cell wall properties.

Lcb. casei N87-AE287 and N87-RS337 shared several variants in a MltA-like hydrolase; MltA includes lytic enzymes involved in peptidoglycan degradation, as well as in cell division and elongation (Vermassen et al., 2019). N87-AE287 and N87-RS337 had improved growth performances compared to wt-N87, and the possible modification of protein functionality (stop codons were generated) may have contributed to this behaviour (e.g. reduced cell lysis compared to wt-N87 and N87-AE51). The genome of N87-AE284 harbours a nucleotide transition in LPXTG-motif cell wall anchor domain-containing protein. Cell surface proteins with a LPXTG-like motif are involved in the adhesion capability of LAB (Järvå et al., 2020) and, therefore, further investigation should be carried-out to verify the real impact of this mutation on some strain features (e.g. growth, cellular adhesion, biofilm formation).

The respiratory phenotype N87-RS337 had a synonymous SNP on a LacI family transcriptional regulator; members of LacI family regulate the expression of operons involved in sugar metabolism (e.g. *lac* operon); CcpA, the master regulator of catabolite repression in Gram+ bacteria, for example, belongs to LacI family. Although the above SNP generated a silent impact on protein sequence (no amino acid change), we also considered it because recent studies (Bailey et al., 2021) showed that synonymous mutations may have beneficial effect on strain fitness and may drive adaptive evolution being involved in changes of transcription and translation processes (e.g. gene expression, splicing, mRNA stability, protein folding). Moreover, since previous studies (Gaudu et al., 2003; Mazzeo et al., 2012; Zotta et al., 2012) demonstrated that the global regulator CcpA had significant role in the aerobic and respirative metabolism of LAB, investigations on other LacI family members might be useful to elucidate regulation mechanisms in AE and RS phenotypes. On the other hand, a nucleotide

transition was found in serine/threonine kinase PrkC, a protein belongs to the Hanks-type kinases involved in different biological processes (e.g. sporulation and biofilm formation in *Bacillus subtilis*, antimicrobial resistance, GIT adherence, cell division), including the regulation of catabolite repression via phosphorylation of the CcpA co-repressor HPr (Janczarek et al., 2018). A further SNP in CDSs of N87-RS337 was found in the magnesium and cobalt efflux protein CorC, involved in the transport of divalent cations Mg^{2+} and Co^{2+} (cofactors of several enzymes) across the membrane. Interesting is also the frameshift deletion found in N87-RS337 in the intergenic region between valyl-tRNA synthetase (involved in transcriptional regulation, translation and protein biosynthesis) and transcriptional repressor Rex. Rex plays a central role in the regulation of carbon and energy metabolism in Gram+ bacteria, by sensing the $NADH/NAD^+$ ratio; its DNA-binding affinity, in fact, is modulated by intracellular $NADH/NAD^+$ levels, and it is stable in presence of high concentrations of the oxidized cofactor. Rex regulates the transcription of respiratory genes (i.e. NADH dehydrogenase, cytochrome *bd* oxidase, heme biosynthesis enzymes) by repressing them when $NAD^+/NADH$ ratio is high (Sevilla et al., 2019). Rex activity, thus, is strongly affected by O_2 level and respiration rate. Probably, the mutation on Rex sequence positively affected N87-RS337, improving its respiratory phenotype. On the other hand, a SNP was found in the upstream region of Mn-dependent catalase, one of the main H_2O_2-degrading enzymes (together to heme-dependent catalase) in *Lcb. casei* N87. Although, respiratory growth enhanced the activity of heme-catalase because of heme supplementation during cultivation, recently Ricciardi et. al (2018) demonstrated that AE conditions also improved gene expression and activity of the Mn-dependent enzyme.

Regarding the weakened mutant N87-AE51, a genomic variant was identified on nicotinate phosphoribosyltransferase. This protein (PncB), together to nicotinamidase-related amidase (PncA), is involved in salvage pathway for NAD^+ biosynthesis. Actually, in bacteria there are two major pathways for NAD generation: through *de novo* synthesis (from L-aspartic acid) and through the salvage/recycling pathway (from nicotinic acid or nicotinamide; Johnson et al., 2015). The salvage genes *pncA-pncB* are present in several LAB, including the genome of *Lcb. casei* N87. Mutation in

