

Amino acids other than glutamate affect the expression of the GAD system in Listeria monocytogenes enhancing acid resistance

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1 **Amino acids other than glutamate affect the expression of the GAD system in *Listeria***
2 ***monocytogenes* enhancing acid resistance**

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ABSTRACT

The Glutamate Decarboxylase (GAD) system is important for survival of *L. monocytogenes* and other microorganisms under acidic conditions. Environmental conditions influence the function of the GAD system. Until now, the only conditions known to lead to increased transcription of the GAD system are the stationary phase in rich media and [anoxic](#) conditions. Previously, we showed that transcription of the GAD system requires unidentified compounds other than glutamate present in rich media. Following a test looking at various compounds we identified for first time that peptone, tryptone and casamino acids activate the GAD system under [oxic](#) conditions suggesting that amino acid(s) other than glutamate and/or peptides are important for the above process. The defined medium, where the GAD system is inactive, once it is supplemented with the above compounds results in an active intracellular and extracellular GAD system and increased acid resistance. Through functional genomics we show that these compounds are required for GadD2 activity and although we previously showed that GadD3 is active part of the intracellular GAD system, the supplementation did not activate this gene. The above is explained by the fact that only *gadD2* transcription was upregulated by these compounds while the transcription of *gadD1* and *gadD3* remained unaffected. Together our results show that the *L. monocytogenes* GadD2 decarboxylase is activated in the presence of amino acids or peptides other than glutamate, a finding that has important implications for acid tolerance and food safety.

Keywords

[Glutamate decarboxylase](#) system; *L. monocytogenes*; [peptone](#); [tryptone](#); [casaminoacids](#); [γ-aminobutyrate](#); [acid tolerance](#); [GABA](#)

52

53 INTRODUCTION

54

55 The Glutamate Decarboxylase System (GAD) system is present in a wide variety of living
56 organisms from all kingdoms of life, playing different roles ranging from brain functions in
57 mammals, to response under stress conditions such as temperature shock and hypoxia in plants
58 (Shelp et al., 1999) and to acid resistance in microorganisms. The main role of the GAD system
59 in microorganisms is protection from acidic conditions (Capitani et al., 2003; Cotter et al.,
60 2001a; Su et al., 2011) and in some of them such as *L. monocytogenes* (Cotter et al., 2001a)
61 and *Escherichia coli* (Capitani et al., 2003; Foster 2004) it is the most important acid resistance
62 mechanism. The GAD system is present in many other bacteria such as *Shigella flexneri*
63 (Waterman and Small, 2003), *Mycobacterium tuberculosis* (Cole et al., 1998; Cotter et al.,
64 2001a), *Lactobacillus reuteri*, *Lactococcus lactis*, *Lactobacillus plantarum* (Su et al., 2011)
65 and *Brucella* spp. (Damiano et al. 2014). *L. monocytogenes* normally possesses three
66 decarboxylases GadD1, GadD2 and GadD3 and two antiporters GadT1 and GadT2 (Cotter et
67 al., 2005; Karatzas et al., 2012). The five corresponding genes are arranged in three separate
68 operons namely *gadD1T1*, *gadT2D2* and *gadD3* (Cotter et al., 2005). The *gadD1T1* operon has
69 been shown to enhance growth under mild acidic conditions (Cotter et al., 2005), while
70 *gadD2T2* plays important role in survival under severe acidic conditions (Cotter et al., 2001a;
71 Cotter et al., 2005). Furthermore, we have shown that the GAD system comprises two distinct
72 parts i) the extracellular glutamate decarboxylase system (GAD_e) and ii) the intracellular
73 glutamate decarboxylase (GAD_i) system (Karatzas et al., 2012). The GAD_e is the part of the
74 GAD system that imports glutamate from the extracellular environment through the antiporters
75 and the decarboxylases convert it to γ -amino butyric acid (GABA) and CO₂. This reaction
76 consumes a proton resulting in an increase of intracellular pH. Subsequently, the GABA is

77 exported via the antiporters GadT in exchange for a further glutamate molecule. The GAD_i has
78 previously been described converting intracellular pools of glutamate to intracellular GABA
79 (GABA_i; (Karatzas et al., 2010; Karatzas et al., 2012). Subsequently, GABA_i can directed
80 through the GABA shunt and converted to succinate semi aldehyde and succinate (Feehily et
81 al., 2013). Most strains of *L. monocytogenes* such as 10403S and LO28 utilize both GAD_i and
82 GAD_e (Karatzas et al., 2010) whereas EGD-e which is the most studied *L. monocytogenes*
83 strain, utilizes only the GAD_i system resulting in a highly acid sensitive phenotype (Feehily et
84 al., 2013).

