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1 Manganese modulates metabolic activity and redox homeostasis in translationally-blocked
2 *Lactococcus cremoris*, impacting metabolic persistence, cell-culturability, and flavor
3 formation.

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14 **Abstract**

15 Manganese (Mn) is an essential trace element that is supplemented in microbial media with varying
16 benefits across species and growth conditions. We found that growth of *Lactococcus cremoris* was
17 unaffected by manganese omission from the growth medium. The main proteome adaptation to
18 manganese omission involved increased manganese transporter production (up to 2000-fold), while the
19 remaining 10 significant proteome changes were between 1.4 and 4 fold. Further investigation in
20 translationally-blocked (TB), non-growing cells showed that Mn supplementation (20 μM) led to
21 approximately 1.5X faster acidification compared to Mn-free conditions. However, this faster acidification
22 stagnated within 24 hours, likely due to draining of intracellular NADH that coincides with substantial loss
23 of culturability. Conversely, without manganese, non-growing cells persisted to acidify for weeks, albeit at
24 a reduced rate, but maintaining redox balance and culturability. Strikingly, despite being unculturable, α-
25 keto acid-derived aldehydes continued to accumulate in cells incubated in the presence of manganese,
26 whereas without manganese cells predominantly formed the corresponding alcohols. This is most likely
27 reflecting NADH availability for the alcohol dehydrogenase-catalyzed conversion. Overall, manganese
28 influences the lactococcal acidification rate, and flavor formation capacity in a redox dependent manner.
29 These are important industrial traits especially during cheese ripening, where cells are in a non-growing,
30 often unculturable state.

31

32 ## Introduction

33 Growth and survival of microorganisms heavily relies on the environmental availability of metal cofactors,
34 particularly for essential alkaline earth and transition metals such as magnesium, calcium, manganese,
35 iron, cobalt, copper, and zinc. In this group, manganese is especially important because of its relatively
36 high solubility, abundance, and distinctive redox abilities (1). In comparison to other biologically important

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37 redox-active metals, i.e Fe^{2+}, Mn^{2+} is a weaker electron donor or reducing agent (1). Consequently, cells
38 can accumulate and tolerate high cytoplasmic concentration of free Mn^{2+} (2) without negative redox
39 outcomes under conditions that will normally promote formation of toxic free radicals through Fenton-type
40 reactions (3). Based on structural similarity among other transition metals, only manganese is able to
41 replace magnesium in its cofactor binding site and activate the corresponding enzymes which are
42 ubiquitous in carbon, nucleic acid and protein metabolism.(1, 4, 5).

43 Therefore, intracellular manganese homeostasis is essential for optimal cellular activities. In bacteria, the
44 intracellular Mn^{2+} concentration is typically maintained relative to other metals as an inverse of the Irving-
45 Williams series (Mg^{2+} and Ca^{2+} (weakest cofactor binding) < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+})
46 (6, 7). This universal order predicts the stabilities of (transitional) metal complexes independent of the
47 ligands (7), and highly influences the metal competition to cofactor binding sites that depends on both its
48 abundance and affinity. To ensure specific metal cofactors are inserted to metalloenzymes, a cell finely
49 tunes its intracellular metal pools by employing cytosolic metal sensors and transporters. In the case of
50 manganese, two main transporters are reported in lactic acid bacteria (LAB), which are an ABC transport
51 system (*mtsCBA*) and Nramp transporters (*mntH*) (8). Typically, intracellular manganese is maintained at
52 micromolar levels, which is 1,000- 10,000-fold lower than intracellular Mg2+ but 10,000-100,000-fold
53 higher than other metals in the Irving-Williams series, with an exception for iron that is commonly present
54 in comparable amount to manganese (6). The maintenance of intracellular manganese levels is especially
55 relevant for cellular bioenergetics where various enzymes related to carbon metabolism, e.g., lactate
56 dehydrogenase, phosphoglycerate mutase & fructose-1,6-bisphosphate phosphatase are either strictly
57 Mn-dependent or highly stimulated by manganese (1, 9). Many bacterial superoxide dismutases which act
58 as scavenger of reactive oxygen species also incorporates manganese in their active site (10). Therefore,
59 manganese is generally considered to be crucial not only in survival under oxidative stress conditions, but
60 also in ATP generation (10, 11).

61 In Lactic Acid Bacteria, manganese supplementation has frequently been shown to contribute to cell
62 growth and functionality during fermentation applications. The bioavailability of manganese has been
63 found to enhance *in vitro* formation of flavors such as benzaldehyde (12) and the aldehydes derived from
64 α-keto acids e.g. 3-methylbutanal (13). In the latter case, the conversion of branched-chain α-keto acids
65 (BCAAs) was reported to serve as a redox sink and the utilization of BCAAs can result in a marked increase
66 of biomass, possibly due to additional ATP formation (14). On the other hand, lactococci grown without
67 manganese supplementation have shown higher survival following a heat shock (15). Although the
68 underlying mechanism to this observation is not known, it is plausible that manganese deprivation leads
69 to stress responses that provide cross-protective resistance (16). Nonetheless, manganese

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70 supplementation is generally favored for microbial cultivation media despite these variations in
71 physiological consequences in various species and growth conditions (17). While various studies have
72 investigated the effect of manganese on growth and stress resistance, no studies investigated its effect
73 on the metabolism of non-growing cells, which have distinct metabolic strategies and requirements. A
74 non-growing state is commonly encountered in various biotechnological applications such as in the
75 production of pharmaceuticals (18, 19), fermented foods (20), or biofuels (21). It is especially relevant in
76 various long-term fermentation processes such as cheese ripening where a significant portion of volatile
77 flavor metabolites are generated for up to years after cell growth has ceased.

78 In the present study, we investigated the physiological and molecular (proteome) adaptation of
79 *Lactococcus cremoris* to the presence and absence of manganese supplementation to a chemically defined
80 medium. Furthermore, we investigated the role of manganese in cellular survival and metabolic activity in
81 growing and translationally-blocked (TB) cells. The results indicate that cells in the absence of manganese
82 can maintain their growth rate with relatively modest adjustment in cytoplasmic proteins compared to
83 membrane transporters. However, in non-growing, TB-cells manganese omission led to a striking
84 prolongation of acidification capacity, cell-survival as well as maintenance of redox homeostasis. These
85 observations demonstrate that manganese omission strongly influences the *L. cremoris* metabolism under
86 TB conditions, while it does not appear to have apparent consequences for growth or physiology of *L.*
87 *cremoris* during cultivation.

88 Methods

89 Strain and Mn-omission cultivation

90 *Lactococcus cremoris* NCDO712 (22), MG1363 (pNZ5519), MG1363 (pAK80) (23),
91 MG1363(pCPC75::atpAGD) (23), and MG1363 (pNZ5519) (this study, Supplementary Methods 1) were
92 grown on chemically defined medium for prolonged cultivation (CDMPC) (24) at 30°C without aeration.
93 Strains were pre-cultured in the presence or absence of Mn for 25 generations (4 direct transfers with a
94 100-fold dilution) to minimize carry-over effects. Details on strain-specific ingredients and growth rate
95 measurement and can be found in Supplementary Methods 2 and 3.

96 Proteome analysis

97 Proteome samples were harvested in quadruplicates from exponentially growing cultures of strain
98 NCDO712 that was precultured for 20 generations in the presence or absence of manganese. Protein
99 extraction and analysis were performed as described previously (25). Details and modifications to the
100 proteomics methods can be found in Supplementary Methods 4. The mass spectrometry proteomics data

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101 have been deposited to the ProteomeXchange Consortium via the PRIDE (26) partner repository with
102 the dataset identifier PXD030123

103 Acidification and luminescence measurements of TB-cells

104 Long term analysis of metabolite production was performed as described earlier (27). Exponentially
105 growing cells pre-cultured in the presence or absence of Mn were harvested and resuspended at a density
106 between 1E+07 and 2.5E+07 cells/mL in their corresponding growth medium supplemented with
107 erythromycin (5µg/mL) and 10µM 5(6)-carboxyfluorescein (Sigma-Aldrich 21877), with or without Mn
108 (20µM). For volatile production measurement, aliquots of TB-cultures were transferred to sterile GC-MS
109 vials. Time-course samples were analyzed for organic acids, volatiles, viability, and membrane integrity
110 (Supplementary Methods 5 and 6)

111 Luminescence measurement was performed as described earlier (28). Aliquot of TB-*L. lactis* MG1363
112 harbouring pNZ5519 (1E+07 cells/mL, precultured without manganese) was concentrated at selected time
113 points of incubation by centrifugation and resuspension in less volume of supernatant to a concentration
114 of 1E+08 cells/mL. Nonanal 1% in silicon oil was supplied as reaction substrate and added either into
115 empty wells or the empty space between the wells of the microplate. Luminescence was determined at
116 20-min intervals over a period of 6 hours after nonanal addition in a Genios microplate reader (Tecan,
117 Zurich, Switzerland). The gain was set at 200 and integration time was set at 1000 ms.

