

A novel approach in acidic disinfection through inhibition of acid resistance mechanisms; Maleic acid-mediated inhibition of glutamate decarboxylase activity enhances acid sensitivity of Listeria monocytogenes

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1 **A novel approach in acidic disinfection through inhibition of acid resistance**
2 **mechanisms; Maleic acid-mediated inhibition of glutamate decarboxylase activity**
3 **enhances acid sensitivity of *Listeria monocytogenes***

4

5 Ranju Paudyal^a Ruth H. Barnes^a and Kimon Andreas G. Karatzas*^a

6 ^a Department of Food and Nutritional Sciences, The University of Reading, PO Box 226,
7 Whiteknights, Reading RG6 6AP, UK.

8

9

10 * Corresponding author

11

12

13

14 Corresponding author address:

15 School of Chemistry, Food and Pharmacy

16 Department of Food & Nutritional Sciences,

17 University of Reading,

18 Reading

19 RG6 6AD

20 UK

21

22 Tel. +44 118 378 6678

23 Fax. +44 118 931 0080

24 Email: k.karatzas@reading.ac.uk

25

26 **Keywords:** *Listeria monocytogenes*, maleic acid, glutamate decarboxylase, inhibition, GAD, biofilm

27 **ABSTRACT**

28 Here it is demonstrated a novel approach in disinfection regimes where specific molecular
29 acid resistance systems are inhibited aiming to eliminate microorganisms under acidic
30 conditions. Despite the importance of the Glutamate Decarboxylase (GAD) system for
31 survival of *Listeria monocytogenes* and other pathogens under acidic conditions its potential
32 inhibition by specific compounds that could lead to its elimination from foods or food
33 preparation premises has not been studied. The effects of maleic acid on the acid resistance of
34 *L. monocytogenes* were investigated and found that it has a higher antimicrobial activity
35 under acidic conditions than other organic acids, while this could not be explained by its pKa
36 or Ki values. The effects were found to be more pronounced on strains with higher GAD
37 activity. Maleic acid affected the extracellular GABA levels while it did not affect the
38 intracellular. Maleic acid had a major impact mainly on GadD2 activity as also shown in cell
39 lysates. Furthermore, it was demonstrated that maleic acid is able to partly remove biofilms
40 of *L. monocytogenes*. Maleic acid is able to inhibit the GAD of *L. monocytogenes*
41 significantly enhancing its sensitivity to acidic conditions and together with its ability to
42 remove biofilms, make a good candidate for disinfection regimes.

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54 1. INTRODUCTION

55

56 Listeriosis caused by *Listeria monocytogenes* is the leading cause of death due to a food
57 borne illness in the UK (Mook et al., 2011) and as such is a serious problem of Public Health
58 affecting the Food Industry. *L. monocytogenes* is a facultative anaerobic bacterium that can
59 be isolated from soil, water, animal feed, faeces and tissues from various invertebrates and
60 vertebrate animals including humans (Cooper and Walker, 1998). This bacterium has the
61 ability to proliferate in a wide range of temperatures even below zero (Hudson et al. 1994)
62 while it can persist (Fagerlund et al., 2016; Holch et al., 2013) and it is difficult to control in
63 food processing environments (Salyers and Whitt, 2002). Normally, sodium hypochlorite or
64 benzalkonium chloride are used while to a lesser extend acidic disinfectants are also used
65 (Barker and Park 2001; Zhang and Farber 1996). However, a major factor affecting the
66 popularity of a specific class of disinfectants is their antimicrobial activity and here we
67 present a concept that could significantly increase the antimicrobial activity of class of acidic
68 disinfectants. This novel approach that could be used to eliminate *L. monocytogenes* involves
69 specifically inhibiting acid resistance mechanisms in combination with acidic conditions. One
70 such cellular target is the glutamate decarboxylase (GAD) system which is the most
71 important acid resistance system in *L. monocytogenes* (Cotter et al., 2001a) that comprises
72 three decarboxylases (GadD1, GadD2 and GadD3) and two antiporters, (GadT1 and GadT2;
73 Cotter et al., 2005). The antiporters import extracellular glutamate, which is converted to
74 GABA and CO₂, with a subsequent removal of protons, pH increase and export of GABA in
75 exchange of another glutamate molecule (Paudyal and Karatzas, 2016). Furthermore, the
76 decarboxylases can remove protons through processing intracellular glutamate through the
77 intracellular GAD system (GAD; Karatzas et al., 2012). Therefore, affecting the activity of
78 the GAD system could enhance the sensitivity to acid treatments, resulting in successful

79 elimination from food processing environments and food contributing to the reduction of
80 foodborne disease.