85 It is well known that the GAD system is expressed in rich and complex media like Brain Heart
86 Infusion (BHI; Hersh et al., 1996; Penfound et al., 1999). However, we have shown that the *L.*
87 *monocytogenes* GAD system is not being upregulated under acidic conditions, as somebody
88 would expect and its expression depends on unknown signalling compounds present in rich
89 growth media. To date, little is known about the regulation and expression of the GAD system
90 in *L. monocytogenes*. GadD2, the most potent part of the GAD system is known to be
91 upregulated under anoxic conditions (Feehily et al., 2013; Jydegaard-Axelsen et al.,
92 2004), during stationary phase (Cotter et al., 2001b) and in gastric fluid (Cotter et al., 2001a)
93 while we are not aware of any other conditions leading to its upregulation. *L. monocytogenes*
94 requires specific media for growth and for the GAD system to be expressed (Cotter et al.,
95 2001b; Penfound et al., 1999). The inability of the GAD system to be expressed in a defined
96 medium (DM) supplemented with glutamate (Glt; DMG) has been previously shown by
97 Karatzas et al., (2010). In the same study, it was shown that the transcription of the GAD genes
98 was minimal in a defined medium supplemented with glutamate (DMG; Karatzas et al., 2010).
99 Therefore, this medium could be used to study environmental conditions and compounds that
100 trigger the expression of the GAD system, in order to gain insights into how this important acid
101 resistance mechanism is regulated in *L. monocytogenes*.

102 The main aim of this study was to identify compounds that are essential for the expression and
103 function of the GAD system through the use of DMG medium as a basis. During these
104 experiments a number of compounds and mixtures of components were tested and tryptone,
105 peptone and casamino acids were identified as activators of the GAD system. Furthermore, we
106 identified the impact of these mixtures of compounds on survival under acidic conditions, on
107 the GAD_e and GAD_e system activity, the role of each one of the GAD genes and on
108 transcription of the GAD genes.

109 Knowledge of the environmental conditions that activate or inhibit the GAD system can help
110 to understand the acid resistance of this pathogen since the GAD system is the most important
111 mechanism of acid resistance. This knowledge could help us predict the survival of *L.*
112 *monocytogenes* in various acidic foods or environments or even help us manipulate its acid
113 resistance through interventions that could affect the availability of these activators. These
114 findings could have a major economic impact on the food industry and lead to new methods
115 reducing the incidence of listeriosis whose prevalence in the EU and UK has increase
116 significantly the last years (EFSA and ECDC, 2018). Furthermore, since *L.*
117 *monocytogenes* is a model microorganism for the study of the GAD system in Gram-positive
118 bacteria, that is distinctly different from that of Gram-negative bacteria such as *E. coli*, it could
119 contribute to the wider understanding of acid resistance in Gram-positive organisms.

120

121

122 MATERIALS AND METHODS

123

124 *Bacterial strains and growth conditions*

125 Mutants in the three GAD decarboxylases of 10403S and their isogenic WT were used in this
126 study (**Table 1**). All strains were stored at –80°C in cryovial tubes with 7% DMSO. Stock

