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1 **Title: A plasmid-encoded putative glycosyltransferase is involved in hop tolerance and**

2 **beer spoilage in *Lactobacillus brevis*.**

3 **Running title: A glycosyltransferase involved in beer spoilage in *Lb. brevis***

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1

15   **Abstract**

16   *Lactobacillus brevis* beer-spoiling strains harbor plasmids that contain genes such as *horA*,

17   *horC* and *hitA*, which are known to confer hop tolerance. The *Lb. brevis* beer-spoiling strain

18   UCCLBBS124, which possesses four plasmids, was treated with novobiocin resulting in the

19   isolation of UCCLBBS124 derivatives exhibiting hop-sensitivity and an inability to grow in

20   beer. One selected derivative was shown to have lost a single plasmid, designated here as

21   UCCLLBS124_D, which harbors the *UCCLBBS124_pD0015* gene, predicted to encode a

22   glycosyltransferase. Hop   tolerance   and   growth   in   beer   was   restored   when

23   *UCCLBBS124_pD0015* was introduced in one of these hop-sensitive derivatives on a

24   plasmid. We hypothesize that this gene modifies the surface composition of the

25   polysaccharide cell wall conferring protection against hop compounds. Furthermore,

26   introduction of this gene *in trans* in *Lb. brevis* UCCLB521, a strain that cannot grow in and

27   spoil beer, was shown to furnish the resulting strain with the ability to grow in beer while its

28   expression also conferred phage-resistance. This study underscores how the acquisition of

29   certain mobile genetic elements plays a role in hop tolerance and beer spoilage for strains of

30   this bacterial species.

31   **Importance**

32   *Lactobacillus brevis* is a member of the lactic acid bacteria and is often reported as the

33   causative agent of food or beverage spoilage, in particular that of beer. Bacterial spoilage of

34   beer may result in product withdrawal or recall with concomitant economic losses for the

35   brewing industry. A very limited number of genes involved in beer spoilage have been

36   identified and primarily include those involved in hop resistance such as *horA*, *hitA*, and

37   *horC*. However, since none of these genes are universal, it is clear that there are likely (many)

38   other molecular players involved in beer spoilage. Here, we report on the importance of a

39   plasmid-encoded glycosyltransferase associated with beer spoilage by *Lb. brevis* that is

40   involved in hop tolerance. The study highlights the complexity of the genetic requirements to

41   facilitate beer spoilage and the role of multiple key players in this process.

42 **Introduction**

43   *Lactobacillus brevis* is a major threat for commercial and amateur brewers as strains

44 of this species are the predominant bacterial contaminants associated with beer spoilage (1).

45 Such *Lb. brevis* strains can grow in beer despite the presence of ethanol, low pH and the

46 depletion of oxygen and nutrients (2). Moreover, hop compounds added to beer for bitter

47 flavor development during the fermentation process also exert antibacterial activity through

48 the presence of *iso*-α-acids (1, 2). *Lb. brevis* beer-spoiling (BS) strains appear to have

49 acquired chromosomally- or plasmid-derived genetic content to survive and grow in beer (2).

50 *Lb. brevis* resistance to ethanol (up to 10 %) and pH lower than the optimal growth

51 conditions (pH 4-6) seems to be associated with chromosomal genes, possibly due to the

52 general stressors they represent (3, 4). However, *Lb. brevis* BS strains are also known to

53 harbor plasmids that are associated with their beer-spoilage phenotype and more specifically

54 with hop tolerance (5-8). Plasmid-derived genes that underpin hop-resistance in *Lb. brevis*

55 include *horA, horC, hitA* and *orf5$_{ABBC45}$* (1, 2). The genes *horA* and *horC* encode multidrug

56 transporter proteins driven by ATP and proton motive force (PMF), respectively, and were

57 identified as being involved in *iso*-α-acid extrusion from the bacterial cell (5, 7). The gene

58 *hitA* encodes a transmembrane protein involved in the transport of divalent cations such as

59 Mn^{2+} in exchange of protons released from hop bitter acids (8). The *orf5$_{ABBC45}$* gene was

60 identified in *Lb. brevis* BS strain ABBC45 which was unable to grow in beer after it had lost

61 a plasmid carrying this gene. The *orf5$_{ABBC45}$* gene encodes a predicted transmembrane protein

62 resembling a PMF-dependent multidrug transporter, which is presumed to be responsible for

63 *iso*-α-acid export (9).

64   However, these genes are not always indicative of BS ability as the presence of such

65 genes can be found among *Lb. brevis* strains that are unable to grow and consequently spoil

66 beer (designated here as NBS strains) (10). Indeed, *horA* is present in the *Lb. brevis* NBS

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67　　strain UCCLB556 (10). Moreover, genes identified as conferring hop-resistance are not

68　　always simultaneously present in BS strains, e.g. the BS strain UCCLBBS124 carries

69　　plasmids harboring *horA* and *horC*, however it does not possess *hitA* (10). Analysis of BS

70　　strain *Lb. brevis* BSO 464 has highlighted the importance of plasmids and genes on mobile

71　　genetic elements for bacterial growth in beer and beer spoilage ability (6). Recently, a gene

72　　predicted to encode a glycosyltransferase was identified among BS strains responsible for

73　　excess β-glucan formation (11). This gene is also present on the genome of *Lb. brevis* BS

74　　strain UCCLBBS124, while it is absent in that of BS strain UCCLBBS449 (10). This

75　　indicates that beer spoilage is not uniquely governed by the presence of a few genes, but

76　　rather a combination of genes acting in concert to confer beer resistance to the strain. It also

77　　suggests that other plasmid-encoded genes involved in beer spoilage are yet to be discovered.