118 Results

119 1. Manganese omission did not lead to changes in growth characteristics or
120 metabolic end products

121 Growth rate changes in many organisms are accompanied by metabolic shifts and they are suggested to
122 reflect re-allocations in cellular protein investment and constraints on microbial growth (29, 30). In *L.*
123 *lactis* such metabolic shift has been described, where homofermentative acidification predominated by
124 lactic acid occurs at high growth rate but switches to heterofermentative acidification at low growth rate
125 (31). To investigate the requirement of manganese on growth, we removed manganese from the
126 preparation of our standard chemically-defined medium for lactococci. Remarkably, changes in the growth
127 of strain NCDO712 was not detected in the absence of manganese. Since a carry-over effect from the
128 previous growth medium might be sufficient to compensate for the lack of manganese, we further
129 cultivated NCDO712 for 4 subcultures and a total of 25 generations to ensure complete removal of
130 manganese. At the end of this subculturing, no apparent effect on the growth rates of NCDO712 was seen

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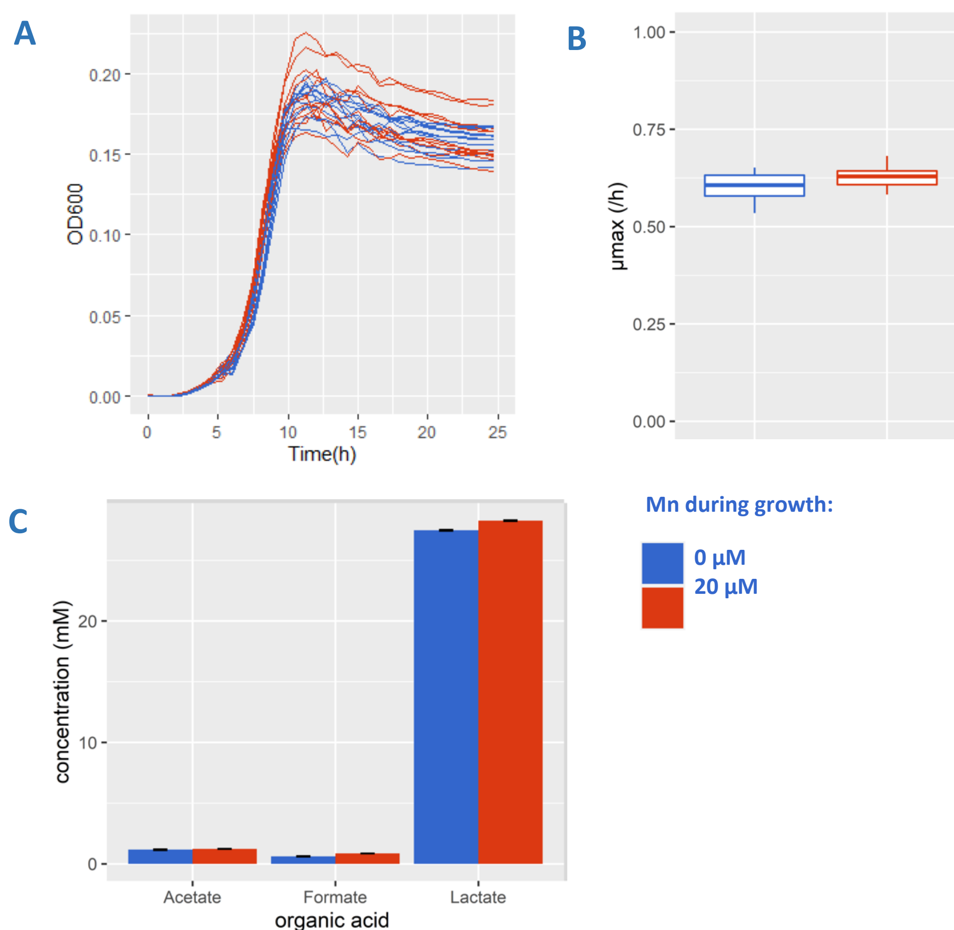
131 in relation to manganese availability (Figure 1A). The growth rate remained high in the absence of
132 manganese throughout the transfers and under excess or limited supply of lactose (Supplementary Figure
133 S1), which implies that cells can grow with minute amounts of manganese available in their environment.
134 In line with the high growth rate, the composition of produced organic acids remained unchanged and was
135 predominated by lactic acid when manganese was omitted (Figure 1B). These results suggest that Mn
136 omission lead to no changes in flux (32) through the central energy generation pathway, i.e., glycolysis
137 coupled to pyruvate conversion to lactic acid by lactate dehydrogenase (LDH), nor in the energy and redox
138 state of the cells i.e., ADP/ATP ratio, or NAD+/NADH ratio (33).

139



140
141
142
143 **Figure 1** *Lactococcus lactis* NCDO712 was serially propagated 4 times (25 generations) in defined medium
144 supplemented with lactose at excess (12.5 mM – growth stops due to acid accumulation) concentration in
145 the presence (red) and absence (blue) of manganese (20µM). The growth curve (panel A, n=8), maximum
146 specific growth rate (panel B, n=3) and concentrations of organic acids (panel C, n=2) are shown. Error
147 bars indicate the standard deviation from the stated n biological replicates.

148

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149 ## 2. Proteome adjustments to manganese omission mainly up-regulated Mn
150 transporters

151 Growth data suggests that cells are apparently unaffected by manganese deprivation. To characterize the
152 cellular responses to manganese omission from the growth medium, we performed a global proteome
153 analysis. Manganese omission led to only 17 significantly differentially expressed proteins, encompassing
154 11 up-regulated and 6 down-regulated expression levels (Table 1). By far the most prominent proteome
155 adaptation occurred in the expression of membrane transporters such as MntH, MtsA, and MtsB. There an
156 up to 2000-fold increase of expression was observed for the ABC transporter MtsA upon the omission of
157 manganese. The expression of proton dependent NRAMP-related manganese transporter MntH (plasmid
158 encoded) increased under manganese omission by 4-fold. Next to these transporters, the putative
159 manganese transporter llmg_1024 and llmg_1025 increased by 4-fold. This is comparable to the fold-
160 change observed for MntH and it may potentially be involved in the regulation of intracellular Mn
161 concentration. Additionally, the genome-encoded MntH (*llmg*_1490) was not captured by differential
162 analysis (Table 1) due to its level being below the detection limit with Mn supplementation. When taking
163 into account the estimated minimum detection level in our proteomics data (4.8 LFQ intensity), this
164 genome-encoded MntH increased by at least 20-fold upon Mn omission to an average LFQ intensity of 6.1,
165 which is comparable with the upregulation of plasmid-encoded $MntH_P$ (Supplementary Table S1). The
166 upregulation of these Mn transporters indicates that cells detected Mn shortage despite no apparent
167 changes in growth rate or acidification profile. However, it cannot be excluded that trace amounts of Mn
168 that might be present as contaminants of medium constituents are compensating or masking any effects
169 of Mn omission on growth. Moreover, it is unknown whether the upregulated transport functions would
170 allow the cells to accumulate these trace amount to a sufficient intracellular level for growth.

171 Aside from these transporters, manganese omission also led to 12 more modest, but significant changes
172 in cytoplasmic protein levels (ranging from 1.4 and 4-fold changes). Notably, the majority of these
173 proteins are associated with redox metabolism and many catalyze NAD-dependent reactions. Manganese
174 omission from the growth medium increased the expression of 2-dehydropantoate 2-reductase (3-fold)
175 and a putative pyridoxamine 5'-phosphate oxidase (2.5-fold), while the expression of NADH oxidase (0.27-
176 fold), aldehyde-alcohol dehydrogenase (0.41-fold), and a putative ferredoxin protein (0.48-fold)
177 decreased. Moreover, manganese availability seems to correspond to changes in a few proteins related to
178 stress response. For example, expression of the peptide methionine sulfoxide reductase (PMSR), which
179 catalyzes the reduction of methionine sulfoxide in proteins back to methionine. This enzyme may protect
180 cells against oxidative stress (34). It was found to be approximately 2-fold decreased in the absence of
181 manganese. Conversely, manganese omission led to more than 3-fold increased expression of universal

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182 stress protein UspA, which is associated with resistance against various stresses. Intriguingly, aside from
183 PMSR, differential expression of superoxide dismutase (MnSOD) and/or other oxidative stress related
184 functions (Supplementary Table S1) were not observe in the absence of manganese, illustrating a lack of
185 prominent changes in the oxidative stress levels experienced by these cells. Overall, the changes in
186 proteome data are dominated by major changes in Mn-transport proteins, and more modest changes in a
187 number of cytosolic proteins that suggest that cells might experience a shift in the redox balance.