81 Previously, maleic acid has been shown to inhibit the GAD system of *E. coli* (Fonda, 1972)
82 while we are not aware of any similar work on other bacteria. Lately, maleic acid has been
83 proposed to substitute the more toxic EDTA in dentistry for plaque removal during
84 implementation of root canals (Ballal et al., 2009b) while it has been shown to eradicate *E.*
85 *faecalis* biofilms (Ferrer-Luque et al., 2010). Organic acids are commonly used in food
86 preservation and in disinfection because of their antimicrobial effects and their low toxicity.
87 An example is lactic acid and its salts that are widely used as antimicrobials in various food
88 products, particularly in meat and poultry (Dibner and Buttin, 2002). However, the
89 antimicrobial effectiveness of maleic acid and its mode of action have not been thoroughly
90 investigated and this is what was attempted in the present study. Firstly, various organic acids
91 such as succinic, acetic, lactic and maleic acid for their inhibitory effects against the growth
92 of *L. monocytogenes* were investigated and maleic acid ranked last. Despite that, it ranked
93 first in bactericidal activity against the same organism under acidic conditions. Subsequently,
94 its mode of action was investigated through functional genomics and protein activity of the
95 GAD system activity and its ability to remove biofilms of this organism as it has been shown
96 to do in dental biofilms (Ballal et al., 2009a).

97

98 **2. MATERIALS AND METHODS**

99

100 ***2.1. Bacterial strains and growth conditions.***

101 All strains (Table 1) were stored in cryovials with 7% DMSO at -80°C . Stock cultures from $-$
102 80°C were passed onto Brain Heart Infusion (BHI) agar (LABM, Lancashire UK) and
103 incubated at 37°C overnight. Three colonies from each plate were transferred with a loop in 3

104 ml of sterile BHI (LAB M, Lancashire UK) and incubated overnight at 37°C with shaking
105 (140 rpm). Subsequently, the overnight cultures were used to inoculate 20 ml of sterile BHI
106 medium (1% inocula) in 250 ml conical flasks and incubated overnight (~ 18 h) at 37°C with
107 shaking (140 rpm). These overnight cultures were used for all acid challenges and assays
108 described below.

109

110 ***2.2. Determination of Minimum Inhibitory Concentrations (MICs)***

111 Concentrations ranging from 0.5-6.9 mg/ml (4.31-60.30 mM) of maleic, succinic, lactic and
112 acetic acid were prepared. BHI Broth prepared with different acids was inoculated with 1%
113 inoculum of overnight cultures and 200 µl of that were placed on 96-well plates. The growth
114 was measured overnight in a Sunrise machine (Tecan, Mannedorf, Switzerland) operated by
115 Magellan software (Tecan, Mannedorf, Switzerland) at 620_{nm} with 20 min time intervals
116 between measurements at 37°C to identify the MIC.

117

118 ***2.3. Survival under acidic conditions***

119 Twenty ml cultures were prepared in BHI and grown in 250 ml flasks overnight at 37°C with
120 shaking. Acid challenge took place with the addition of 4.31 mM of succinic, acetic, lactic,
121 HCl and maleic acid or no acid with the subsequent adjustment of the pH to 3.3 with the
122 addition of 1 M HCl for EGD-e WT and its *gad* mutants. For 10403S WT and *gad* mutants
123 the concentration used was 8.6 mM with adjustment of pH to 3, as the above conditions used
124 for EGD-e did not affect the survival of this strain which has previously been shown to be
125 highly acid tolerant (Karatzas et al., 2012). Samples were obtained prior to pH adjustment
126 and thereafter every 20 min up to 60 min and used to prepare 10-fold serial dilutions which
127 were plated onto BHI agar and incubated at 37°C overnight, and subsequently, colonies were

128 counted to assess survival under lethal acidic conditions. All experiments were performed in
129 triplicate.