78　　　　　In the present study we generated plasmid-cured derivatives of *Lb. brevis* BS strain

79　　UCCLBBS124 using novobiocin. This approach has been successfully employed previously

80　　to cure plasmids from lactic acid bacteria (LAB) isolates (6, 12). Plasmid-cured derivatives

81　　were assessed for their ability to grow in the presence of hop and in beer. A derivative that

82　　showed inability to grow in beer was selected and analyzed to ascertain which plasmids were

83　　responsible for this phenotype. Bioinformatic analysis of the genetic content of such plasmids

84　　revealed candidate genes required for growth in beer. These genes were used in

85　　transformation experiments to revert the NBS phenotype.

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88 **Results and Discussion**

89 *Derivatives with impaired growth in beer reveal loss of plasmid UCCLBBS124_D*

90 The beer-spoiling *Lb. brevis* strain UCCLBBS124 (abbreviated here as UCC124) possesses

91 four plasmids carrying genes of interest for bacterial beer spoilage (Table 3). Following

92 exposure to novobiocin, surviving *Lb. brevis* UCC124 cells were plated and fifty isolated

93 colonies (10) were randomly selected for further analysis. Thirty four of these fifty colonies

94 displayed impaired growth in beer. PCR-based identification of the hop-resistance gene *horA*

95 revealed the loss of this gene, located on plasmid UCCLBBS124_D (abbreviated here as

96 UCC124_D) in 33 out of the 34 isolates. One derivative, designated here as MB569, was

97 selected for genome sequencing, after which its sequence was compared to that of the WT,

98 confirming that plasmid UCC124_D had been lost from strain MB569.

99 *Tolerance of MB569 to iso-α-acids, ethanol and low pH*

100 The inability of strain MB569 to grow in beer highlights the apparent importance of plasmid

101 UCC124_D in conferring a beer spoilage phenotype on strain UCC124 (Figure 1). Beer is a

102 harsh environment incorporating a number of stresses such as low pH, lack of nutrients, and

103 the presence of ethanol and hop compounds. In order to understand which of these stresses

104 imposed a negative impact on growth of MB569, the WT strain and MB569 were grown in

105 MRS broth and mimicking conditions encountered in beer, e.g. pH4, 5.4 % ethanol, and 30

106 ppm *iso*-α-acids. Strain MB569 was shown to be capable of growth in MRS broth at neutral

107 pH and at pH 4, while it can also grow in the presence of ethanol comparable to the WT

108 strain (Figure 1). However, MB569 is incapable of growth in the presence of *iso*-α-acids

109 unlike the WT strain UCC124 (Figure 1). This indicates that plasmid-cured derivative

110 MB569 has lost the ability to spoil beer due to its sensitivity to the antimicrobial compounds

111 present in hops. Therefore, based on this phenotype and the finding that MB569 lacks

112   plasmid UCC124_D (when compared to its parental strain), it indicates that this plasmid is

113   linked to hop tolerance and thus contributing to the ability of strain UCC124 to cause beer

114   spoilage.

115   *Identification and functional annotation of genes present on plasmid UCC124_D*

116   Plasmid UCC124_D is 21 kb in size and is predicted to encompass 16 genes. Interestingly, a

117   7 kb region of this plasmid, contains six genes that are uniquely present among the plasmids

118   of *Lb. brevis* BS strains (Table 4) (10). In order to assess the possible role of these genes in

119   beer spoilage, the BS plasmid-specific genes *UCCLBBS124_pD0014* (abbreviated here as

120   *UCC124_D14)*, encoding a predicted cytosine deaminase, *UCCLBBS124_pD0015* (renamed

121   *gtf$_{D15}$*), encoding a predicted glycosyltransferase, *UCCLBBS124_pD0016* (designated here as

122   *UCC124_horA*), which encodes HorA (Table 4), were individually cloned into plasmid

123   pNZ44 prior their transformation into NZ9000. The resulting plasmids were then introduced

124   into strain MB569 to determine the ability of the obtained recombinant strains to grow in beer

125   (where MB569 itself is unable to do so). Genes with locus tags *UCCLBBS124_pD0017*

126   (abbreviated as UCC124_D17), *UCCLBBS124_pD0018* (abbreviated as UCC124_D18) and

127   *UCCLBBS124_pD0019* (abbreviated as UCC124_D19) and encoding acyl-sn-glycerol-3-

128   phosphate acyltransferases and a glycosyltransferase (Table 4) were cloned together as a

129   cluster (as present in plasmid UCC124_D) in pNZ44 prior their introduction into NZ9000

130   and, subsequently, MB569.

131   Introduction of the genes *UCC124_D14*, *UCC124_horA*, *UCC124_D17, UCC124_D18* and

132   *UCC124_D19* in MB569 did not enable any obvious improvement of growth in the presence

133   of *iso*-α-acid (30 ppm) or beer (when compared to strain MB569) (data not shown).