188 **Table 1** Significant differentially expressed proteins in manganese omitted compared to manganese supplemented cultures. Proteins were selected based on
189 cutoff parameters of s0 = 0.01 and a False Discovery Rate (FDR) of 0.05. LFQ (Label Free Quantitation) values represent the average from 4 biological
190 replicates.

Protein names	Gene names	LFQ (+Mn)	LFQ (-Mn)	Fold Change	P-value
Manganese ABC transporter substrate binding protein	mtsA llmg_1138	4.9±0.04	8.26±0.11	2269.43	0.00000
Manganese ABC transporter ATP binding protein	mtsB llmg_1136	5.72±0.22	7.77±0.1	110.67	0.00001
Mn2+/Fe2+ transporter, NRAMP family	mntH pNZ712_01	6.43±0.13	7.05±0.04	4.19	0.00021
Uncharacterized protein	llmg_1025	6.5±0.1	7.12±0.04	4.15	0.00007
Putative membrane protein	llmg_1024	6.4±0.09	6.97±0.03	3.78	0.00005
Universal stress protein UspA	UspA	7±0.05	7.5±0.06	3.20	0.00003
2-dehydropantoate 2-reductase (EC 1.1.1.169) (Ketopantoate reductase)	panE llmg_1131	5.52±0.15	5.99±0.08	2.99	0.00324
Uncharacterized protein	llmg_2395	5.7±0.1	6.11±0.07	2.55	0.00120
Ribonuclease J (RNase J) (EC 3.1.-.-)	rnj llmg_0876	7.2±0.01	7.51±0.07	2.02	0.00024
Lipoprotein	plpB llmg_0336	6.82±0.03	7.06±0.05	1.75	0.00050
Ribonuclease J (RNase J) (EC 3.1.-.-)	rnj llmg_0302	7.32±0.02	7.47±0.03	1.40	0.00063
NADH oxidase (EC 1.6.-.-)	noxC llmg_1770	6.76±0.14	6.19±0.11	0.27	0.00171
Aldehyde-alcohol dehydrogenase	adhE llmg_2432	8.1±0.05	7.72±0.13	0.41	0.00294
Putative electron transport protein	llmg_1916	6.9±0.1	6.58±0.04	0.48	0.00229
Peptide methionine sulfoxide reductase MsrA (Protein-methionine-S-oxide reductase) (EC 1.8.4.11)	pmsR msrA llmg_2281	6.25±0.06	5.97±0.07	0.52	0.00197
Glycine betaine/proline ABC transporter (EC 3.6.3.32)	busAA llmg_1048	8.17±0.04	7.92±0.02	0.56	0.00006
Glycine betaine-binding protein	busAB llmg_1049	7.7±0.03	7.46±0.02	0.59	0.00002

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192 **3. Acidification is maintained for a prolonged period in TB-cells only in the**
193 **absence of manganese**

194 The proteome data implied that cells cultured without Mn addition might be in an altered physiological
195 state, which may depend on the intracellular Mn levels and/or NAD^+/NADH availability to maintain
196 metabolic fluxes. We investigated if cellular adaptation to manganese omission affects central metabolism,
197 which we performed in a previously described model system of non-growing cells with inhibited protein
198 synthesis (Nugroho, Kleerebezem, and Bachmann 2020). In this system we can follow acid production of
199 non-growing cells with continuous measurements for up to three weeks. Inhibition of protein synthesis
200 with the antibiotic erythromycin enables the preservation of protein levels, e.g., manganese transporters.
201 It allows comparing of cells grown in the presence or absence of manganese, while manganese
202 concentration in the assay medium can be precisely adjusted. In line with the growth data, lactic acid was
203 still the predominant fermentation end-product, making up more than 90% (Cmol-based) of the produced
204 organic acids (Supplementary Figure S2A) during prolonged incubation irrespective of the treatments. The
205 calculated lactic acid production from the continuous pH measurement (Figure 2B) is in good agreement
206 with the HPAEC measurements (Figure 2A).

207 In the presence of manganese, acidification by TB-cells was initially approximately 1.5-fold faster
208 (Supplementary Figure S2B) but stagnated within 24 hours (Figure 2A & 2B, left panel) reaching a
209 relatively low final lactate concentration (~ 4 mM), and a final pH of 6.16. In contrast, when cells were
210 transferred to assay medium containing no manganese, acidification continued at a lower rate for more
211 than a week, reaching drastically higher final lactic acid concentrations (~ 25 mM) and a lower final pH of
212 ~ 4.0. The latter conditions (25mM lactic acid and pH 4.0) are likely the environmental conditions that
213 prevented further acidification (Figure 2A, 2B). Moreover, in the absence of Mn in TB-cell assay (Figure
214 2B), Mn-precultured cells produced 20 mM lactic acid in 100h compared to 150h when cells were
215 precultured without Mn, potentially as a result of trace amount of Mn carry over. Importantly, these results
216 demonstrate that the absence of manganese in these acidification assays prominently changes the
217 persistence of flux through the central energy-generating pathway. This seems to be irrespective of the
218 accompanying cellular proteome changes as demonstrated by preculture both in the presence or absence
219 of Mn showing prolonged activity following transition to Mn-omitted non-growing assay medium (Figure
220 2A,2B middle and right panel). Despite of the prominent role of manganese in oxidative stress tolerance
221 in many organisms, there were no indications that this stagnated acidification was explained by substantial
222 difference in oxidative stress levels in the absence or presence of Mn in this TB assay (Supplementary
223 Figure S3). Overall this data shows that the omission of manganese during sugar conversion of

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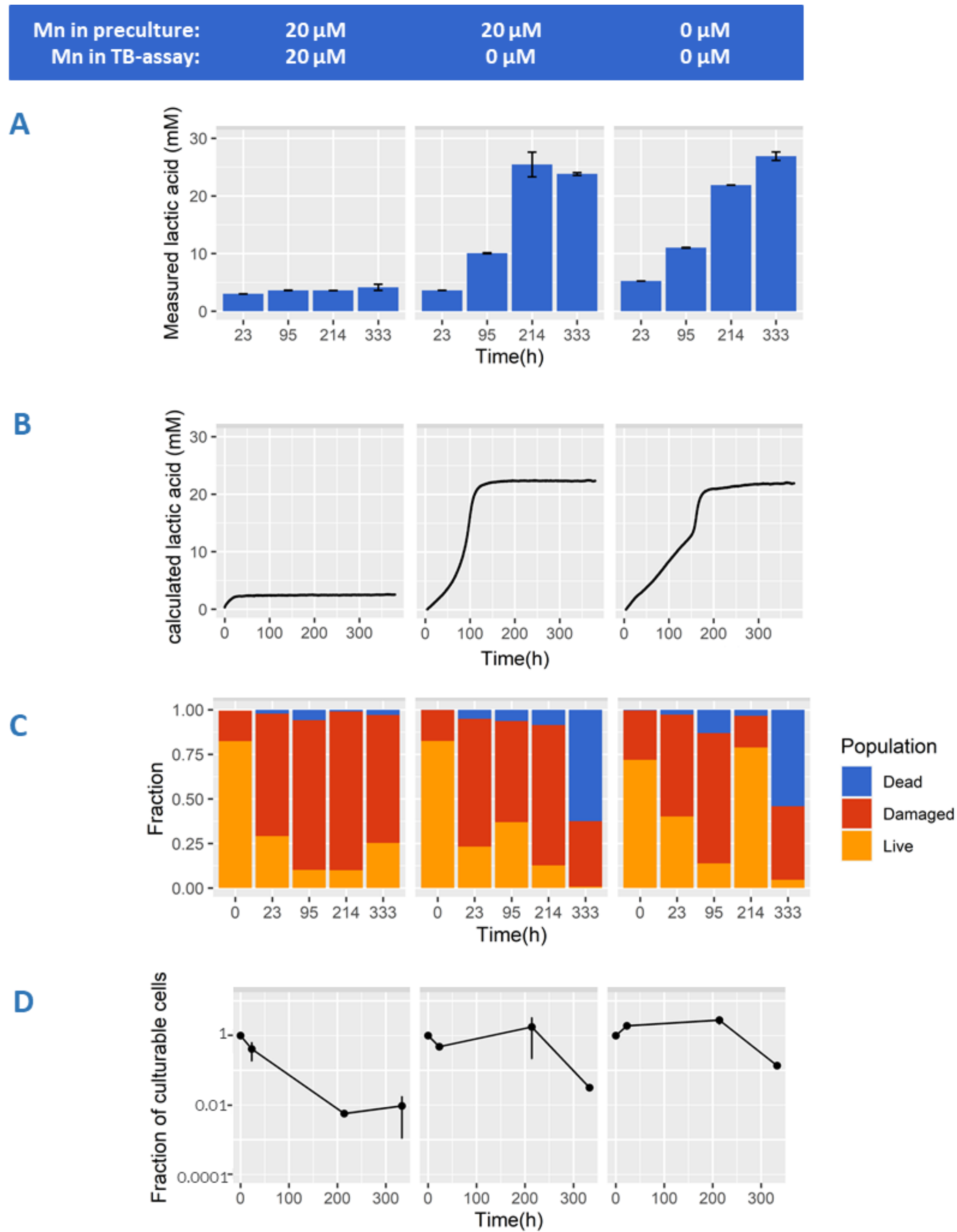
224 translationally blocked cells allows for a much longer persistence of acidification and therefore a higher
225 total product formation.