19

pncB sequences probably affected protein functionality, reducing NAD^+ biosynthesis and recycling of NAD-derived products and impairing growth fitness of N87-AE51. A frameshift deletion (that generated a truncated amino acid sequence) in CDS of uncharacterized SAM-binding protein YcdF was also found in the same phenotype, but information about biological function of this protein are scarce. Among the intergenic regions, a variant was identified between an amino acid permease (involved in the amino acid transport across cell membrane) and MutS; the latter protein, together to MutL (adjacent in *Lcb. casei* N87 genome) are involved in DNA mismatch repair (MMR) mechanisms to ensure fidelity in replication processes and genome stability. During adaptive evolution, therefore, the correct functionality of MutS and MutL is crucial to avoid high mutation rates. However, although mutations in MutSL system have been associated with hypermutable phenotypes, some authors (Willems et al., 2003) demonstrated that amino acid substitutions in MutSL sequences did not generate hypervariable mutants in some *Enterococcus faecium* strains. Consequently, the effect of MutS upstream variant on the features of N87-AE51 should be elucidated. The number of mutations in *Lpb. plantarum* C17-m58 was comparatively low. However, the few observed variants fell within intergenic regions neighbour to sequences encoding for the key enzyme of aerobic metabolism, i.e. pyruvate oxidase (POX), and for some relevant transcriptional regulators (i.e Rrf2, MarR, Spx). In aerobic-growing LAB, POX together to acetate kinase (ACK), drives the oxidation of pyruvate to acetate with production of CO_2, H_2O_2 (POX activity) and extra ATP generation (ACK activity; Zotta et al., 2017). Rrf2 (located between *pox4* and *pox5* genes in *Lpb. plantarum* WCFS1 genome), is a winged helix-turn-helix repressor family that includes several transcriptional regulators involved in redox sensing and regulation (i.e. NsrR, nitric oxide metabolism and detoxification; IscR, iron/sulfur homeostasis in cells; RirA, iron limitation; CymR, cysteine availability; RsrR, redox status in cells; Sevilla et al., 2019). MarR family includes regulators involved in antibiotic resistance and regulation of antioxidant enzymes and thiol reduction systems (detoxification of peroxides and ROS; Sevilla et al., 2019), while Spx family regulates the expression of genes related to the maintenance of thiol redox balance (e.g. thioredoxin/thioredoxin reductases,

518 thiol peroxidase, FMN-dependent oxidoreductases) in Gram+ bacteria (Rojas-Tapias and Helmann,

519 2019). Probably, the detected SNPs affected the activity of POX and transcriptional regulators,

520 improving growth, pyruvate conversion, enzymatic levels of POX and other antioxidant enzymes, as

521 well as the oxidative stress tolerance of *Lpb. plantarum* C17-m58, as already demonstrated by

522 Ricciardi et al. (2015a).

523

524 **Conclusions**

525 This study confirmed that AE and RS conditions (that can occur in different environments) might

526 contribute   to the evolution of LAB, with development of strains with respiratory features, improved

527 fitness, and different genetic equipment, as already demonstrated (Zotta et al., 2017). For several

528 LAB, respiratory phenotypes are more robust than those growing anaerobically and, in this study, we

529 maximized their benefits (at least for some mutants) with an ALE approach. To date, the metabolic

530 and physiological advantages of O_2-tolerant and respiratory phenotypes (in term of biomass yield and

531 reduction of costs for fermentative processes) have been exploited by the Chr. Hansen A/S company

532 for the production of a mixed phage resistant *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis*

533 culture (direct-to-vat F-DVS pHageControl™ R-604), actually used as starter for the production of

534 several cheeses (i.e. Cheddar, Feta, Cottage).

535 The strains of *Lcb. casei* and *Lpb. plantarum*, contrarily to the dairy-associated *Lc. lactis*, are more

536 versatile and suitable for different purposes (i.e. starters and/or adjuncts for dairy, vegetable, cereal-

537 based foods, probiotics, cell factories for metabolite production) and, then, robust phenotypes with

538 improved growth, stress survival and metabolic features may be of practical relevance. Specifically,

539 the use of non-recombinant mutants (e.g. N87-AE284, N87-RS337 and C17-m58) could have

540 multiple advantages in health and food-related fields.

541 The capability to intake exogenous heme (e.g. for the assembly of cytochrome oxidase and catalase),

542 for example, may reduce the amount of this compound in human host and may prevent some heme

543 iron-associated diseases (e.g. colon cancer, inflammatory bowel diseases, intestinal microbial

544 dysbiosis, due to consumption of heme in iron-rich foods); moreover, sequestration of heme may

545 reduce survival of pathogens in human gut, as many of them use heme as growth and virulence factor.

546 From a technological point of view, the naturally boosted ROS/O_2-tolerant phenotypes may be used

547 as antioxidant adjuncts to reduce oxidative processes in foods (Reale et al., 2016 already verified this

548 potential in Cheddar-type cheeses). On the other hand, the generation of the aerobic phenotype N87-

549 AE284 may reduce the costs of hemin and menaquinone as supplements for biomass production, and

550 the strain could be exploited for applications in which the use of animal-derived hemin is not allowed

551 (e.g. kosher and halal diets).

552 The selected mutants demonstrated a good phenotype stability, showing the same growth behaviour

553 in the different assays. At the genomic level, the WGS analysis revealed random mutations in some

554 proteins and transcriptional regulators involved in central metabolism, redox balance and oxidative

555 stress (this is consistent with the type of selective pressure applied) and cell surface features, but their

556 actual role in the generation and maintenance of evolved phenotypes should be better investigated at

557 transcriptomic and proteomic level.

558

559 **Author contributions**

564

565 **Declaration of Competing Interest**

566 The authors declare no competing interests

567

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571

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Figure legends

Fig. 1: Ratio between optical density at 650 nm (OD_{650}) of mutants and parental strain *Lcb. casei* wt-N87 (OD ratio mutant/wt) cultivated in anaerobic (AN), aerobic (AE) and respiratory (RS) conditions. Symbols and colours: red circles, mutants collected from AE propagations; blue triangles, mutants collected from RS propagations. Dotted lines: ± 1.5 fold-changes (significant threshold) compared to parental strain *Lcb. casei* wt-N87.

Fig. 2: Growth (OD_{650nm}) of *Lcb. casei* wt-N87, N87-AE51, N87-AE284, N87-RS337 and *Lpb. plantarum* wt-C17 and C17-m58, cultivated in aerobic condition at 35°C (*Lpb. plantarum*) or 37°C (*Lcb. casei*) for 16 h, without heme and menaquinone supplementation. *, wild-type strains.