134   Interestingly, expression of *gtf$_{D15}$* in MB569 was shown to confer a positive effect on its

135   ability to grow in MRS broth containing 30 ppm *iso*-α-acids, with a significant (P value <

7

136   0.05) growth increase after 72 h compared to the non-complemented strain or MB569

137   carrying the control plasmid pNZ44 (Figure 2A). When *Lb. brevis* MB569 pNZ44:*gtf$_{D15}$* was

138   cultivated in beer, it also exhibited an ability to grow in beer that was significantly better than

139   that of MB569 itself (P value < 0.05) (Figure 2B). Provision of *gtf$_{D15}$ in trans* in MB569 did

140   not restore its growth in beer to the same level as the WT strain (i.e. the strain from which

141   MB569 was derived), but nonetheless allowed survival and growth in beer for this

142   recombinant strain across 96 h. MB569 and MB569 pNZ44 are still able to survive in the

143   presence of *iso*-α-acids or beer after culture for 72 h (Figure 2A and 2B) which might be due

144   to the presence of plasmid UCCLBBS124_C carrying the gene *horC* (Table 3). The *gtf$_{D15}$*

145   gene is predicted to encode a glycosyltransferase based on BLAST analysis and a HHPred

146   analysis (13) predicted the protein to belong to the glycosyl transferase family 8 associated

147   with cell wall glycosylation (99.9 % probability and E-value < 10^{-28}). Further sequence

148   scrutiny suggests that the Gtf$_{D15}$ protein is a membrane-associated protein (TMHMM Server

149   2.0 (14)) with a predicted signal peptide in its N-terminus that may act as a membrane anchor

150   for the protein (http://phobius.sbc.su.se/ (15)). These predictions suggest that GtfD15 is a cell

151   envelope-associated protein that confers protection against certain environmental stressors

152   such as hop compounds.

153   *Introduction of gtf$_{D15}$ in NBS Lb. brevis strains allows growth in beer*

154   The introduction of *gtf$_{D15}$* in MB569 was shown to significantly improve growth of the strain

155   in MRS broth containing hop compounds (30 ppm *iso*-α-acids) and in beer indicating the

156   importance of this gene for beer spoilage by *Lb. brevis* strain UCC124. In order to assess the

157   potential growth-promoting effect of this gene for an NBS strain when inoculated in beer,

158   *gtf$_{D15}$* when cloned into pNZ44 (pNZ44:*gtf$_{D15}$*) was introduced into the NBS *Lb. brevis* strain

159   UCCLB521 (renamed here as UCC521) (Table 1). Remarkably, the presence of pNZ44:*gtf$_{D15}$*

160   in the NBS strain *Lb. brevis* UCC521 permitted the strain to grow significantly better (P

161 value < 0.05) in MRS broth containing 30 ppm *iso*-α-acids and in beer compared to the strain

162 carrying an empty plasmid which is incapable of survival or growth in these environments

163 (Figure 2C and D). These observations reinforce our results above and highlight the

164 significance of the *gtf*$_{D15}$ gene in hop tolerance and beer spoilage. An alternative, though in

165 our opinion less likely explanation is that strains for which we obtained no or reduced CFUs

166 had entered a viable, but non-culturable (so-called VBNC) state as has been previously

167 observed for beer-passaged *Lb. brevis* strains (16).

168 Introduction of pNZ44:*gtf*$_{D15}$ into ATCC 367, another NBS strain, did not allow improved

169 survival or growth in the presence of hop compounds or in beer (data not shown). This

170 suggests that a strain-specific mechanism and involvement of other genes that are absent in

171 ATCC 367 are responsible for increased hop tolerance. Among beer spoilage-related genes,

172 UCC521 possesses the *orf5*$_{ABBC45}$ gene previously identified as involved in hop tolerance (9),

173 unlike ATCC 367 which does not harbor known genes involved in beer spoilage. Moreover,

174 UCC521, although a non-beer spoiler strain, was isolated from the brewery environment,

175 unlike ATCC 367 which was isolated from silage (10). UCC521 may have acquired genes

176 (such as *orf5*$_{ABBC45}$) or plasmids (UCC521 harbors five plasmids) throughout its presence in

177 the brewery environment, which confer hop tolerance when combined with *gtf*$_{D15}$. This

178 scenario has previously been observed, indicating that *Lb. brevis* strains can only survive and

179 grow in beer when multiple beer spoilage-related genes are present in a particular

180 combination (17). Selection pressures of the beer environment determine the genetic content

181 of beer-spoiling strains. The identification of diagnostic marker genes (DMGs) are important

182 in distinguishing BS from NBS strains, as well as predicting the ability of a given strain to

183 grow in beer (17, 18). In the study from Bergsveinson and Ziola, proposed DMGs were not

184 related to hop tolerance and no genes encoding glycosyltransferases were identified as

185 DMGs. However, a glycosyltransferase-encoding gene located on the plasmid of the BS *Lb.*

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186   *brevis* strain BSO 464 which showed more than 99 % nt similarity to gtf_{D15} was described as

187   unique when compared to a NBS *Lb. brevis* strain KB290 and a BS *Pediococcus damnosus*

188   strain Pc344^T (17). From these observations and knowing that gtf_{D15} is highly prevalent in BS

189   *Lb. brevis* strains (Table 4), we propose to include this gene as a DMG to assess the beer

190   spoiling potential of *Lb. brevis* strains.