226

227 **Figure 2** *Lactococcus lactis* NCDO712 was precultured in the presence (left and middle panel) and
228 absence (right panel) of manganese (20µM). Cells (2.5E+07 cells/mL) were transferred into fresh medium
229 containing erythromycin (5µg/mL) and 20µM manganese (left panel) or 0µM manganese (middle and right
230 panel). Concentration of lactic acid (panel A) was measured with HPAEC at selected time points.
231 Continuous measurement of medium pH to calculate lactic acid production overtime can be seen in panel
232 B. Average population fractions based on membrane integrity (panel C) was measured for dead (blue),

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233 damaged (red), and live (orange) cells throughout incubation. Fractions of culturable cells based on plate
234 counts can be seen in panel D. Error bars indicate the standard deviation from 3 biological replicates.

235

236 4. Manganese induces the appearance of viable but non-culturable (VBNC) 237 populations in TB-cells after acidification stagnates

238 The observed stagnation of acidification within 24 hours for Mn supplemented conditions implies that the
239 generation of ATP through glycolysis would also stagnate, which could also affect the viability and integrity
240 of the cells. In this context it is relevant to note that *L. lactis* is known to remain metabolically-active for
241 prolonged periods of carbon starvation, e.g., more than 3.5 years, in a viable but non-culturable (VBNC)
242 state (36). To investigate the influence of manganese on cellular integrity and culturability during
243 acidification in TB-cells, we determined the colony forming unit and membrane integrity over time. Within
244 9 days of prolonged incubation, non-growing cells incubated with manganese showed an approximate
245 100-fold reduction in culturable cells (Figure 2D). This is sharply contrasted by results obtained for cells
246 incubated without manganese, where culturability was maintained close to 100% in the same timeframe.
247 Under the conditions used, rapidly declining culturability of the cells incubated in absence of manganese
248 was only observed after 2 weeks of incubation, which is likely the consequence of the combined stress of
249 low pH and increased lactic acid concentrations. These results are in good agreement with our previous
250 observations that an approximate 40-fold decline of viability was observed under similar conditions after
251 approximately 2 weeks (27). Analogous to the acidification observations presented above, the presence
252 or absence of manganese during the preculturing and the corresponding proteome adaptations did not
253 significantly influence the culturability results we obtained.

254 Membrane integrity analysis (Figure 2C) of the same time series revealed a prominent decline of the
255 subpopulation with an apparent intact membrane ("live") that increasingly progressed towards the
256 subpopulation characterized by slightly damaged ("damaged") or severely damaged ("dead") membrane
257 integrity over the course of incubation. Under all conditions, the damaged subpopulation became more
258 predominant over time and on average ranged from 60% to 80% of the total population in cells incubated
259 between 23h and 214h. Notably, in the absence of manganese the subpopulation classified as 'dead'
260 increased to approximately 60% of the total population after 333h of incubation, whereas less than 5% of
261 the total population was classified as 'dead' when incubated for the same time in media containing
262 manganese. Analogous to the culturability results, this drastic decline of viability is likely due to prolonged
263 exposure to low pH and high lactic acid concentrations inducing increasing cell damage that coincides with
264 loss of culturability (and viability). Conversely, the observed decline in culturability of cells incubated in
265 the presence of Mn was very poorly reflected by an increasing subpopulation of cells characterized as

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266 'dead' according to these membrane-staining procedures. Apparently, the loss of culturability of these
267 cells is not related to loss of cellular integrity but could be related to an unbalanced metabolism because
268 of their high rate of acidification. Potential metabolic consequences of rapid acidification could induce
269 stagnation of acidification and loss of culturability. This could be related to an excessive increase of the
270 ATP/ADP balance, where the depletion of the intracellular ADP pool halts glycolytic flux. Alternatively, the
271 disruption of the intracellular redox balance (NADH/NAD+ ratio) could lead to depletion of either form of
272 this cofactor that would also effectively halt glycolytic flux and/or lactate formation. Loss of either ATP/ADP
273 or NADH/NAD+ homeostasis may also negatively affect culturability by creating an inability to re-initiate
274 energy-generation or biosynthesis pathways required for regrowth.

275

276 **5. Manganese leads to NADH depletion in acidifying TB-cells**

277 To investigate whether manganese induces the proposed ADP depletion and thereby stagnates
278 acidification in TB-cells, we used strain MG1363 (a prophage-cured and plasmid-free derivative of strain
279 NCDO712) harbouring pCPC75::atpAGD (23). This strain constitutively overexpresses the F(1) domain of
280 the membrane-bound F(1)F(0)-ATPase, which modulates intracellular energy levels by accelerating the
281 ATP to ADP conversion (23). However, in the presence of manganese TB-cells of this F(1)-ATPase
282 overexpressing strain displayed the same stagnation of acidification within 24 hours as the control strain
283 (MG1363 harbouring the empty vector pAK80), whereas manganese omission allowed continued
284 acidification at a reduced rate for weeks (Figure 3A). These results demonstrated that preventing the
285 postulated ATP accumulation by its conversion to ADP through the F(1)-ATPase failed to sustain prolonged
286 acidification. This indicates that the disruption of the ATP/ADP homeostasis is not the mechanism
287 underlying the observed phenomenon.

288 To investigate whether a redox balance disruption is able to explain the manganese-induced
289 stagnation of acidification, we used strain MG1363 harbouring pNZ5519 (37) which constitutively
290 expresses *luxAB*, a bacterial luciferase of *Vibrio harveyi*. This luciferase catalyzes the reaction of a long
291 chain aldehyde, e.g., nonanal, oxygen, and reduced flavin mononucleotide ($FMNH_2$) to form a carboxylic
292 acid, FMN, and light (490 nm). Regeneration of intracellular $FMNH_2$ is dependent on the availability of
293 NADH, which is thereby required to maintain the luciferase reaction and the corresponding detection of
294 the luminescence signal. In an initial experiment, the LuxAB substrate nonanal was provided immediately
295 after transferring cells to the TB acidification conditions. In this experiment, the initial luminescence signal
296 was approximately equal in absence and presence of manganese, and subsequently declined over time,
297 to become undetectable after approximately 6 hours (Figure 3B). This result indicates that the high NADH
298 demand of the luminescence reaction effectively drains the cellular NADH pool, which leads to the rapid
299 decline of the luminescence signal over time. Notably, the decline rate of the luminescence signal was