Fig. 3: Dose-response curves to hemin and menaquinone supplementation (mg/L) for *Lcb. casei* wt-N87, N87-AE51, N87-AE284, N87-RS337 and *Lpb. plantarum* wt-C17 and C17-m58, grown in aerobic condition at 35°C (*Lpb. plantarum*) or 37°C (*Lcb. casei*) for 16 h. *, wild-type strains. The response was calculated as (I0-I)/I0, where I0 is the response of control (as OD_{650nm} at 0 mg/L hemin) and I the response at any given concentration of hemin.

Fig. 4: Concentration of hemin (mg/L) causing the maximum stimulation of growth (minx) as a function of menaquinone levels (mg/L), in *Lcb. casei* wt-N87, N87-AE51, N87-AE284, N87-RS337 and *Lpb. plantarum* wt-C17 and C17-m58. *, wild-type strains. Minx values (y axes) were obtained by using the Hill-five equation of R-package mixtox (Zhu, 2017).

Fig. 5: Sequence annotation and gene organization (bp position, + or - strand) of the genes involved in heme uptake and transport on the genome of *Lcb. casei* N87 (genomic fractions were retrieved from Integrated Microbial Genome database). The genes of interest are coloured according to COG categories: light blue, inorganic ion transport and metabolism; light purple, coenzyme transport and metabolism; light green, cell wall/membrane/envelope biogenesis. % of identity between *Lcb. casei* N87 sequences (query; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and those of *Lpb. plantarum* C17 are also reported.

798 **Table 1:** Serial batch propagations of *Lacticaseibacillus casei* wt-N87 propagated in aerobic (AE)
799 and respiratory (RS) conditions
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Propagation [a]	Growth [b]	Inoculum % (v/v) [c]	Incubation time (h)	N gen [d]	X [e]	O_2 uptake [f]	N col [g]
Lcb. casei wt-N87	AN	1	24	-	1.25	>180	-
P0	AE	1	24	-	1.31	50	-
	RS	1	24	-	1.50	45	-
P1	AE	20	4.5	2.32	0.52	105	10
	RS	20	4.5	2.32	0.62	105	10
P2	AE	20	4.5	2.38	0.55	105	10
	RS	20	4.5	2.24	0.51	105	10
P3	AE	20	15	2.26	1.01	100	10
	RS	20	15	2.11	0.99	100	10
P4	AE	20	4.5	2.26	0.73	100	10
	RS	20	4.5	2.33	0.76	100	10
P5	AE	20	4.5	2.51	0.72	95	10
	RS	20	4.5	2.51	0.71	95	10
P6	AE	20	15	3.54	0.91	90	10
	RS	20	15	3.33	0.90	90	10
P7	AE	2	8.0	3.82	0.68	80	10
	RS	2	8.0	4.72	0.73	80	10
P8	AE	2	14.5	4.93	1.29	70	10
	RS	2	14.5	5.76	1.38	70	10
P9	AE	2	8.0	6.51	0.89	60	10
	RS	2	8.0	5.77	0.89	60	10
P10	AE	2	14.5	6.76	1.20	50	10
	RS	2	14.5	6.44	1.19	50	10
P11	AE	1	24	6.65	1.32	50	10
	RS	1	24	7.33	1.37	45	10
P12	AE	1	24	6.69	1.34	45	20
	RS	1	24	7.23	1.41	40	20
P13	AE	1	24	6.71	1.36	40	25
	RS	1	24	7.54	1.48	35	25
P14	AE	1	24	7.15	1.42	35	25
	RS	1	24	7.39	1.53	35	25
P15	AE	1	24	7.22	1.49	35	25
	RS	1	24	7.43	1.57	30	25
P16	AE	1	24	7.22	1.52	30	150
	RS	1	24	7.69	1.61	30	150

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802 [a] Propagation: *Lcb. casei* N87, anaerobic culture used as control and as inoculum for aerobic (AE) and respiratory (RS)
803 propagations; P0-P16, propagation code. [b] Type of cultivation: AN, anaerobiosis; AE, aerobiosis (air); RS, respiration
804 (air, supplementation with 2.5 µg/mL haemin and 1 µg/mL menaquinone). [c] Inoculum: indicates the % of inoculum taken
805 from the previous culture. [d] N gen: The number of generations was calculated as (log Nt-log N_0)/0.30, where Nt and N_0
806 are, respectively, the final and initial cell number. [e] X: Biomass production (X-X_0; g/L) was calculated as difference
807 between the final biomass of each propagation (X) and the biomass of inoculum (X_0). [f] O_2 consumption was correlated
808 to the time (min) of resazurin discolouration (Ricciardi et al., 2014). [g] N col: Number of colonies collected from WMA
809 for each propagation, and used for mutant selection.