191   *Effect on phage sensitivity*

192   As demonstrated above gtf_{D15} was observed to play a role in hop and beer tolerance and is

193   predicted to encode a glycosyltransferase. Since the protein is predicted to be involved in

194   biosynthesis or modification of a cell surface-associated saccharidic polymer, the possible

195   role of this protein in bacteriophage infection was investigated. *Lb. brevis* strain UCC521 is

196   sensitive to *Lb. brevis* phages 3-521 and 521B (19). Plaque assays employing these phages

197   and *Lb. brevis* UCC521 harboring the empty vector pNZ44, or strain UCC521 containing

198   pNZ44:gtf_{D15} displayed similar EOP (Efficiency Of Plaquing) values with no significant

199   difference to the WT (Table 5). However, notable differences in plaque morphology were

200   observed, where plaques were faint and hard to distinguish on the bacterial lawn of UCC521

201   pNZ44:gtf_{D15}. Moreover, overnight incubation of the different strains with the two phages led

202   to complete lysis-in-broth of UCC521 and UCC521 containing pNZ44 with an approximately

203   1000-fold increase of phage titre after overnight propagation (Table 5). In contrast, UCC521

204   pNZ44:gtf_{D15} did not show visible lysis and was able to grow after overnight incubation with

205   just a ten-fold increase in phage numbers after overnight propagation (Table 5). These results

206   reinforce the role of the protein Gtf$_{D15}$ in bacterial protection against diverse environmental

207   hazards such as hop compounds or bacteriophages.

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210 **Conclusions**

211 In this study, we identified a novel genetic component required for beer spoilage and more

212 specifically for hop tolerance. This gene is located on plasmid UCC124_D of *Lb. brevis* BS

213 strain UCC124, validating the importance of plasmids to confer a beer spoilage phenotype.

214 Moreover, this gene had been highlighted previously as common among BS strains (10).

215 Genes required for hop tolerance have all been identified on plasmids (5, 7, 8), reinforcing

216 the importance of such mobile genetic elements in adaptation to the specific hurdles imposed

217 by the beer environment. A derivative of UCC124, MB569 showed impaired growth in beer

218 after the loss of plasmid UCC124_D and despite the presence of plasmids UCC124_B and

219 UCC124_C which carry several genes of interest in beer spoilage. Introduction of gtf_{D15} in

220 strain MB569 restored the hop tolerance phenotype of the strain which ultimately allowed it

221 to grow in beer. Similar results were observed when the gene was introduced into a NBS

222 strain confirming the notion that gtf_{D15} is required for the development of hop tolerance and

223 beer spoilage. Furthermore, this gene impacts on phage sensitivity of its host. This gene

224 seems a unique trait shared among BS strains of *Lb. brevis* and we propose gtf_{D15} as a DMG

225 for the detection of potential bacterial contamination of beer. The gene is predicted to encode

226 a glycosyltransferase and analysis of its topology suggests that it is a membrane-anchored

227 protein involved in the biosynthesis or modification of a cell surface-associated saccharidic

228 polymer. BS strains of *Lb. brevis* have been shown to increase higher molecular weight

229 lipoteichoic acids (LTA) in their cell wall, in the presence of hop bitter acids, thus believed to

230 confer resistance to the bacteria by enhancing the barrier functions of the cell wall and

231 preventing intrusion of hop compounds (20, 21). Moreover, lipoteichoic acids have been

232 described as phage receptors among lactobacilli phages as seen for *Lactobacillus delbrueckii*

233 phages LL-H and JCL1032 (22) but also for *Lactobacillus plantarum* ATCC8014-B2 (23).

234 Therefore, we speculate that this glycosyltransferase is involved in replacing alanine residues

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235   with sugar residues on teichoic acids thereby changing their charge and preventing *iso*-α-

236   acids to penetrate the membrane as well as affecting phage adsorption and/or DNA injection.

237   This predicted glycosyltransferase shows only limited similarity (36 % amino acid similarity

238   in 20 % query cover) with the glycosyltransferase identified in a previous study as

239   responsible for β-glucan formation (11), and is thus believed to play a different role in beer

240   spoilage. Future studies will focus on defining the mechanism that underpins hop tolerance

241   and on determining how the genes identified to date (5, 7, 8) are linked to each other.

242   Moreover, located on the same plasmid as gtf_{D15} are genes predicted to encode a

243   glycosyltransferase and acyltransferases (Table 2) suggesting a common action on teichoic

244   acids with the acyltransferases involved in the acylation of alanine residues or the lipid

245   moiety of the lipoteichoic acids (24). Follow-up work may therefore focus on determining the

246   precise function of the glycosyltransferase (and other associated genes) in the modification of

247   the cell wall and/or cell surface. Another question to be addressed is if and how hop tolerance

248   is enhanced when these genes are present in a certain combination, and how such tolerance is

249   influenced by their expression level.

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251

252 **Materials and Methods**

253 *Bacterial strains and cultivation media*

254 Bacterial strains used in this study are listed in Table 1. *Lb. brevis* strains were grown in

255 MRS broth (Oxoid Ltd., UK) at 30 ˚C while *Lactococcus lactis* NZ9000 was grown in M17

256 broth (Oxoid Ltd., UK) supplemented with 0.5 % glucose. 5 μg/mL chloramphenicol (Cm5)

257 was added to the medium when indicated.