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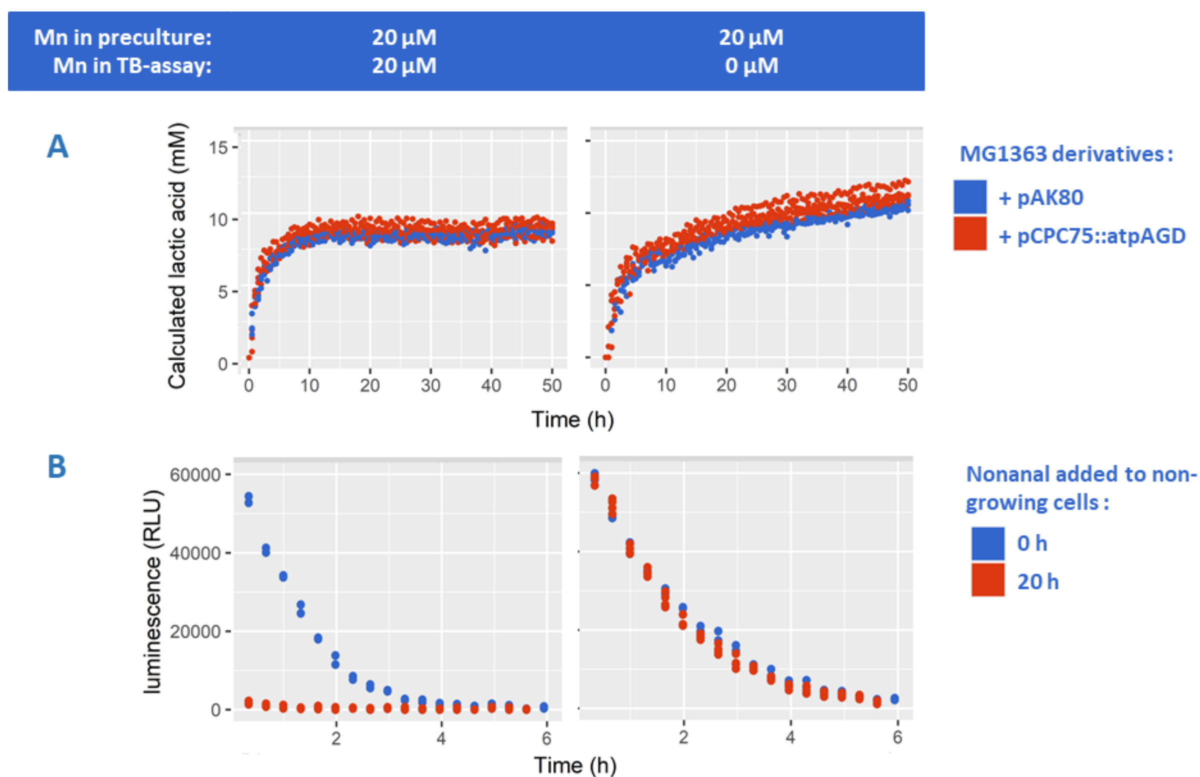
300 higher when manganese was supplemented and reached undetectable luminescence levels 2 hours earlier
301 compared to the condition when manganese was omitted, suggesting that NADH is depleted more rapidly
302 when manganese is present. In a follow up experiment, TB-cell suspensions were left to acidify for 20
303 hours prior to the addition of nonanal to initiate the luminescence reaction (Figure 3B). Under these
304 conditions, the impact of manganese presence in the incubation medium was very pronounced, where the
305 condition lacking manganese generated an initial luminescence level and a subsequent signal-decline curve
306 that were very similar to those observed when nonanal was added from the start, whereas in the presence
307 of Mn there was hardly any detectable luminescence signal (Figure 3B). These results suggest that NADH
308 is depleting during acidification in the presence of Mn, whereas intracellular levels of NADH and redox
309 homeostasis are maintained in the absence of manganese. Taken together these experiments show that
310 manganese-mediated acceleration of acidification correlates with a disruption of redox homeostasis rather
311 than energy homeostasis (ATP/ADP), leading to depletion of NADH and thereby stagnating acidification
312 and possibly inducing a VBNC state. This loss of redox homeostasis is not seen in absence of manganese
313 where NADH pools are apparently kept constant and acidification can be sustained for weeks (27) in these
314 TB-cell suspensions.
315



316

317 **Figure 3** Cells precultured with 20µM Mn were transferred into TB assay with 20µM Mn (left column) or
318 0µM Mn (right column). (Panel A) *Lactococcus cremoris* MG1363 that harbors empty vector pAK80 (upper)
319 or F1-ATPase encoding pCPC75::atpAGD (lower) at 2.0E+07 cells/mL were analyzed for continuous
320 measurement of medium pH to calculate lactic acid production overtime. (Panel B) *Lactococcus lactis*
321 MG1363 (pNZ5519) encoding bacterial *luxAB* luciferase was analysed for luminescence signal maintenance

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322 when starting the reaction after 0h (blue) and 20h (red) of incubation with TB. Cells were incubated at
323 1.0E+07 cells/mL and concentrated to 1E+08 cells/mL prior to luminescence detection. Experiment was
324 carried out at least with 3 biological replicates.

325

326 6. NADH-dependent conversion of aldehydes to alcohols increases in TB-cells 327 upon manganese omission.

328 Next to acidification, NADH depletion might influence or re-route other (industrially-relevant) metabolic
329 pathways that are dependent on the redox state of the cell, such as branched-chain amino acid catabolism.
330 This catabolic pathway is initiated by the transamination of the amino acid leading to the formation of the
331 corresponding α-keto-iso-caproic acid (KICA), which serves as the major metabolic precursor that can be
332 converted to 3 different intermediate products. Only the keto acid conversion to the corresponding
333 aldehyde intermediate, e.g., 3- or 2-methylbutanal, is independent of NAD+ or NADH as a cofactor and is
334 also enhanced by Mn (13). The other keto acid conversions include the NADH-dependent conversion to α-
335 hydroxyisocaproic acid via hydroxyacid dehydrogenase and the NAD+ and CoA dependent conversion to
336 isocaproyl-CoA via ketoacid dehydrogenase.

337 Volatile analysis from TB-cultures shows that the production of 3- and 2-methylbutanal within the first 23
338 to 95 hours was comparable throughout all conditions (Figure 5). However, in the presence of Mn, 3- and
339 2-methylbutanal continued to accumulate during 2 weeks of incubation to reach an average peak area of
340 7.5E+06 (arbitrary unit), which is approximately 3-fold higher than the maximum level reached in the
341 absence of Mn. In contrast, BCAA-derived aldehydes were not further accumulating after the first 23 hours
342 in absence of Mn, and actually declined after 95 hours, suggesting their utilization in aldehyde-consuming
343 reactions. A known reaction for aldehyde conversion leads to formation of the corresponding alcohol (3-
344 and 2-methylbutanol) catalyzed by aldehyde-alcohol dehydrogenase, which is NADH dependent. We found
345 that in the absence of manganese, the maximum peak area of 3-methylbutanol and 2-methylbutanol were
346 increased approximately 10-fold and 5-fold, respectively, in the conditions without manganese compared
347 to those where manganese was supplemented. In the presence of Mn, it is apparent that the reaction
348 cascade stalls at the aldehyde formation, and fails to convert to the alcohol, which agrees with the
349 proposed NADH depletion.

350

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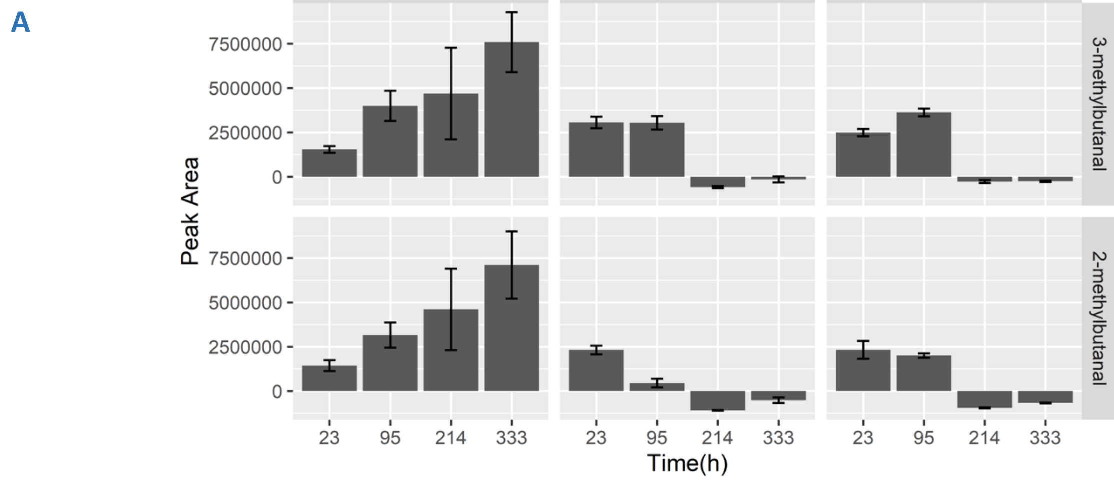
Mn in preculture:	20 µM	20 µM	0 µM
Mn in TB-assay:	20 µM	0 µM	0 µM