130

131 **2.4. GABase assays**

132 GABase assay was used to determine the concentrations of intracellular GABA (GABA_i) in
133 10403S and EGD-e and extracellular GABA (GABA_e) in 10403S and LO28. GABA_i was
134 quantified as described by O'Byrne et al., (2011) while GABA_e was quantified according to
135 the method of Tsukatani et al., (2005) as modified by Karatzas et al., (2010). The GABase
136 reaction was monitored by the measurement of absorbance at 340nm every 2 min for 3 h at
137 37°C using a Sunrise spectrophotometer (Tecan, Mannedorf, Switzerland) operated by
138 Magellan software (Tecan, Mannedorf, Switzerland). All reagents used for the GABase assay
139 were obtained from Sigma-Aldrich (Steinheim, Germany).

140

141 **2.5. GAD activity in protein lysates**

142 Cultures of 10403S and EGD-e were grown in BHI overnight and they were transferred in 50
143 ml centrifuge tubes supplemented with 10 µg/ml chloramphenicol to prevent any further
144 protein translation and were centrifuged at 12,000 X g for 15 min. Cell pellets were washed
145 with sonication buffer as described previously (Abram et al., 2008; Boura et al., 2016) and
146 final cell suspensions were incubated for 30 min with shaking at 37°C. An Eppendorf tube
147 was then filled with acid-washed glass beads (106 µm diameter; Sigma-Aldrich, Steinheim,
148 Germany) and 1ml of cell suspension was transferred to it. Samples were disrupted thrice by
149 a Mini-Beadbeater (Biospec, Bartesville, USA) for 1 min and cooled for 1 min on ice. Then
150 0.1% DNase1 (Sigma-Aldrich, Saint Louis, USA) was added to the cell lysate, incubated at
151 37°C for 30 min with shaking and 1 ml was transferred into Eppendorf tubes and centrifuged
152 at 5,000 X g for 15 min. The supernatant was then transferred to sterile Eppendorf tubes and

153 40 μ l of this was mixed with 450 μ l of pyridine hydrochloride buffer (P-HCl; Fonda, 1972)
154 adjusted at pH 4.5 and supplemented with 30 mM glutamate with or without 8.6 mM maleic
155 acid. Subsequently, GABA levels were measured through GABase assays as described above.
156 Previously, with the use of standard concentrations of GABA it was shown that maleic acid
157 does not inhibit the activity of GABase.

158

159 ***2.6. Determination of GABA by GC-MS***

160 As the activity of the GABase enzyme could be affected by various molecules present in the
161 cultures or the supernatant or the bacterial lysates, GABA concentrations in randomly
162 selected samples were also determined by gas chromatography – mass spectrometry as
163 described previously by Elmore et al., (2005). Results were compared with those by GABase
164 assay and in all cases levels were \pm 5%.

165

166 ***2.7. Biofilm removal by maleic acid***

167 Biofilm formation and its removal by maleic acid was tested on 96-well flat bottom plates.
168 Biofilm assay protocol was adapted from Borucki et al., (2003) as modified by Harvey et al.,
169 (2007) based on the original work of O'Toole and Kolter, (1998), with modifications. 10403S
170 WT was inoculated in BHI at 1% and 200 μ l of that were placed in each well of the 96 well
171 plates and incubated at 30°C static for 4 days. Subsequently, liquid cultures were discarded
172 and biofilms were treated with different maleic acid concentrations (0 - 17.23 mM) at 30°C
173 for 2 min. Then, plates were washed twice with distilled water and were inverted on a paper
174 towel to remove excess moisture followed by drying in a laminar flow hood. Biofilms were
175 stained for 20 min with 0.1% aqueous solution of crystal violet and washed with distilled
176 water three times followed by drying for 20 min. The adhered biofilms were dissolved in

177 95% ethanol and the optical density was measured at 620 nm in a Tecan Sunrise microplate
178 reader (Tecan UK Ltd, Theale, RG7 5AH UK) operated by Magellan software.