258 *Plasmid curing and plasmid content analysis*

259 The overall experimental approach is presented in Figure S1. Plasmid curing of the BS strain

260 UCC124 was achieved using novobiocin treatment (25). A 1 % inoculum of a WT strain

261 overnight culture was used to inoculate 10 mL MRS broth containing 0.25 μg/mL

262 novobiocin. Cultures were incubated at 26 °C for 72 h. After incubation, cells were diluted

263 and plated on MRS agar. After 3 days of incubation at 26 °C, isolated colonies were

264 randomly selected and derivatives with impaired growth in beer (no growth observed after 72

265 h) were checked for the presence or loss of hop-resistance genes *horA*, *horC* and *orf5$_{ABBC45}$*

266 (Table 2). A derivative showing loss of hop-resistance gene was selected and sequenced

267 using Illumina sequencing technology. Paired-end sequence reads were generated using an

268 Illumina HiSeq2500 system (read length 2 x 250 bp). FASTQ sequence files were generated

269 using the Illumina Casava pipeline version 1.8.3. After Illumina sequencing the obtained

270 sequences were mapped back against the WT reference sequence to detect mutations by

271 single nucleotide polymorphism (SNP) or plasmid content loss. SNP analysis was performed

272 by aligning Illumina raw reads against a reference sequence using Bowtie2 V 2.3.5 (26). The

273 reads were then sorted using Samtools (27) and VarScan v2.3.9 was applied for the detection

274 of variants (28). A minimum allelic variation frequency cut-off of 0.25 was applied.

275 *Construction of plasmid vectors*

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276 Genes of interest were amplified by PCR (Table 2) and cloned into the expression vector

277 pNZ44 (29). PCR products and pNZ44 plasmid DNA were digested with the appropriate

278 enzymes (Roche, USA) at 37 ˚C for at least 4 h, following the manufacturer's instructions

279 (Table 2). A ratio of (3:1) was applied for the ligation of the PCR product with pNZ44 using

280 T4 DNA ligase (Promega, USA). The mixture was incubated at room temperature for at least

281 4 hours prior to electrotransformation into *L. lactis* NZ9000 competent cells.

282 *Preparation of competent cells and electrotransformation*

283 Competent cells of *L. lactis* NZ9000 were prepared as previously described (30). Competent

284 cells of *Lb. brevis* UCC124 were prepared using an adapted version of a previously described

285 protocol (31): An overnight culture was transferred (1 % inoculum) to 10 mL MRS broth

286 containing 1 % glycine and incubated overnight at 30 ˚C. 5 mL of the overnight culture was

287 transferred to fresh MRS broth containing 1 % glycine (50 mL final volume) and cells were

288 grown to an OD_{600nm} of 0.6. Cells were harvested by centrifugation at $4,000 \times g$ for 15 min at

289 4 ˚C and washed in ice-cold wash buffer (0.5 M sucrose, 10 % glycerol). The wash step was

290 repeated twice and the cells were finally resuspended in 200 µL wash buffer prior to storage

291 at -80 ˚C and/or electroporation (see below). All constructs were generated using *L. lactis*

292 NZ9000 as the cloning host, verified by sequencing after PCR amplification using the

293 primers pnz44F and pnz44R (Table 2) prior to their transfer into *Lb. brevis* strains.

294 Electrotransformation was performed using freshly prepared competent cells as described

295 above, where 45 µL of cells and 5 µL of plasmid construct were mixed into a pre-chilled 2

296 mm electroporation cuvette (Cell Projects, Kent, England) and subjected to electroporation at

297 1.5 kV (*Lb. brevis*) or 2.0 kV (*L. lactis*), 200 Ω, 25 µF. Following electroporation, 950 µL

298 recovery broth was added (MRS broth supplemented with 0.5 M sucrose and 0.1 M $MgCl_2$

299 (*Lb. brevis*) or GM17 broth supplemented with 20 mM $MgCl_2$ and 2 mM $CaCl_2$ (*L. lactis*)).

300 Cells were recovered at 30 ˚C for 3 h (*Lb. brevis*) or 2 h (*L. lactis*) prior to spread plating on

14

301 MRS (*Lb. brevis*) or GM17 (*L. lactis*) agar supplemented with Cm5. Presumed transformants

302 were purified on MRS agar + Cm5 and colonies were checked by sequencing after PCR

303 amplification using the primers pnz44F and pnz44R (Table 2) and applied to growth assays

304 as described below.

305 *Growth assays*

306 Growth profiles of the wild-type strain and its derivative were obtained by transferring an

307 overnight culture (1 % inoculum) to MRS broth, MRS broth supplemented with 30 ppm *iso-*

308 α-acids or beer (fresh Heineken lager 5 % ethanol, pH 4, 23 ppm *iso*-α-acids). Cultures were

309 incubated at 30 ˚C for 72 hours. One mL of culture was retrieved after 24, 48, 72 and 96

310 hours, diluted in Ringer's solution and plated on MRS agar plates. Plates were incubated at

311 30 ˚C anaerobically for 48 hours prior to colony counting. The number of viable bacteria of

312 each strain was assessed after CFU/mL calculation. Non-inoculated controls were used in all

313 the experiments as blank measurements. These measurements were then subtracted from each

314 experimental condition to produce the values represented on growth curves. Statistical

315 differences were calculated using unpaired *t* test method (32).