A



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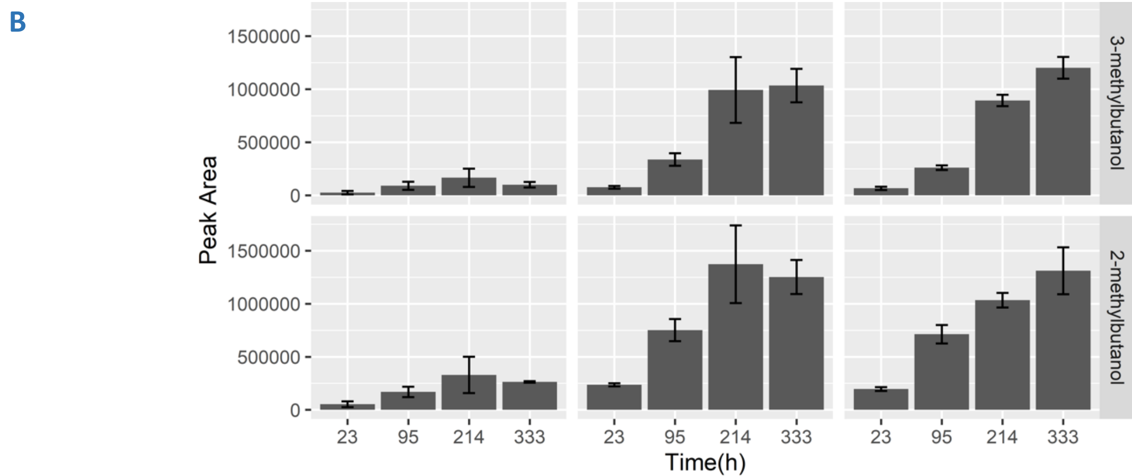

351

352 **Figure 5** *Lactococcus lactis* NCDO712 was precultured in the presence (left and middle panel) and absence
353 (right panel) of manganese (20µM). Cells (2.5E+07 cells/mL) were transferred into fresh medium
354 containing erythromycin (5µg/mL) and 20µM manganese (left panel) or 0µM manganese (middle and right
355 panel). GC-MS peak areas of 3-methylbutanal and 2-methylbutanal (panel A) as well as 3-methylbutanol
356 and 2- methylbutanol (panel B) were measured throughout incubation. Error bars indicate the standard
357 deviation from 3 biological replicates.

358

359

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360 **Discussion**

361 In this study, we evaluated how manganese influenced the physiology of *L. lactis*, and show that
362 manganese omission from the growth medium did not impose a measurable growth rate reduction, while
363 bringing a substantial survival advantage upon translational blocking. We observed that the adaptation of
364 the cellular proteome to growth conditions that lack manganese mainly involves the upregulation of Mn
365 importers. This may allow for the accumulation of minute levels of Mn contaminations from medium
366 constituents inside the cell to achieve Mn levels that support a high growth rate. Although we did not
367 determine intracellular Mn concentrations, it has previously been reported that *Lactococcus lactis* MG1363,
368 accumulated up to 0.7 mM Mn intracellularly during growth in the medium that was also employed in this
369 study (38). The study implied that manganese is required for lactococcal enzyme activities, making our
370 finding that we can omit this metal from the medium without consequences in terms of growth or central
371 energy metabolism even more striking. In addition, the results we report here are also contrasting the
372 apparent dependency for Mn in other lactic acid bacteria species, to sustain rapid growth and oxygen
373 tolerance (39, 40).

374 Cytoplasmic manganese is important in lactococci, which is supported by the extensive transport systems
375 dedicated for Mn homeostasis. Four Mn transport systems were suggested from the MG1363 genome
376 which includes the chromosomal NRAMP-family transporter (encoded by *mntH)*, an ABC
377 transporter(encoded by *mtsAB* -- see below*)*, and the putative Mn-Fe importer (encoded by *llmg1024-*
378 *1025* - see below). Additionally, *L. lactis* strain NCDO712 used in this study also harbors 6 plasmids , one
379 of which encodes an extra copy of NRAMP-family transporter MntHp (encoded by $mntH_p$). Next to the
380 characterized transport systems, the proteins encoded by *llmg*_1024-1025 (domain PF01988; detected
381 with E-value 3.1E-41 and 3.1E-40, respectively) show the presence of a VIT1 domain that is also found in
382 various Fe2+/Mn2+ import systems. The upregulation we found for these genes support the role of this
383 protein in Mn import. From an evolutionary perspective, dairy lactococci need to acquire manganese, of
384 which the availability is limited in their natural environment. In bovine milk, Mn concentrations are
385 reported to vary between 4 and 6.5 μM (41) which is in the same order of the concentration supplied in
386 the present study. The total concentration of Mn in cheese was found at a similar level, which was between
387 5 and 20 μmole per kg cheese (42), but its bioavailability might be influenced by the pH, salt concentration,
388 association with other (metal) ions or casein micelles, water activity and various other factors (43). The
389 availability of redundant transporter systems are likely to ensure sufficient uptake under dynamic
390 environmental conditions.

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391 It was unexpected that in non-growing cells, we found that manganese supplementation at a physiological
392 concentration for growth led to rapidly stagnating acidification and a culturability decline. This effect on
393 acidification may result from the manganese-mediated modulation of the activity of enzymes in the central
394 energy generating pathway. Within this metabolic pathway, various enzymes were reported to be
395 dependent or activated by manganese such as. FBPase, PGM and LDH (1, 9, 44). As a result, manganese
396 addition might affect the homeostasis of this pathway. The balance of ADP/ATP and NAD+/NADH are
397 especially important as both have been implicated in controlling metabolic flux as well as fermentation
398 end-product profiles from homolactic to mixed acids (33, 45). We demonstrated that in our setup the
399 effects on central metabolism were associated with NAD+/NADH rather than ADP/ATP disbalance.

400 Manganese deficiency might not be fully compensated by overproduction of Mn importers as demonstrated
401 by the changes of NADH-utilizing enzymes in the absence of Mn. If NADH is a rate-limiting factor, such
402 changes might be an effort to tune the flux through those reactions and thereby preventing an excessive
403 drain of the intracellular NADH pool. Alternatively, regulating the level of these enzymes at low intracellular
404 NADH concentrations may be necessary to maintain their flux at the required level. In contrast,
405 translational blocking disallows proteome changes that may serve as cell's coping mechanism against NAD
406 imbalance. Without proteome adjustment, a slight disbalance in NAD(H) regeneration might lead to a
407 substantial depletion of NAD(H) over a prolonged period of time, which potentially explains the stagnating
408 acidification of non-growing cells. Consequently, the lack of NADH also blocks other pathways that rely on
409 its availability such as alcohol production in BCAA catabolism as well as $FMNH_2$ regeneration required for
410 luminescence reaction (Figure 6). Nevertheless, it is remarkable that the long term conversion of keto
411 acids to aldehydes in this study was maintained even when central energy-generating pathway has halted
412 for an extended period, implying that cells are likely to maintain a sufficiently high energetic state to
413 import amino acids and that NADH depletion rather than ATP depletion corresponds to the emergence of
414 VBNC state. In yeasts, it has been reported that NAD(P)H depletion is associated with VBNC state resulting
415 from sulfite exposure (46, 47). In non-growing cells of retentostats, where the carbon source is
416 continuously supplied albeit rapidly utilized (48), the production of various aldehydes from amino acid
417 degradation such as benzaldehyde, benzeneacetyladehyde, 3-(methylthio)-propanal was not only highly
418 correlated with the low growth rate but also with the increased loss of culturability on agar. While
419 intracellular NADH concentration was not measured in these retentostat studies, it could be that NADH