179

180 ***2.8. Calculation of undissociated acids using Henderson-Hasselbalch equation***

181 The undissociated percentage of acid was determined according to the equation of
182 Henderson-Hasselbalch as adapted by Wemmenhove et al., (2016).

$$183 \quad [\text{Undissociated acid}] = [\text{Total acid}] / 1 + 10^{(\text{pH}-\text{pKa})} \quad (1)$$

184 Molarities of total acid of solutions were pre-set, pH values were those values determined
185 prior and after incubation. pKa values were used as described in literature. pKa 1.9, 6.07 for
186 maleic acid, 5.61,4.2 for succinic acid, 4.76 for acetic acid and 3.86, 15.1 for lactic acid.

187

188 ***2.9. Statistical analysis***

189 In all cases, experiments were run at least in triplicate (unless stated), and the results were
190 assessed with paired Student *t* test. *P* values lower than 0.05 indicated results that were
191 statistically significant.

192

193 **3. RESULTS**

194

195 ***3.1. MICs of different organic acids.***

196 The MICs of WT 10403S for maleic, succinic, lactic and acetic acid were 34 mM, 25 mM, 31
197 mM and 30 mM which corresponded to pH values prior to growth of 4.84, 5.14, 5.32 and
198 5.02 respectively (**Table 2**). Of all compounds tested, maleic acid was the least inhibitory
199 despite acting at a lower pH (4.84) while succinic was the most inhibitory.

200

201 ***3.2. Acid survival of WT 10403S and EGD-e in the presence of different acids.***

202 To investigate the effect of different acids on acid survival, the most acid resistant (10403S)
203 and the weakest (EGD-e) strain were challenged with 8.6 mM and 4.3 mM of each organic
204 acid at pH 3 and 3.3 respectively. On both strains, maleic acid was the most bactericidal (**Fig.**
205 **1A, B**).

206

207 **3.3. Survival of WT 10403S, LO28 and EGD-e in the presence of maleic acid.**

208 Once it was determined that maleic acid was the most bactericidal, the survival of 10403S
209 and two other strains of *L. monocytogenes* (LO28 WT and EGD-e) was assessed against 4.3
210 mM maleic acid at pH 3. In the absence of maleic acid, 10403S WT was the most resistant
211 strain while LO28 WT intermediate and EGD-e WT the most sensitive (**Fig. 2A, B, C**).
212 However, in the presence of maleic acid results were reversed, with the highest antimicrobial
213 effect seen on 10403S WT followed by LO28 and EGD-e WT (**Fig. 2A, B, C**). As the GAD
214 system is known to be the most important acid resistance mechanism in *L. monocytogenes*
215 (Cotter et al., 2001b), the GABA_e production of these strains was assessed and it was found
216 that 10403S had the most active GAD_e followed by LO28 and by EGD-e, with the latter
217 producing no GABA_e (**Fig. 2D**).

218

219 **3.4. The role of GAD genes in the presence of maleic acid in EGD-e**

220 In the absence of maleic acid, there was no significant difference between the strains at pH
221 3.3 after 60 min (**Fig. 3A, B**) while when 4.3 mM maleic acid was added, EGD-e WT,
222 $\Delta gadD1$, $\Delta gadD2$ and $\Delta gadD3$ showed 2.46, 2.79, 3.8 and 5.2 log reduction respectively
223 (**Fig. 3A**). Thus, $\Delta gadD3$ was the most sensitive strain suggesting that GadD3 is the most
224 important GAD system component for survival either in the presence of maleic acid (**Fig.**
225 **3A**) or in its absence (**Fig. 3B**). This coincides with the lower level of GABA_i in the $\Delta gadD3$
226 while there was no statistically significant difference in the levels of GABA_i between the

227 other mutants and the WT (**Fig. 3C**). Interestingly, although the presence of maleic acid
228 enhanced the acid sensitivity of all strains (**Fig. 3A**), it did not seem to affect the GABA_i
229 levels (**Fig. 3C**).