316 *Phage activity against Lb. brevis strains and transformants*

317 To assess phage sensitivity of *Lb. brevis* strains, transformants carrying genes of interest were

318 compared to the wild-type (WT) strain using plaque assays, as previously described (33). A

319 10 µL volume of the appropriate phage dilution and 200 µL of *Lb. brevis* culture were added

320 to 4 mL of soft agar supplemented with 10 mM $CaCl_2$, mixed and poured onto an MRS agar

321 plate supplemented with 10 mM $CaCl_2$ and 0.5% glycine. Plates were incubated at 30 °C

322 overnight and the resulting plaques were enumerated. Phage titre was determined as plaque-

323 forming units per mL (PFU/mL). The ability of phages to propagate and multiply within the

324 host cell was also tested. *Lb. brevis* strains were grown to early exponential phase (OD_{600nm} ~

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325 0.25), at which point phages were added to the culture (T0) at a MOI (multiplicity of

326 infection) of 1, along with 10 mM CaCl$_2$. The mix was further incubated at 30 °C overnight

327 (T1). The number of phages present in the medium (i.e. following removal of bacterial cells

328 by centrifugation) at T1 was then determined by plaque assay. Phage propagation efficiency

329 on a given host was then determined by dividing the phage titre (PFU/mL) at T1 by the phage

330 titre (PFU/mL) at T0.

331 *GenBank accession numbers*

332 *Lb. brevis* UCCLBBS124: CP031169, *Lb. brevis* UCCLBBS124_A: CP031170, *Lb. brevis*

333 UCCLBBS124_B: CP031171, *Lb. brevis* UCCLBBS124_C: CP031172 and *Lb. brevis*

334 UCCLBBS124_D: CP031173.

335 **Author Contributions:** MF performed experiments and genomic analysis. DS, JM, TS and

336 VB provided materials and strains. MF, JM, TS and DS were involved in project design and

337 wrote the manuscript. All authors read and approved the final manuscript.

338 **Conflicts of Interest:** The authors declare that VB and TS are employees of Heineken.

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451   **Table 1.** Bacterial strains and plasmids used in this study.

Strain / Plasmid	Description	References
***Lb. brevis* strains**		
UCCLBBS124 (UCC124)	Beer-spoiling strain isolated from spoiled beer keg (Singapore)	(10)
UCCLBBS449	Beer-spoiling strain isolated from unpasteurized spoiled beer (The Netherlands)	(10)
MB569	Non-beer spoiling strain derivative of UCCLBBS124	This study
UCCLB521 (UCC521)	Non-beer spoiling strain isolated from brewery environment (The Netherlands)	(10)
MB569 pNZ44	MB569 carrying pNZ44	This study
MB569 pNZ44:*gtf*$_{D15}$	MB569 carrying pNZ44 with *gtf*$_{D15}$	This study
UCCLB521 pNZ44:*gtf*$_{D15}$	UCCLB521 carrying pNZ44 with *gtf*$_{D15}$	This study
***L. lactis* strains**		
NZ9000	Transformation host	(34)
Plasmids		
pNZ44	Transformation vector, chloramphenicol resistance gene	
pNZ44:*gtf*$_{D15}$	pNZ44 harboring *gtf*$_{D15}$	This study

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453   **Table 2.** PCR primers used in this study. Incorporated restriction sites are indicated in capital
454   letters.

Primer name	Sequence (5' - 3')	Target	GenBank accession no.
horAF	cgcaactgaggctaacttct	*horA* gene in UCCLBBS124	CP031173
horAR	ggcttgctatgctaggata	*horA* gene in UCCLBBS124	CP031173
horCF	gtatgcctaagtgacgt	*horC* gene in UCCLBBS124	CP031172
horCR	cattctctgcctctatac	*horC* gene in UCCLBBS124	CP031172
orf5F	ctggattgaggtgaggg	*orf5* gene in UCCLBBS124	CP031172
orf5R	gctgtaaagggtagtgattg	*orf5* gene in UCCLBBS124	CP031172
pNZ44F	aacaattgtaacccatac	pNZ44 promoter	
pNZ44R	gaacgtttcaagccttgg	pNZ44 MCS	
pD14F	aaaaaaCTGCAGgtccgaacagcgttcggatt	Gene *UCCLBBS124_pD0014* in UCCLBBS124_D	CP031173
pD14R	aaaaaaTCTAGAttaatcttcgaaatagtt	Gene *UCCLBBS124_ pD0014* in UCCLBBS124_D	CP031173
pD15F	aaaaaaCCATGGgcggtttggatattttatact	Gene *UCCLBBS124_ pD0015* in UCCLBBS124_D	CP031173
pD15R	aaaaaaTCTAGAtcactcagttttcaattccc	Gene *UCCLBBS124_ pD0015* in UCCLBBS124_D	CP031173
pD16F	aaaaaaCTGCAGaggcttgctatgctagg	Gene *UCCLBBS124_ pD0016* in UCCLBBS124_D	CP031173
pD16R	aaaaaaTCTAGAtcacccgttgctcgt	Gene *UCCLBBS124_ pD0016* in UCCLBBS124_D	CP031173
pD17-19F	aaaaaaCCATGGggggtagaatggttctgtt	Gene *UCCLBBS124_ pD0017-19* in UCCLBBS124_D	CP031173
pD17-19R	aaaaaaTCTAGAttattgataatgaccagcaa	Gene *UCCLBBS124_ pD0017-19* in UCCLBBS124_D	CP031173

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462   **Table 3.** *Lb. brevis* UCC124 plasmids and genes of interest for beer spoilage.

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UCC124 plasmids	Size (bp)	ORFs no.	Accession no.	Gene(s) of interest	References
UCCLBBS124_A (UCC124_A)	49,560	42	CP031170		
UCCLBBS124_B (UCC124_B)	23,078	20	CP031171	*gtf family 2*	(11)
UCCLBBS124_C (UCC124_C)	22,370	27	CP031172	*horB, horC, orf5*	(7, 9)
UCCLBBS124_D (UCC124_D)	20,971	16	CP031173	*horA*	(5)

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468   **Table 4.** Presence and absence of genes of UCCLBBS124_D among *Lb. brevis* BS strains.