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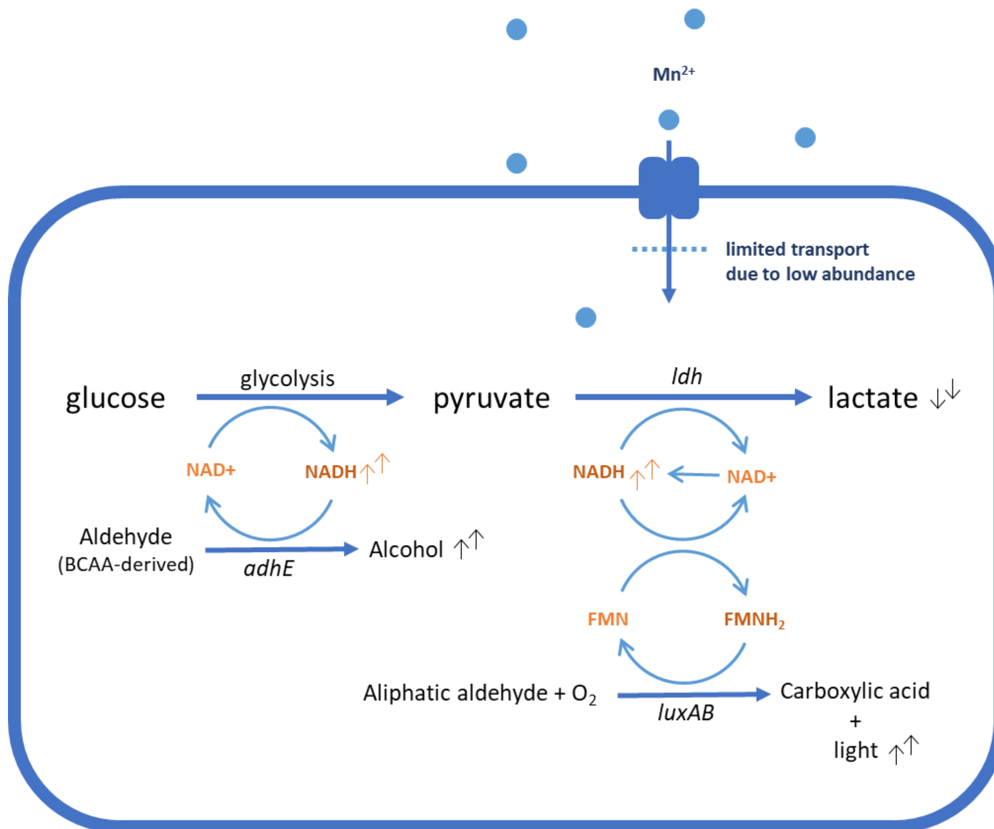
420 disbalance coincides with the emergence of VBNC populations and coinciding accumulation of aldehyde-
421 volatiles under those conditions.



422

423 **Figure 6** Schematic simplification of the effect of manganese omission on the metabolism of TB-*L. lactis*
424 NCDO712. Limited manganese import might reduce glycolysis flux or LDH activity, but prevents NADH
425 depletion, and allows other NADH-dependent reactions to take place for a prolonged period. Increase (↑↑
426) or decrease (↓↓) of metabolic compounds measured upon manganese omission are indicated.

427

428 Our study is relevant for biotechnological and fermentation purposes, especially when the metabolism of
429 non-growing cells that rely on cofactor recycling is of interest. As demonstrated, the prolonged stability of
430 acidification and NADH regeneration are potentially crucial for the transition toward non-culturable state.
431 Such transition is potentially relevant for various applications where cells are stored under suboptimal
432 conditions such as commercial starters and probiotic products. In line with other investigations of
433 lactococci at near-zero growth (49), the distinct end-metabolite and its accumulated formation by viable
434 but non-culturable lactococci substantiate their potential important role in flavor formation during ripening.
435 This was exemplified by BCAA-derived volatiles that are particularly critical for the flavor characteristics
436 of fermented foods. Aldehyde intermediates from BCAA catabolism are more potent flavor volatiles than
437 their alcohol derivatives with an odor threshold that differs by two orders of magnitude (50). The prolonged
438 aldehyde formation in cells that no longer acidify and presumably no longer generating substantial levels

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439 of ATP imply that this metabolite formation is uncoupled from cellular energy status and growth and
440 potentially very suitable for cell factory applications. Next to volatile production, the present study further
441 highlights the relevance of our high-throughput non-growing model system for the investigation of starter
442 cultures in cheese ripening where flavor formation by non-acidifying VBNC cells with limited protein
443 synthesis can be mimicked. Finally, understanding the factors that influence the stability of prolonged
444 metabolism through the presented TB model may provide new approaches in modulating the yield of
445 desired compounds produced by non-growing cells.

446 Conflict of Interest

447 The project is organized by and executed under the auspices of TiFN, a public - private partnership on
448 precompetitive research in food and nutrition. HB is employed by NIZO Food Research. The authors have
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451 The Netherlands) and the Top-sector Agri&Food.

452 Author Contributions

453 ADWN, MK and HB conceived and designed the study, ADWN, BvO, SB, SAB carried out the
454 experiments, all authors analyzed the data; ADWN, MK and HB wrote the paper;

455 Funding

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462 discussion.

463 Associated data

464 Supplementary Materials

465 Text S1: Supplementary methods, information and additional data

466 Text S1, DOCX file, 2.85MB

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467 Supplementary Text S1.docx

468

469 Table S1: Complete list of proteins measured in manganese omitted compared to manganese
470 supplemented cultures.

471 Table S1, XLSX file, 0.4MB

472 Supplementary Table S1.xlsx

473

474 **References**

475 1. Kehres DG, Maguire ME. 2003. Emerging themes in manganese transport, biochemistry and
476 pathogenesis in bacteria. FEMS Microbiol Rev. Elsevier.

477 2. Archibald FS, Duong MN. 1984. Manganese acquisition by Lactobacillus plantarum. J Bacteriol 158.

478 3. Nies DH. 2016. The biological chemistry of the transition metal transportome of: Cupriavidus
479 metallidurans. Metallomics. Royal Society of Chemistry.

480 4. Cowan JA. 2002. Structural and catalytic chemistry of magnesium-dependent enzymes. BioMetals.
481 Springer.

482 5. Vernon WB. 1988. The role of magnesium in nucleic-acid and protein metabolism. Magnesium.

483 6. Foster AW, Osman D, Robinson NJ. 2014. Metal preferences and metallation. J Biol Chem. American
484 Society for Biochemistry and Molecular Biology Inc.

485 7. Irving H, Williams RJP. 1948. Order of stability of metal complexes. Nature 162:746–747.

486 8. Nierop Groot MN, Klaassens E, de Vos WM, Delcour J, Hols P, Kleerebezem M. 2005. Genome-based in
487 silico detection of putative manganese transport systems in Lactobacillus plantarum and their genetic
488 analysis. Microbiology 151:1229–1238.

489 9. Holland R, Pritchard GG. 1975. Regulation of the L-Lactate Dehydrogenase from Lactobacillus casei by
490 Fructose-1,6-Diphosphate and Metal IonsJOURNAL OF BACTERIOLOGY.

491 10. Archibald FS, Fridovich I. 1981. Manganese, superoxide dismutase, and oxygen tolerance in some lactic
492 acid bacteria. J Bacteriol 146.

493 11. Barnese K, Gralla EB, Valentine JS, Cabelli DE. 2012. Biologically relevant mechanism for catalytic
494 superoxide removal by simple manganese compounds. Proc Natl Acad Sci U S A 109:6892–6897.

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495 12. Nierop Groot MN, De Bont JAM. 1999. Involvement of manganese in conversion of phenylalanine to
496 benzaldehyde by lactic acid bacteria. Appl Environ Microbiol 65:5590–5593.

497 13. Smit BA, Engels WJM, Alewijn M, Lommerse GTCA, Kippersluijs EAH, Wouters JTM, Smit G. 2004.
498 Chemical Conversion of α-Keto Acids in Relation to Flavor Formation in Fermented Foods. J Agric Food
499 Chem 52:1263–1268.

500 14. Ward DE, Van Der Weijden CC, Van Der Merwe MJ, Westerhoff H V., Claiborne A, Snoep JL. 2000.
501 Branched-chain α-keto acid catabolism via the gene products of the bkd operon in Enterococcus
502 faecalis: A new, secreted metabolite serving as a temporary redox sink. J Bacteriol 182:3239–3246.

503 15. Dijkstra AR. 2015. Spray drying of starter cultures: Diverse solutions within Lactococcus lactis to
504 improve robustness.

505 16. Rangel DEN. 2011. Stress induced cross-protection against environmental challenges on prokaryotic
506 and eukaryotic microbes. World J Microbiol Biotechnol. Springer.

507 17. Anjem A, Varghese S, Imlay JA. 2009. Manganese import is a key element of the OxyR response to
508 hydrogen peroxide in *Escherichia coli*. Mol Microbiol 72:844–858.

509 18. Masuda A, Toya Y, Shimizu H. 2017. Metabolic impact of nutrient starvation in mevalonate-producing
510 Escherichia coli. Bioresour Technol 245:1634–1640.