230

231 ***3.5. The role of GAD genes in the presence of maleic acid in 10403S***

232 In presence of 8.6 mM maleic acid at pH 3, WT, $\Delta gadD1$, $\Delta gadD2$, $\Delta gadD3$ showed 2.0,
233 2.55, 2.61 and 3.28 log reduction respectively (**Fig. 4A**). In the presence of maleic acid,
234 $\Delta gadD2$ showed a rapid inactivation resulting in approximately 3.2 log reduction of the
235 CFU/ml the first 20 min, followed by a minor reduction of 0.5 log CFU/ml the next 40 min.
236 $\Delta gadD2$ was the most sensitive strain followed by $\Delta gadD3$ (**Fig. 4A**) and the relative
237 sensitivity between strains was similar in the absence of maleic acid (**Fig. 4B**).

238 To understand the role of the components of the GAD system genes in the presence of maleic
239 acid, the GAD activity of the 10403S WT and its isogenic GAD mutants was assessed.
240 $\Delta gadD2$ was found to produce no GABA_e (**Fig. 4C**) and very low levels of GABA_i (**Fig. 4D**)
241 both in the presence or absence of maleic acid. All other gene deletions did not seem to have
242 any major effect on GABA_i levels, while similarly to EGD-e, maleic acid did not seem to
243 have any significant effect on GABA_i levels (**Fig. 4D**). However, maleic acid significantly
244 reduced GABA_e levels by 61% ($P < 0.05$; paired T-test), 30% and 38% in the WT, $\Delta gadD1$
245 and $\Delta gadD3$ respectively, while $\Delta gadD2$ was unable to produce any GABA_e (**Fig. 4C**).

246

247 ***3.6. GAD activity in protein lysates.***

248 GAD activity was determined in protein lysates through the assessment of GABA production
249 in the presence of glutamate. Our results show that in 10403SWT, maleic acid resulted in a
250 statistically significant decrease of GAD activity by 21% ($P = 0.0053$; **Fig. 5**). It was also
251 attempted unsuccessfully to setup a similar assay for EGD-e but despite trying different pH

252 values we were unable to detect any GABA (data not shown). This might be due various
253 reasons, such as low levels of GadD3 that cannot result in detectable GABA, inability to
254 recover GadD3 or specific unknown conditions that are required for GadD3 function.

255

256 **3.7. Biofilm Formation.**

257 In all cases the OD_(620nm) measuring the concentration of crystal violet corresponding to the
258 concentration of cells in the biofilm present on polystyrene was slightly lower with statistical
259 significance in the presence of maleic acid than the control (**Fig. 6**). This effect was seen even
260 at low maleic acid concentrations (<17.23 mM) suggesting that the latter could partly remove
261 biofilms of *L. monocytogenes*.

262

263 **3.8. Calculation of the percentage of undissociated acid**

264 One of the two pKas of maleic acid is extremely low (1.9) compared to other weak acids
265 resulting in a low percentage of undissociated (8.01 – 0.04%, pH 3 - 5.32) and a significant
266 percentage of dissociated molecules (91.99 – 99.96%, pH 3 - 5.32; **Table 3**). As seen in
267 (**Table 3**), in contrast to the other acids, the majority of the molecules are in dissociated form
268 in all pH values used.

269

270 **4. DISCUSSION**

271 In the present study, a novel and more targeted approach for the disinfection of *L.*
272 *monocytogenes* that might apply to other pathogens is investigated. The concept relies on the
273 inhibition of major acid resistance systems such as the glutamate decarboxylase system in
274 combination with the application of acidic conditions. This approach could be followed in the
275 inactivation of various other pathogenic organisms possessing the GAD system such as *E.*
276 *coli* (Waterman and Small, 2003), *Shigella flexneri* (Waterman and Small, 2003),

277 *Mycobacterium tuberculosis* (Cole et al., 1998; Cotter et al., 2001a), *Clostridium perfringens*
278 (Feehily et al., 2013), *B. abortus* (Roop et al., 2003) or even spoilage organisms such as
279 several species of lactic acid bacteria (Su et al., 2011). Further research could identify more
280 inhibitors of the GAD system or other amino acid decarboxylase systems.