Gene	Predicted function	*Lb. brevis* BS strains						
		UCCLBBS124	UCCLBBS449	UCCLB95	TMW1.2108	TMW1.2111	TMW1.2112	TMW1.2113
UCCLBBS124_pD0014 = UCC124_D14	Cytosine deaminase	+	+	-	+	+	-	+
UCCLBBS124_pD0015 = gtf$_{D15}$	Glycosyltransferase family 8	+	+	-	+	+	+	+
UCCLBBS124_pD0016 = UCC124_horA	HorA	+	+	-	+	+	-	+
UCCLBBS124_pD0017 = UCC124_D17	Acyl-sn-glycerol-3-phosphate acyltransferase	+	+	-	+	+	+	+
UCCLBBS124_pD0018 = UCC124_D18	Glycosyltransferase family 8	+	+	-	+	+	+	+
UCCLBBS124_pD0019 = UCC124_D19	Acyl-sn-glycerol-3-phosphate acyltransferase	+	+	-	+	+	+	+

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476   **Table 5.** Effect of phages 3-521 and 521B on *Lb. brevis* strain UCC521 and derivatives.

		Lb. brevis strains		
		UCC521	**UCC521 pNZ44**	**UCC521 pNZ44:*gtf*$_{D15}$**
	EOP (Efficiency Of Plaquing)	1.00	0.58 ± 0.29	0.64 ± 0.21
Phage 521B	**Plaque morphology**	Small clear plaques	Small clear plaques	Faint plaques
	Phage titre after O/N propagation* (PFU/mL)	2.90E+09	2.30E+09	3.00E+07
	EOP	1.00	1.52 ± 0.20	1.19 ± 0.19
Phage 3-521	**Plaque morphology**	Small clear plaques	Small clear plaques	Faint plaques
	Phage titre after O/N propagation* (PFU/mL)	4.30E+09	1.80E+09	4.80E+07

477   *Overnight propagation (O/N) was realized with a starting phage titre of 10^6 PFU/mL (results

478   are average of triplicate assays).

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A.

B.
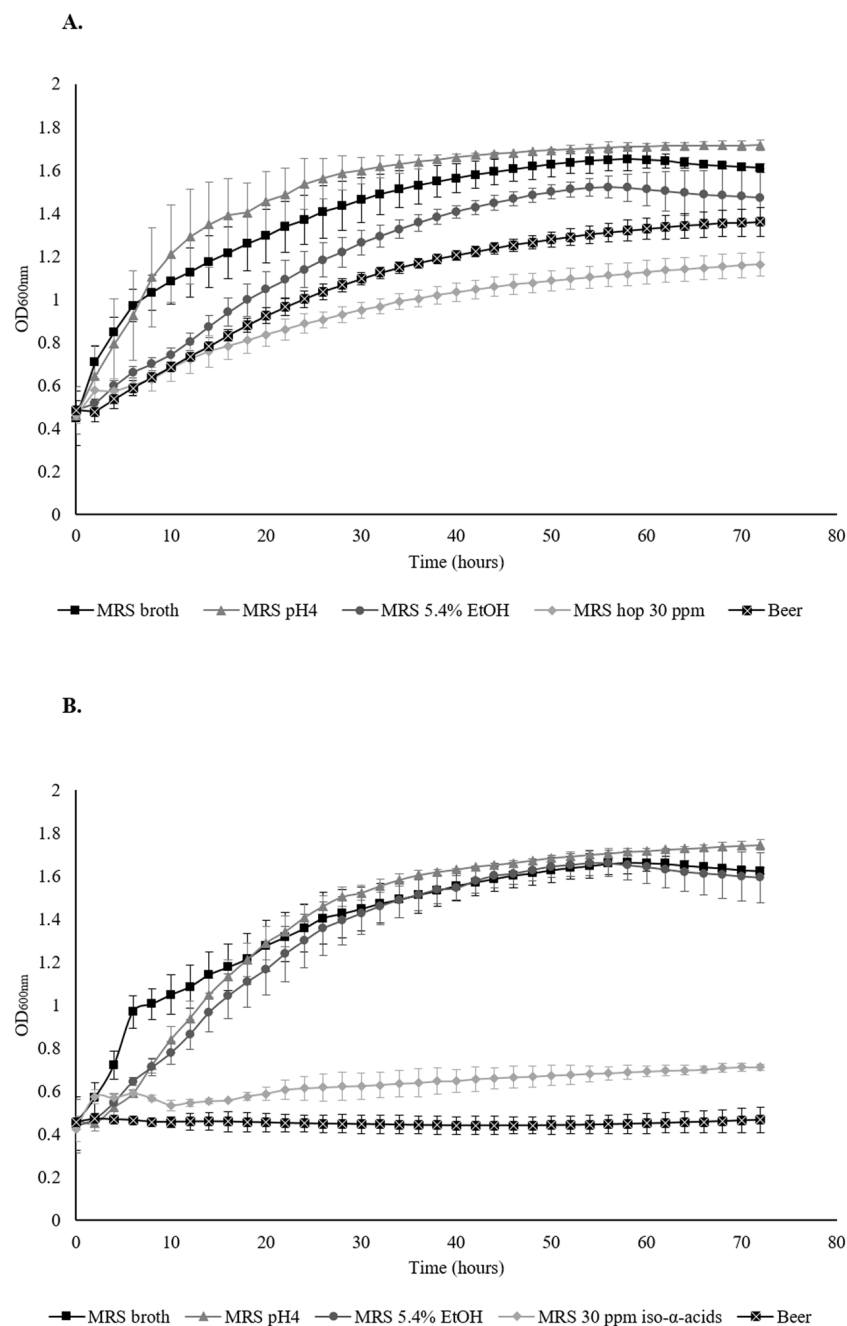
480   **Figure 1.** Growth of the WT beer-spoiling strain *Lb. brevis* UCC124 **(A)** and its plasmid-