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Table 2: Growth parameters, oxygen consumption and H_2O_2 tolerance of *Lacticaseibacillus casei* wt-N87 and 10 selected mutants

Strain	Cycle [a]	Growth [b]	μ_{max} [c]	Biomass [d]	dpH [e]	O_2 consumption [f]		Tolerance of H_2O_2 [g]	
						Exp	Stat	Exp	Stat
wt-N87	P0 (C)	AN	0.276 ± 0.004	1.54 ± 0.01	-2.77 ± 0.01	none	none	6.9	13.7
wt-N87	P0 (C)	AE	0.228 ± 0.009	1.65 ± 0.03	-2.66 ± 0.01	70	150	27.5	55.0
wt-N87	P0 (C)	RS	0.367 ± 0.007	1.87 ±0 .02	-1.49 ± 0.01	40	120	55	55.0
AE51	P6 (AE)	AN	0.333 ± 0.017 [†]	1.59 ± 0.02	-3.15 ± 0.01 [†]	none	none	6.9	13.7
AE51	P6 (AE)	AE	0.068 ± 0.001 [‡]	0.94 ± 0.01 [‡]	-2.62 ± 0.04	150	none	13.7 [‡]	27.5 [‡]
AE51	P6 (AE)	RS	0.123 ± 0.003 [§]	1.41 ± 0.04 [§]	-3.04 ± 0.01 [§]	150	none	13.7 [§]	27.5 [§]
AE169	P14 (AE)	AN	0.285 ± 0.003	1.45 ± 0.02 [†]	-2.62 ± 0.01 [†]	none	none	6.9	13.7
AE169	P14 (AE)	AE	0.292 ± 0.003 [†]	1.99 ± 0.03 [‡]	-2.75 ± 0.02 [‡]	90	150	27.5	55.0
AE169	P14 (AE)	RS	0.288 ± 0.008 [§]	1.94 ± 0.00	-2.67 ± 0.01 [§]	90	150	27.5 [§]	55.0
AE284	P16 (AE)	AN	0.296 ± 0.000 [†]	1.66 ± 0.02 [†]	-3.04 ± 0.00 [†]	none	none	27.5 [†]	55.0 [†]
AE284	P16 (AE)	AE	0.387 ± 0.000 [‡]	2.63 ± 0.04 [‡]	-2.80 ± 0.00 [‡]	35	90	55.0 [‡]	110.0 [‡]
AE284	P16 (AE)	RS	0.428 ± 0.005 [§]	2.29 ± 0.03 [§]	-2.78 ± 0.00 [§]	40	90	55.0	110.0 [§]
AE331	P16 (AE)	AN	0.269 ± 0.019	1.58 ± 0.02	-2.77 ± 0.04	none	none	13.7 [†]	13.7
AE331	P16 (AE)	AE	0.381 ± 0.002 [†]	1.70 ± 0.00	-2.90 ± 0.03 [‡]	80	150	55.0 [‡]	55.0
AE331	P16 (AE)	RS	0.375 ± 0.005	1.68 ± 0.00 [§]	-2.83 ± 0.02 [§]	50	120	55.0	55.0
RS37	P4 (RS)	AN	0.392 ± 0.013 [†]	1.33 ± 0.00 [†]	-3.00 ± 0.01 [†]	none	none	6.9	13.7
RS37	P4 (RS)	AE	0.083 ± 0.001 [‡]	1.29 ± 0.02 [‡]	-3.02 ± 0.02 [‡]	150	none	13.7 [‡]	27.5 [‡]
RS37	P4 (RS)	RS	0.112 ± 0.001 [§]	1.43 ± 0.01 [§]	-3.00 ± 0.03 [§]	150	none	13.7 [§]	27.5 [§]
RS176	P15 (RS)	AN	0.288 ± 0.006 [†]	1.63 ± 0.02 [†]	-2.81 ± 0.01	none	none	13.7 [†]	13.7
RS176	P15 (RS)	AE	0.336 ± 0.013 [‡]	1.86 ± 0.03 [‡]	-2.76 ± 0.03 [‡]	100	150	27.5	27.5 [‡]
RS176	P15 (RS)	RS	0.373 ± 0.005	1.95 ± 0.02 [§]	-2.72 ± 0.06 [§]	60	150	55.0	55.0
RS208	P16 (RS)	AN	0.332 ± 0.006 [†]	1.76 ± 0.04 [†]	-2.75 ± 0.01	none	none	6.9	13.7
RS208	P16 (RS)	AE	0.367 ± 0.006 [‡]	1.85 ± 0.03 [‡]	-2.84 ± 0.01	50	150	27.5	55.0
RS208	P16 (RS)	RS	0.369 ± 0.002	2.01 ± 0.00 [§]	-2.76 ± 0.01 [§]	40	90	27.5 [§]	55.0
RS262	P16 (RS)	AN	0.310 ± 0.010 [†]	1.56 ± 0.04	-2.98 ± 0.01 [†]	none	none	6.9	13.7
RS262	P16 (RS)	AE	0.126 ± 0.003 [‡]	1.92 ± 0.04 [‡]	-2.95 ± 0.02 [‡]	90	140	27.5	55.0
RS262	P16 (RS)	RS	0.230 ± 0.005 [§]	1.96 ± 0.02 [§]	-2.90 ± 0.03	90	140	27.5 [§]	55.0
RS313	P16 (RS)	AN	0.297 ± 0.004 [†]	1.68 ± 0.02 [†]	-2.94 ± 0.01 [†]	none	none	13.7 [†]	13.7
RS313	P16 (RS)	AE	0.351 ± 0.001	1.77 ± 0.02 [‡]	-2.91 ± 0.01 [‡]	60	150	27.5	55.0
RS313	P16 (RS)	RS	0.384 ± 0.004 [§]	2.19 ± 0.09 [§]	-3.01 ± 0.11 [§]	40	130	55.0	55.0
RS337	P16 (RS)	AN	0.305 ± 0.019 [†]	1.86 ± 0.05 [†]	-2.88 ± 0.01 [†]	none	none	13.7 [†]	27.5 [†]
RS337	P16 (RS)	AE	0.379 ± 0.002 [‡]	2.08 ± 0.03 [‡]	-2.96 ± 0.01 [‡]	50	140	55.0 [‡]	55.0
RS337	P16 (RS)	RS	0.427 ± 0.005 [§]	2.35 ± 0.02 [§]	-2.92 ± 0.00 [§]	30	110	55.0	110.0 [§]