281 Initially the growth of 10403S WT in the presence of different organic acids namely succinic,
282 lactic, maleic and acetic was investigated. The MIC of maleic acid was 34 mM which was the
283 highest compared to the MIC of the other acids suggesting that it was the least inhibitory
284 (**Table 3**). This should be expected as one of the pKas of maleic acid is as low as 1.9 and at
285 relatively mild acidic conditions, such as those used in the MIC experiments (pH ~5), only a
286 small percentage of the acid would be in the undissociated form (<0.08%; **Table 3**) which is
287 more antimicrobial. The other carboxyl would have a minor influence in the formation of
288 undissociated molecules as the first carboxyl dissociates almost fully (>92%) in pH values
289 above 3. In contrast, the other acids have a significantly higher percentage of undissociated
290 molecules than maleic acid (**Table 3**).

291 Subsequently, the bactericidal activity of these acids focusing on the survival of two *L.*
292 *monocytogenes* strains EGD-e WT (acid sensitive) and 10403S WT (acid resistant) was
293 studied at pH 3.3 and 3 respectively. (**Fig. 1A, B**). Interestingly, maleic acid possessed a
294 significantly stronger bactericidal activity compared to the other acids which could not be
295 explained by its pKa since at this pH, most of the molecules would be in the dissociated form
296 (>92.65%; **Table 3**). On the contrary, at that pH the pKas of all other acids used, were higher,
297 suggesting that most of their molecules were in the undissociated and therefore more
298 bactericidal form compared to that of maleic acid. Despite that, maleic acid excreted
299 significantly stronger bactericidal activity than the other acids at the same pH. This suggests
300 that maleic acid could be used to enhance the antibacterial effects of acidic environments.

301 Furthermore, the bactericidal effects of maleic acid were not due to the classical mechanism
302 of release of protons intracellularly by undissociated molecules of maleate entering the cell.
303 In further experiments, the bactericidal effect of maleic acid at pH 3 in three different strains
304 of *L. monocytogenes* was investigated. We used *L. monocytogenes* 10403S which is one of
305 the most acid resistant strains described in literature, LO28 which has a moderate acid
306 resistance and EGD-e which is one of the most acid sensitive strains described (Feehily et al.,
307 2014; **Fig. 2**). Interestingly, the bactericidal activity of maleic acid was more pronounced
308 with the most acid resistant strain of *L. monocytogenes* (10403S; **Fig. 2C**) less pronounced
309 with moderately acid resistant strain (LO28; **Fig. 2B**) and least pronounced with the least acid
310 resistant strain (EGD-e; **Fig. 2A**). It could be said, that bactericidal effects seemed to be more
311 pronounced the more acid resistant a strain was, but also the higher its GAD activity was
312 (**Fig. 2D**) as these two latter characteristics corresponded well for these 3 strains (**Fig. 2**).
313 This suggested that maleic acid could be involved with the function of the GAD system in *L.*
314 *monocytogenes*.

315 To investigate this hypothesis, it was attempted to see the effect of maleic acid on strains
316 carrying deletions of the GAD genes. The EGD-e strain does not use the GAD_e system
317 mediated mainly by GadD2 but relies on GAD_i mediated mainly by GadD3 which utilises
318 intracellular pools of glutamate (Feehily et al., 2014; Karatzas et al., 2012). This is reflected
319 in the results as the $\Delta gadD3$ was more sensitive than all other strains in the presence or
320 absence of maleic acid (**Fig. 3A, B**). This is explained by the inability of this mutant to utilise
321 the GAD_i system which mainly comprises GadD3 as seen by the complete absence of
322 intracellular GABA either in the presence or absence of maleic acid (**Fig. 3C**). In agreement
323 with previous work, (Feehily et al., 2014) GadD1 seems to be completely inactive as the
324 corresponding mutant showed similar survival to the wild type (**Fig. 3A**). It should be
325 mentioned here that GadD1 of *L. monocytogenes* is a designated glutamate decarboxylase