481   cured derivative MB569 **(B)** in beer, MRS broth, MRS broth at pH4 and MRS broth

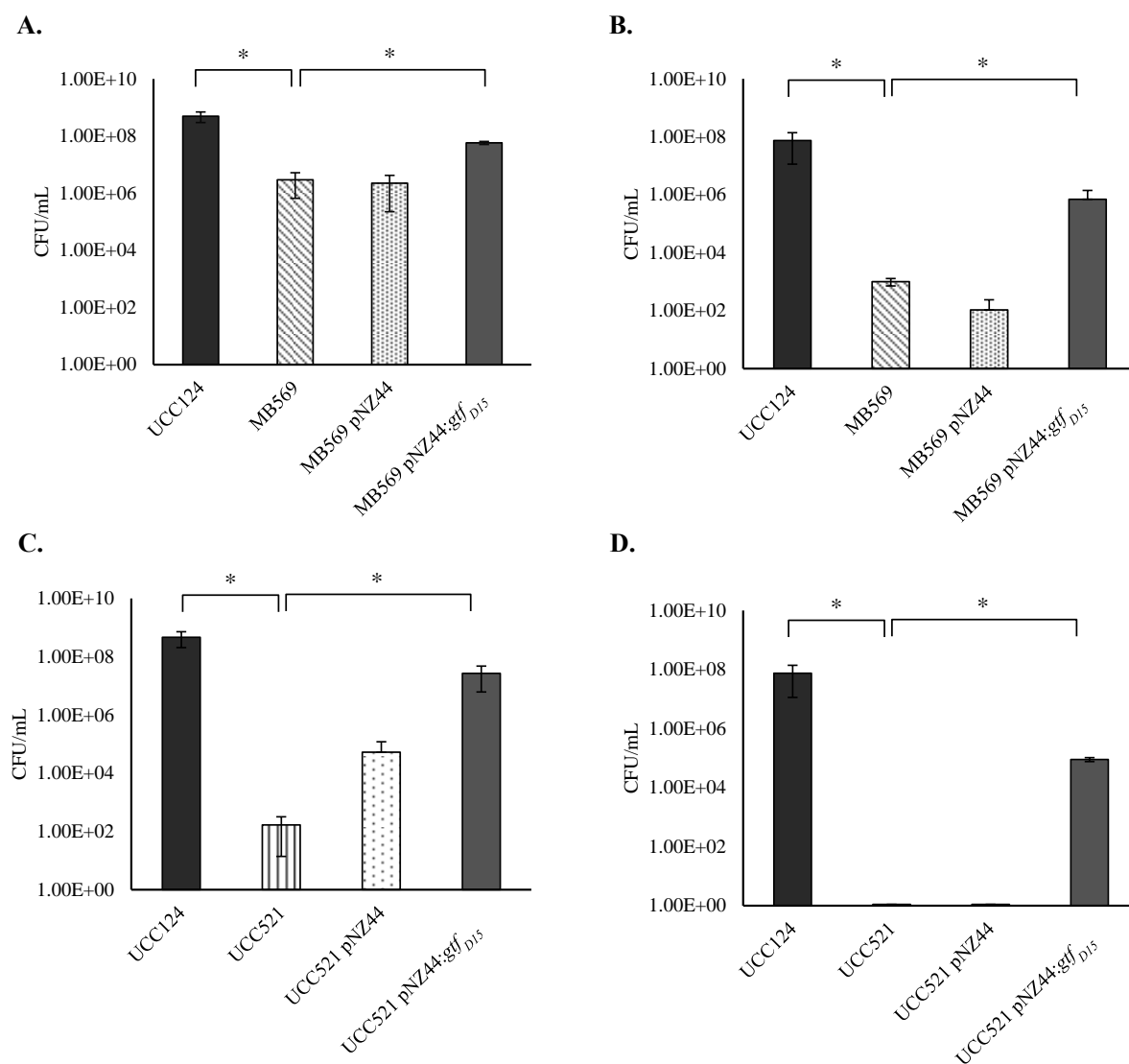482   supplemented with 5.4 % ethanol or 30 ppm *iso*-α-acids.

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483   **Figure 2.** Number of viable bacteria (CFU/mL) of the WT BS strain *Lb. brevis* UCC124, the

484   derivative MB569 +/- the empty plasmid pNZ44 and MB569 carrying the gene gtf_{D15} after

485   growth in **(A)** MRS broth containing 30 ppm *iso*-α-acids for 72 h and **(B)** beer for 96 h

486   (P<0.05). CFU/mL of the WT BS strain *Lb. brevis* UCC124, the NBS UCC521 +/- the empty

487   plasmid pNZ44 and the NBS UCC521 carrying the gene gtf_{D15} after growth in **(C)** MRS

488   broth containing 30 ppm *iso*-α-acids and **(B)** beer for 96 h (P<0.05).

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