821 [a] Cycle: Propagation cycle from which the mutant was collected; code in brackets indicates the type of cultivation during propagations (P0, control growth of wild-type N87; AN,
822 anaerobiosis; AE, aerobiosis; RS, respiration). [b] Growth: type of cultivation (AN, anaerobiosis; AE, aerobiosis; RS, respiration) used to for the estimate the growth parameters of
823 selected mutants. [c] μ_{max}: maximum specific growth rate; estimated mean values $\pm$ standard errors are shown; R^2 of growth modelling ranged from 0.955 to 0.999. [d] Biomass
824 production (X-X_0; g/L) was calculated as difference between final (at 24 h; X) and initial (at 0 h; X_0) biomass values. [d] Decrease in pH was calculated as difference between final
825 (at 24 h) and initial (at 0 h) pH values. [e] Oxygen consumption was correlated to the time (min) of resazurin discolouration (Ricciardi et al., 2014); Exp, exponential growth phase;
826 Stat, stationary growth phase.[f] Tolerance of H_2O_2: concentration of H_2O_2 (mmol/L) causing > 5-log reductions; concentration of stressed cells used to inoculate WMB was 5-log
827 cfu/mL. [†‡§] Significant differences (Tukey's HSD, $p < 0.005$) between mutants and wt-N87, respectively, during AN (†), AE (‡) and RS (§) cultivations. Mutants in bold (AE51,
828 AE284, RS337) were used for heme uptake experiments, whole genome re-sequencing and identification of genomic variants.
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Table 3: Effect of heme (H) and menaquinone (M) on the growth kinetics of *Lacticaseibacillus casei* wt-N87, N87-AE51, N87-RS337 and *Lactiplantibacillus plantarum* wt-C17 and C17-m58

Strains	Growth [a]	X(max) [b]	lag [c]	μ_{max} [d]	R^2 [e]
Lcb. casei wt-N87	0H+0M	4.09±0.02	-	0.492±0.003	0.997±0.00
	0H+1M	4.10±0.02	-	0.496±0.000 [†]	0.997±0.00
	10H+1M	3.38±0.02 [§]	-	0.405±0.001 [§]	0.995±0.00
Lcb. casei N87-AE51	0H+0M	2.00±0.02	2.463±0.387	0.333±0.008	0.991±0.01
	0H+1M	2.02±0.02	2.612±0.047 [†]	0.331±0.005	0.993±0.01
	10H+1M	0.89±0.01 [§]	3.044±0.026 [§]	0.275±0.002 [§]	0.991±0.00
Lcb. casei N87-AE284	0H+0M	4.36±0.01	-	0.492±0.002	0.997±0.00
	0H+1M	4.24±0.00 [†]	-	0.478±0.005 [†]	0.996±0.00
	10H+1M	3.71±0.01 [§]	-	0.418±0.000 [§]	0.998±0.00
Lcb. casei N87-RS337	0H+0M	3.83±0.03	0.813±0.013	0.463±0.000	0.999±0.00
	0H+1M	3.93±0.01 [†]	0.489±0.012 [†]	0.468±0.002 [†]	0.996±0.00
	10H+1M	3.38±0.02 [§]	0.589±0.022 [§]	0.402±0.003 [§]	0.996±0.00
Lpb. plantarum wt-C17	0H+0M	2.14±0.02	0.575±0.000	0.478±0.009	0.998±0.00
	0H+1M	2.08±0.01 [†]	0.683±0.063 [†]	0.483±0.003	0.998±0.00
	10H+1M	1.61±0.01 [§]	- [§]	0.381±0.001 [§]	0.995±0.00
Lpb. plantarum C17-m58	0H+0M	2.36±0.01	0.433±0.016	0.488±0.009	0.998±0.00
	0H+1M	2.25±0.01 [†]	0.513±0.025 [†]	0.497±0.003	0.997±0.00
	10H+1M	1.81±0.01 [§]	- [§]	0.379±0.001 [§]	0.995±0.00

[a] Growth conditions: 0H+0M, 0 µg/ml hemin and 0 µg/ml menaquinone; 0H+1M, 0 µg/ml hemin and 1 µg/ml menaquinone; 10H+1M, 10 µg/ml hemin and 1 µg/ml menaquinone. [b] X(max): maximum biomass production (g/L). [c] lag: duration of the lag phase (h). [d] maximum specific growth rate (1/h). [e] R^2 for growth modelling (Baranyi and Roberts (1994). Mean values ± standard errors of 2 biological replicates are shown. [† §] Significant differences (Tukey's HSD, $p < 0.005$) with unsupplemented aerobiosis (0H+0M), within the same strain.