326 only on the basis of genetic similarity and no worker neither we have ever shown it to
327 possesses such an activity. Therefore, it is still debatable if GadD1 is a glutamate
328 decarboxylase. The survival of the other mutants (**Fig. 3A**) and the levels of GABA_i did not
329 show any possible inhibition by maleic acid. However, it should be stated that GABA_i levels
330 are also affected by its catabolism through the GABA shunt and therefore they do not reflect
331 GAD activity. Similarly, there was no effect of maleic acid or mutations (apart from *gadD2*)
332 on the levels of GAD_i in 10403S.

333 On the other hand, there was a major effect of maleic acid on GAD_i activity in 10403S as all
334 mutants apart from Δ *gadD2*, which does not possess GAD_e activity, showed a reduced GAD_e
335 activity in the presence of maleic acid (**Fig. 4C**). This reduced GAD_e activity resulted in
336 increased inactivation in the presence of maleic acid (**Fig. 4A**). Increased inactivation was
337 observed also in the case of EGD-e, but this was more enhanced with 10403S. This was
338 clearly due to the inhibition of GadD2 in 10403S, while inhibition of maleic acid towards
339 GadD3 might not be so strong.

340 To investigate further the effect of maleic acid on the GAD system, its effect on the GAD
341 activity in lysates of 10403S in the presence or absence of maleic acid was investigated. In
342 these experiments, it was clearly demonstrated that maleic acid inhibits GAD activity by 25%
343 ($P = 0.0053$; **Fig. 5**). Furthermore, our experiments showed that maleic acid acts specifically
344 on the glutamate decarboxylase and not on the antiporter which is a membrane protein and
345 thus was removed in the process of producing the lysate. It has been shown in *E. coli*, that the
346 glutamate decarboxylase enzymes (GadD1 and GadD2) are in the cytosol at neutral pH and
347 undergoes conformational change under low pH triggering their move towards the membrane
348 (Capitani et al., 2003). Previously, it has been shown that maleic acid inhibits glutamate
349 decarboxylase activity in *E. coli* (Fonda, 1972) and in *Curcubita moschata* (Ohno Mei,

350 1962) however, to our knowledge this is the first time it is shown to inhibit the GAD activity
351 in any other bacterium than *E. coli*.

352 The ability of various acids to inhibit the GAD system of *E. coli* has been studied previously
353 (Fonda, 1972) and estimated the K_i of various acids. The K_i is the measure of the inhibition
354 of the GAD enzyme and the K_i of succinate is lower than that of maleic for the GAD of *E.*
355 *coli*. However, in our work maleic showed a much stronger effect on the cells of *L.*
356 *monocytogenes*. However, the final effect is not only the result of the actual inhibition
357 conferred, but also the ability of the compound to enter the cell.

358 Furthermore, since GadD2 is the main determinant of GAD system in 10403S it could be said
359 that maleate that enters the cell inhibits specifically GadD2 activity. Unfortunately, after
360 several attempts it was impossible to identify the optimum pH for the GAD_i system of EGD-e
361 comprising mainly GadD3, to see if there is any inhibitory effect of maleic acid in this strain
362 as well. We speculated that the optimum pH of GAD_i driven mainly by GadD3 in EGD-e is
363 higher than that of GadD2 in 10403S (pH 4.5). However, increasing the pH did not result in
364 any measurable Gad activity.

365 Finally, the possibility of using maleic acid to remove biofilms of *L. monocytogenes* was
366 investigated. Previously, it has been shown that maleic acid is able to remove *E. faecalis*
367 biofilms and it has been suggested to replace EDTA as an irrigant in dentistry due to its lower
368 toxicity (Ferrer-Luque et al., 2010). Furthermore, *L. monocytogenes* is known to form
369 biofilms which are difficult to remove and contribute significantly in the appearance of
370 various outbreaks and individual cases of this pathogen. Our results show that maleic acid is
371 able to partly remove biofilms of *L. monocytogenes* at very low levels (1-2 mM) and in
372 combination with its ability to enhance the sensitivity of this pathogen in acidic environments
373 it could be used in disinfections regimes for this purpose.