511 19. Wang W, Li S, Li Z, Zhang J, Fan K, Tan G, Ai G, Lam SM, Shui G, Yang Z, Lu H, Jin P, Li Y, Chen X, Xia
512 X, Liu X, Dannelly HK, Yang C, Yang Y, Zhang S, Alterovitz G, Xiang W, Zhang L. 2020. Harnessing the
513 intracellular triacylglycerols for titer improvement of polyketides in Streptomyces. Nat Biotechnol
514 38:76–83.

515 20. Graf N, Altenbuchner J. 2014. Genetic engineering of Pseudomonas putida KT2440 for rapid and high-
516 yield production of vanillin from ferulic acid. Appl Microbiol Biotechnol 98:137–149.

517 21. Fuchino K, Kalnenieks U, Rutkis R, Grube M, Bruheim P. 2020. Metabolic Profiling of Glucose-Fed
518 Metabolically Active Resting Zymomonas mobilis Strains. Metabolites 10:81.

519 22. Gasson MJ. 1983. Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci
520 after protoplast-induced curing. J Bacteriol 154:1–9.

521 23. Koebmann BJ, Solem C, Pedersen MB, Nilsson D, Jensen PR. 2002. Expression of genes encoding F1-
522 ATPase results in uncoupling of glycolysis from biomass production in Lactococcus lactis. Appl Environ
523 Microbiol 68:4274–4282.

524 24. Price CE, Branco Dos Santos F, Hesseling A, Uusitalo JJ, Bachmann H, Benavente V, Goel A, Berkhout
525 J, Bruggeman FJ, Marrink SJ, Montalban-Lopez M, De Jong A, Kok J, Molenaar D, Poolman B, Teusink

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526 B, Kuipers OP. 2019. Adaption to glucose limitation is modulated by the pleotropic regulator CcpA,
527 independent of selection pressure strength. BMC Evol Biol 19:15.

528 25. Chen Y, Pelt-KleinJan E van, Olst B van, Douwenga S, Boeren S, Bachmann H, Molenaar D, Nielsen J,
529 Teusink B. 2021. Proteome constraints reveal targets for improving microbial fitness in nutrient-rich
530 environments. Mol Syst Biol 17:e10093.

531 26. Vizcaíno JA, Csordas A, Del-Toro N, Dianes JA, Griss J, Lavidas I, Mayer G, Perez-Riverol Y, Reisinger F,
532 Ternent T, Xu QW, Wang R, Hermjakob H. 2016. 2016 update of the PRIDE database and its related
533 tools. Nucleic Acids Res 44:D447–D456.

534 27. Nugroho ADW, Kleerebezem M, Bachmann H. 2020. A Novel Method for Long-Term Analysis of Lactic
535 Acid and Ammonium Production in Non-growing Lactococcus lactis Reveals Pre-culture and Strain
536 Dependence. Front Bioeng Biotechnol 8.

537 28. Bachmann H, Kleerebezem M, Van Hylckama Vlieg JET. 2008. High-throughput identification and
538 validation of in situ-expressed genes of Lactococcus lactis, p. 4727–4736. *In* Applied and
539 Environmental Microbiology. American Society for Microbiology (ASM).

540 29. Molenaar D, van Berlo R, de Ridder D, Teusink B. 2009. Shifts in growth strategies reflect tradeoffs in
541 cellular economics. Mol Syst Biol 5:323.

542 30. Basan M, Hui S, Okano H, Zhang Z, Shen Y, Williamson JR, Hwa T. 2015. Overflow metabolism in
543 Escherichia coli results from efficient proteome allocation. Nature 528:99–104.

544 31. Goel A, Eckhardt TH, Puri P, de Jong A, Branco dos Santos F, Giera M, Fusetti F, de Vos WM, Kok J,
545 Poolman B, Molenaar D, Kuipers OP, Teusink B. 2015. Protein costs do not explain evolution of
546 metabolic strategies and regulation of ribosomal content: does protein investment explain an anaerobic
547 bacterial Crabtree effect? Mol Microbiol 97:77–92.

548 32. Koebmann BJ, Westerhoff H V, Snoep JL, Solem C, Pedersen MB, Nilsson D, Michelsen O, Jensen PR.
549 2002. The extent to which ATP demand controls the glycolytic flux depends strongly on the organism
550 and conditions for growth. Mol Biol Rep 29:41–45.

551 33. Garrigues C, Loubiere P, Lindley ND, Cocaign-Bousquet M. 1997. Control of the shift from homolactic
552 acid to mixed-acid fermentation in Lactococcus lactis: Predominant role of the NADH/NAD+ ratio. J
553 Bacteriol 179:5282–5287.

554 34. Weissbach H, Etienne F, Hoshi T, Heinemann SH, Lowther WT, Matthews B, St. John G, Nathan C, Brot
555 N. 2002. Peptide methionine sulfoxide reductase: Structure, mechanism of action, and biological
556 function. Arch Biochem Biophys 397:172–178.

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557 35. Nugroho ADW, Kleerebezem M, Bachmann H. 2020. A novel method for long-term analysis of lactic
558 acid and ammonium production in non-growing Lactococcus lactis reveals pre-culture and strain
559 dependence. Front Bioeng Biotechnol 8:1170.

560 36. Ganesan B, Stuart MR, Weimer BC. 2007. Carbohydrate starvation causes a metabolically active but
561 nonculturable state in Lactococcus lactis. Appl Environ Microbiol 73:2498–2512.

562 37. Bachmann H, Santos F, Kleerebezem M, Van Hylckama Vlieg JET. 2007. Luciferase Detection during
563 Stationary Phase in Lactococcus lactis. Appl Environ Microbiol 73:4704–4706.

564 38. Goel A, Santos F, De Vos WM, Teusink B, Molenaar D. 2012. Standardized Assay Medium To Measure
565 Lactococcus lactis Enzyme Activities while Mimicking Intracellular Conditions
566 https://doi.org/10.1128/AEM.05276-11.

567 39. Tong Y, Zhai Q, Wang G, Zhang Q, Liu X, Tian F, Zhao J, Zhang H, Chen W. 2017. System-wide
568 analysis of manganese starvation-induced metabolism in key elements of Lactobacillus plantarum. RSC
569 Adv 7:12959–12968.

570 40. Archibald FS, Fridovich I. 1981. Manganese and Defenses against Oxygen Toxicity in Lactobacillus
571 plantarum Downloaded fromJOURNAL OF BACTERIOLOGY.

572 41. Iyengar G V., Kasperek K, Feinendegen LE, Wang YX, Weese H. 1982. Determination of Co, Cu, Fe, Hg,
573 Mn, Sb, Se and Zn in milk samples. Sci Total Environ 24:267–274.

574 42. Mendil D. 2006. Mineral and trace metal levels in some cheese collected from Turkey. Food Chem
575 96:532–537.

576 43. Du Bray1 EA. Preliminary compilation of descriptive geoenvironmental mineral deposit models.

577 44. Crow' And VL, Pritchard GG. 1977. Fructose 1,6-Diphosphate-Activated L-Lactate Dehydrogenase from
578 Streptococcus lactis: Kinetic Properties and Factors Affecting Activation Downloaded fromJOURNAL OF
579 BACTERIOLOGY.

580 45. Koebmann BJ, Westerhoff H V., Snoep JL, Nilsson D, Jensen PR. 2002. The glycolytic flux in Escherichia
581 coli is controlled by the demand for ATP. J Bacteriol 184:3909–3916.

582 46. Park H, Hwang YS. 2008. Genome-wide transcriptional responses to sulfite in Saccharomyces
583 cerevisiae. J Microbiol 46:542–548.

584 47. Serpaggi V, Remize F, Recorbet G, Gaudot-Dumas E, Sequeira-Le Grand A, Alexandre H. 2012.
585 Characterization of the " viable but nonculturable" (VBNC) state in the wine spoilage yeast
586 Brettanomyces. Food Microbiol 30:438–447.

587 48. van Mastrigt O, Abee T, Lillevang SK, Smid EJ. 2018. Quantitative physiology and aroma formation of a

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588 dairy Lactococcus lactis at near-zero growth rates. Food Microbiol 73:216–226.

589 49. van Mastrigt O, Gallegos Tejeda D, Kristensen MN, Abee T, Smid EJ. 2018. Aroma formation during
590 cheese ripening is best resembled by Lactococcus lactis retentostat cultures. Microb Cell Fact 17:1–8.

591 50. Smit G, Smit BA, Engels WJM. 2005. Flavour formation by lactic acid bacteria and biochemical flavour
592 profiling of cheese products. FEMS Microbiol Rev 29:591–610.

593