Table 4. Genomic variants within the coding sequences of *Lacticaseibacillus casei* mutants

Gene name and information [a]	SNP position [b]	Ref [c]	Alt [c]	Type of SNP [d]	Mutants AE51	Mutants AE284	Mutants RS337	Impact of SNP [e]
Alpha-galactosidase (135 bp, 44 aa; genome position 406079-406079, strand +)/Function: lactose and galactose uptake and utilization	406175	C	T	non-synonymous (Ti)	X	X	X	aa substitution
hypothetical protein (153 bp, 50 aa; genome position 525870-526022, strand +)/Function: unknown	525894	A	C	non-synonymous (Tv)	X	X	X	aa substitution
Hydrolase (MltA/3D domain family (684 bp, 227 aa; genome position 8452-9135, strand +)/Function: cell wall degradation, cell division and elongation	8710	G	T	stop-gain (Tv)		X	X	stop codon, nonsense
	8711	A	G	non-synonymous (Tv)		X	X	
	8713	A	T	stop-gain (Tv)		X	X	
	8714	A	C	non-synonymous (Tv)		X	X	
	8715	A	T	non-synonymous (Tv)		X	X	
nicotinate phosphoribosyltransferase (1446 bp, 481 aa; genome position 463262-464707, strand +)/Function: salvage pathway for NAD$^+$ biosynthesis	464326	A	C	non-synonymous (Tv)	X			aa substitution
Uncharacterized SAM-binding protein YcdF, DUF218 family (YdcF-like family) (1050 bp, 349 aa; genome position 745495-744446, strand -)/Function: unknown	744850	A	-	frameshift deletion	X			stop codon truncated protein
LPXTG-motif cell wall anchor domain-containing protein/KxYKxGKxW signal peptide containing protein (8178 bp, 2725 aa; genome position 654464-646287, strand -)/Function: adhesion capability	646328	C	T	non-synonymous (Ti)		X		aa substitution
LacI transcriptional regulator family (981 bp, 326 aa; genome position 193681-192701, strand -)/Function: DNA-binding transcriptional regulator, carbon catabolite control	193108	G	A	synonymous (Ti)			X	silent

Serine/threonine protein kinase PrkC (1992 bp, 663 aa; genome position 692740-694731, strand -)/Function: regulation of signal transduction	692778	C	T	non-synonymous (Ti)			X	aa substitution
Magnesium and cobalt efflux protein CorC (1389 bp, 462 aa; genome position 36630-38018, strand -)/Function: Mg2+ and Co2+ trnsport	37370	C	T	non-synonymous (Ti)			X	aa substitution

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867 ^a Functional annotation and information of sequence in which the mutation was identified. ^b Position of point mutation (Single Nucleotide Polymorphism, SNP) on the genome of

868 wild-type *Lcb. casei* N87. ^c Nucleotide in the genome of wild-type (Ref) and mutant (Alt) strain. ^d Type of mutation and nucleotide substitution. In brackets: Ti, transition; Tv,

869 transversion. ^e Effect of SNP on protein sequence.

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Table 5. Genomic variants within intergenic regions of *Lacticaseibacillus casei* and *Lactiplantibacillus plantarum* mutants

Intergenic region [a]	Upstream gene/SNP position [b]	Downstream gene 2/SNP position [c]	Ref [d]	Alt [d]	Mutants
290 bp (strand +)	aminoacid permease (1830 bp; 609 aa; genome position 24924-26753, strand +) SNP position: 192 downstream	DNA mismatch repair protein MutS (2574 bp; 857 aa; genome position 27044-29617, strand +) SNP position: 99 upstream	C transversion	A	*Lacticaseibacillus casei* N87-AE51
577 bp (strand -)	Oligo-1,6-glucosidase (1686 bp; 561 aa; genome position 572723-574408, strand -) SNP position: 97 downstream	Mn-dependent catalase (807 bp; 268 aa; genome position 574986-575792, strand -) SNP position: 481 upstream	T transversion	G	*Lacticaseibacillus casei* N87-RS337
517 bp (strand +)	Redox-sensitive transcriptional repressor Rex (669 bp, 222 aa; genome position 311751-312419, strand +) SNP position: 68 bp downstream	Valyl-tRNA synthetase (2643 bp; 880 aa; genome position 312937-315579, strand +) SNP position: 450 bp upstream	T deletion	-	*Lacticaseibacillus casei* N87-RS337
82 bp (strand +)	Rrf2 family transcriptional regulator (1755 bp, 584 aa; genome position 375254-377008 bp, strand +) SNP position: 51 bp downstream	Pyruvate oxidase (1755 bp, 584 aa; genome position 375254-377008 bp, strand +) SNP position: 31 bp upstream	T transition	C	*Lactiplantibacillus plantarum* C17-m58
171 bp (strand +)	Negative regulator of proteolysis (390 bp, 129 aa; genome position 591569-591958 bp, strand +) SNP position: 87 bp downstream	Predicted transcriptional regulator (420 bp, 139 aa; genome position 592129-592548 bp, strand +) SNP position: 84 bp upstream	C transversion	A	*Lactiplantibacillus plantarum* C17-m58

[a] Length (bp) and strand direction of intergenic region in which the mutation was identified. [b] Annotation of sequence upstream of intergenic region (upstream gene). [c] Annotation of sequence downstream of intergenic region (downstream gene); sequence information and SNP position were reported for both up- and downstream genes. [d] Nucleotide in the genome of wild-type (Ref) and mutant (Alt) strain.