374

375 **5. CONCLUSIONS**

376 Overall our work showcases a novel approach in disinfection regimes through inhibition of
377 specific molecular acid resistance systems in combination with acidic conditions. Normally
378 the inhibitor of the acid resistance system and in this specific case, the maleic acid which
379 inhibits the GAD system, could be added in minor concentrations and bring about major
380 reduction in the bacterial numbers. If this property is combined with biofilm removal, it could
381 contribute in the removal of pathogens such *L. monocytogenes* and other microorganisms
382 from food and food premises.

383

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504

505 LEGENDS TO THE FIGURES

506 **Fig. 1** (A) Survival of EGD-e WT in presence of concentration of 0.5 mg/ml maleic,
507 succinate, HCl, acetate and lactate grown in BHI. Subsequently, cultures were acid
508 challenged at pH 3.3 with the addition of 1M HCl. (B) Survival of 10403S WT in presence of
509 1mg/ml concentration of maleic, succinate, HCl, acetate and lactate grown in BHI.
510 Subsequently, cultures were acid challenged at pH 3 with the addition of 1M HCl. Both (A)
511 and (B) cells were grown overnight until stationary phase (~18h) at 37°C with shaking. D.L
512 denotes detection limit of the experimental setup.

513 **Fig. 2** Survival of WT EGD-e (A), LO28 (B), 10403S (C) respectively. Strains were treated
514 without and with 4.3 mM of maleic acid grown in BHI until stationary phase (~18 h) at 37°C
515 with shaking. Subsequently, cultures were acid challenged at pH 3 with the addition of 1M
516 HCl. (D) Extracellular GABA_e through time (min) following the adjustment of pH 4.2 of
517 overnight cultures of WT EGD-e, LO28 and 10403S grown until stationary phase in BHI.

518 **Fig. 3** Survival of stationary phase cultures of EGD-e WT and its *gad* mutants after exposure
519 at pH 3.3 for 60 min in the presence or absence of 4.3 mM of maleic acid (A), or in the
520 absence of maleic acid at pH 2.5 (B). (C) GABA_i of overnight stationary phase cultures of
521 WT EGD-e and its isogenic *gad* mutants following exposure at pH 4.2 for 60 min, in the
522 presence or absence of 4.3 mM maleic acid. Cultures were grown in BHI for ~18 h at 37°C

523 with shaking, pH was adjusted with the addition of 1 M HCl, while asterisks represent a
524 statistically significant difference ($P < 0.05$).

525

526 **Fig. 4** Survival of stationary phase cultures of 10403S WT and its isogenic *gad* mutants at pH
527 3 in the presence or absence of 8.6 mM of maleic acid after 40 min (A) or, in the absence of
528 maleic acid at pH 2.5 for 60 min (B). Concentration of GABA_e (C) and GABA_i (D) in
529 stationary phase cultures of 10403S WT and its *gad* mutants after 60 min at pH 4.2 in the
530 presence or absence of 8.6 mM maleic acid. Intracellular GABA_i. Cultures were grown in
531 BHI for ~18 h at 37°C with shaking, pH was adjusted with the addition of 1 M HCl, while
532 asterisks represent a statistically significant difference ($P < 0.05$).

533

534 **Fig. 5** 10403S WT cells grown overnight until stationary phase (~18 h) at 37°C with shaking.
535 Lysates were produced and GAD activity was monitored in them through GABA
536 measurements. Asterisk represents statistically significant difference ($P < 0.05$).

537

538 **Fig. 6** Average optical densities of crystal violet-stained biofilms of *L. monocytogenes*
539 10403S WT after growth in 96 well plates at 30°C without shaking. Error bars represent
540 standard deviation from triplicate observations and asterisks represents statistically
541 significant difference ($P < 0.05$) with the control containing no maleic acid.

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