Figure 1
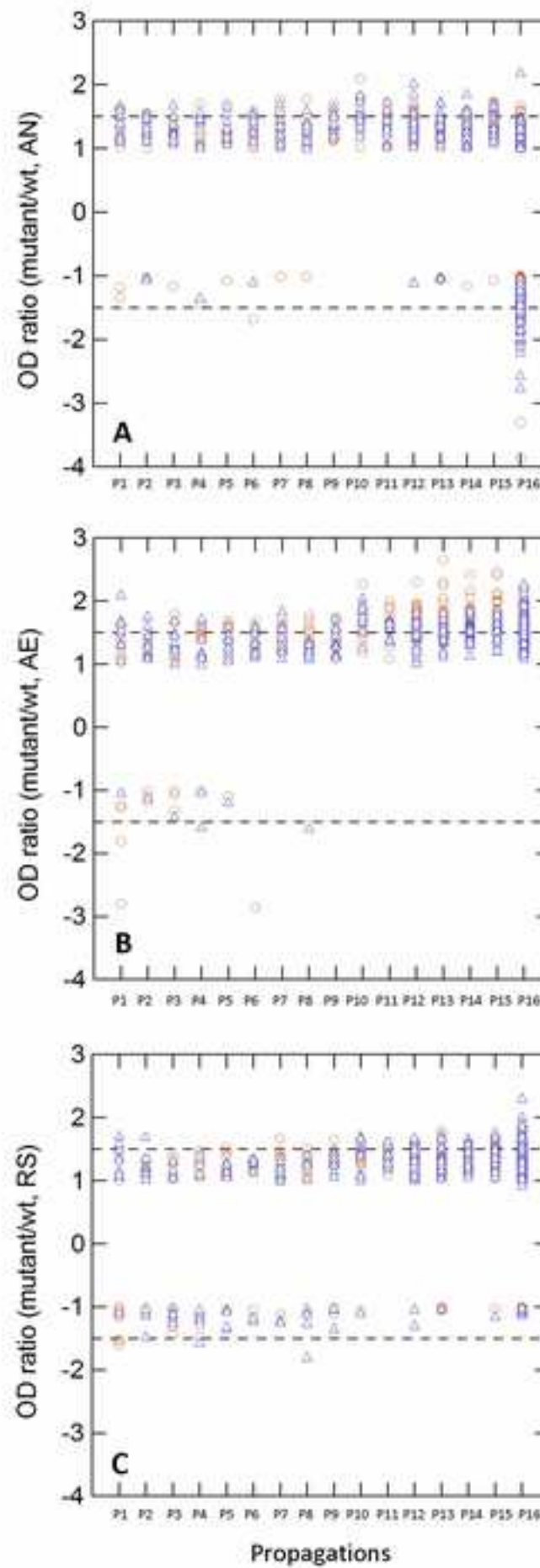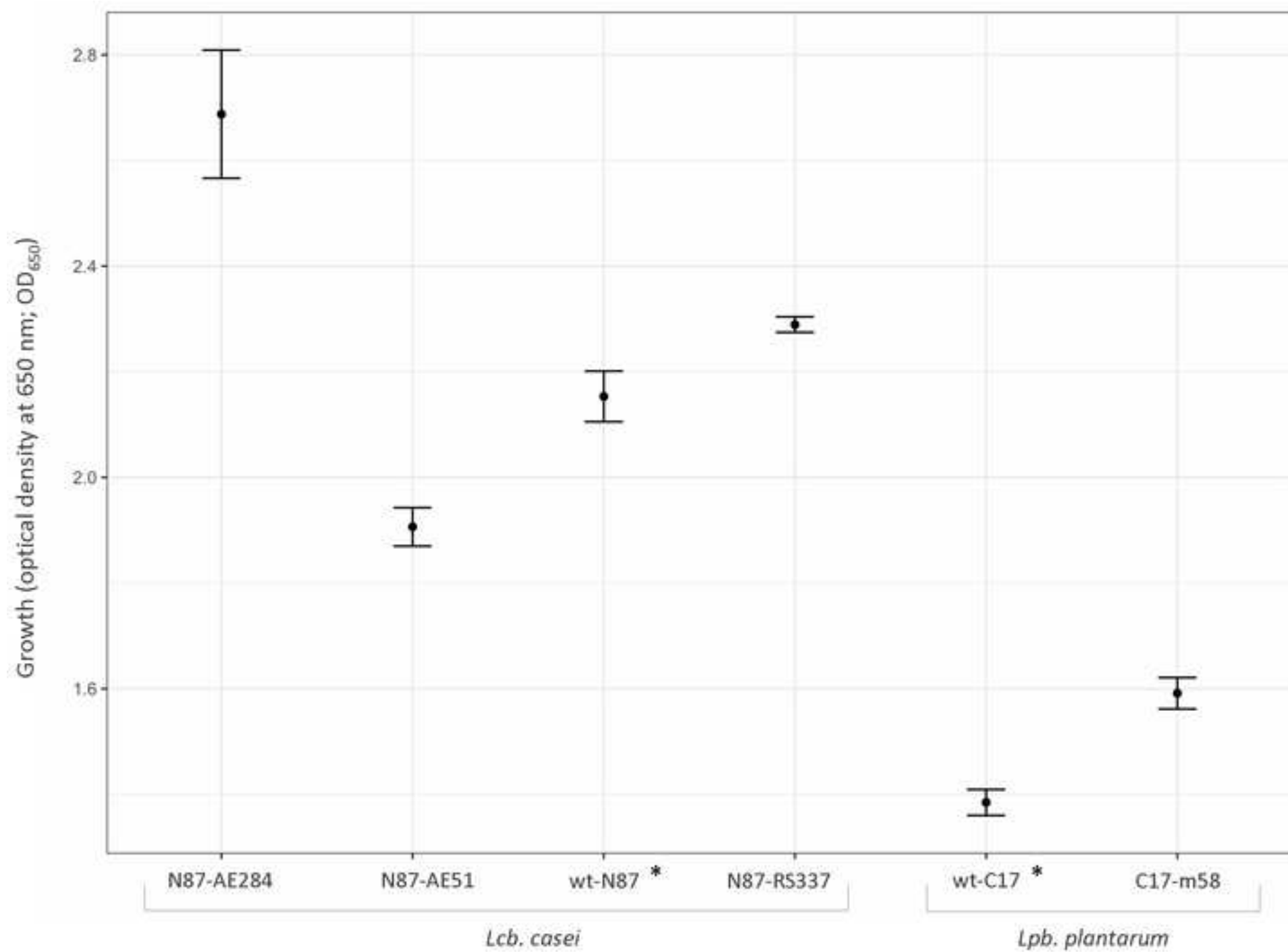
Figure 2

Figure 3

Figure 4

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Figure 5
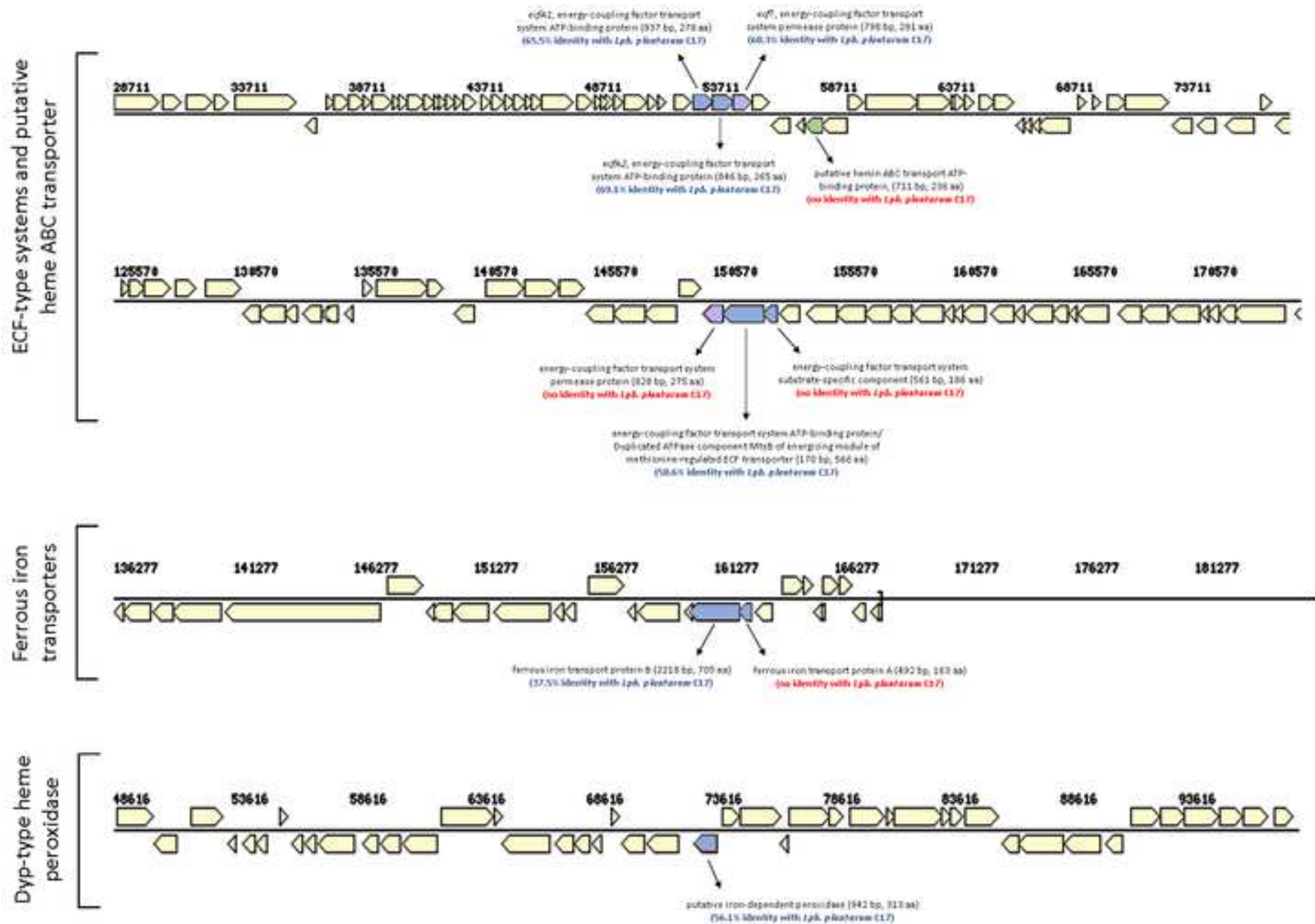
Conflict of Interest

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.
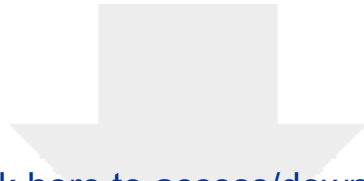
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