127 cultures from –80°C were grown in BHI Agar (LAB M, Lancashire, UK) and plates incubated
128 at 37°C overnight. Three colonies from each plate were transferred into 3 ml of sterile BHI
129 (LAB M, Lancashire, UK) and incubated overnight at 37°C with shaking at 140 rpm. DMG
130 (Amezaga et al., 1995) was prepared by supplementation of 6.8% of either tryptone (Thermo
131 Scientific Oxoid, LP0042, Basingstoke, UK), peptone (Thermo Scientific Oxoid, CM009,
132 Basingstoke, UK) and casamino acids (Difco, Michigan, USA). Similarly, for other
133 experiments DMG was prepared with 3.6% peptone or 10% casamino acids. Subsequently, the
134 overnight cultures were used to inoculate 20 ml of sterile DMG medium (1% inoculum) either
135 with or without supplementation in 250 ml conical flasks and incubated at 37°C with shaking
136 at 140 rpm overnight (~24 h). Subsequently, these overnight cultures were used in all other
137 experiments. As described below in GABase Assay, GABA_e was measured in DMG under
138 oxic and anoxic conditions in presence of 0.5 mg/ml of sodium chloride, 0.5
139 mg/ml maleic acid, 0.5 mg/ml succinic acid, 0.5 mg/ml acetic acid, 0.5 mg/ml lactic acid, 0.1%
140 sodium deoxycholate, 5% oxgall, 5% bile salts, 2%, 10% casamino acids, 3.6% peptone and
141 6.8% tryptone was also used to measure GABA_e.

142

143 ***Survival under acidic conditions***

144 Acid survival experiments were performed in DMG. Strains were grown as described above in
145 the presence or absence of 6.8 % tryptone, or peptone, or casamino acids. The 10403S WT
146 strain and its isogenic *gad* mutants $\Delta gadD1$, $\Delta gadD2$ and $\Delta gadD3$ were grown in the presence
147 or absence of 6.8%, 3.6%, 10% tryptone, peptone and casamino acids respectively. Different
148 concentrations were used depending on the *gad* mutants and to achieve measurable rate of
149 death at similar pH. To achieve death of cells at measurable rate, the pH of the overnight
150 cultures was adjusted to 2.4 and 2.5 depending on the experiments performed and challenged
151 with HCl (1M). Samples were obtained prior to the pH adjustment and thereafter every 20 min

up to 60 min decimal serial dilutions were prepared from those samples and plated onto BHI agar. Subsequently, plates were incubated at 37°C overnight, and subsequently, colonies were counted to assess survival under lethal acidic conditions. All experiments were performed in triplicate.

GABase assay

A commercial preparation known as GABase was used to determine the intracellular (GABA_i) and extracellular (GABA_e) concentrations of GABA. GABA_i was quantified as described by O’Byrne et al., (2011), while GABA_e was quantified according to the method of Tsukatani et al., (2011) as modified by Karatzas et al. (2010). The pH of the cultures was adjusted to 4.2 to quantify the levels of GABA_i and GABA_e. This pH was chosen as it does not cause any cell death, which is essential when activity of a cellular system is measured, while it is low enough to ensure GAD system activation (Karatzas et al., 2012).

The GABase reaction was monitored by measurement of the absorbance at 340 nm every 2 min for 3 h at 37°C using a Sunrise spectrophotometer (Tecan, Mannedorf, Switzerland) operated by Magellan software (Tecan, Mannedorf, Switzerland). All reagents used for the GABase assay were obtained from Sigma-Aldrich (Steinheim, Germany).

Real-time PCR determination of gad gene expression

Transcription of the *gad* genes (*gadD1*, *gadD2* and *gadD3*) was assessed as described previously by Karatzas et al., (2010) by real time reverse transcription-PCR (RT-PCR). Transcription of the antiporter-encoding genes (*gadT1* and *gadT2*) was not assessed because their transcription is similar to the corresponding glutamate decarboxylases (*gadD1* and *gadD2* respectively) that belong to the same operon, as shown previously (Karatzas et al., 2012). Efficiencies of the primer pairs *gadD1F-gadD1R*, *gadD2F-gadD2R*, *gadD3F-gadD3R* and

16SF-16SR were 2.12, 2.09, 2.03 and 2.27 respectively (Karatzas et al., 2010). Overnight cultures of 10403S WT were grown for ~24 h in DMG in the presence or absence of 6.8% tryptone or peptone or casamino acids. Samples were taken at 28 h and relative expression of the data was calculated as a ratio between expression of each of the target genes and that of 16S rRNA which was used as the reference gene for each cDNA sample. The advanced relative quantification settings of the Light Cycler 480 SW 1.5.1 software programme were used, with PCR efficiency as described previously (Karatzas et al., 2010). Relative expression of each gene was calculated by dividing the values obtained for this gene with those for the 16S rRNA gene. Subsequently the relative expression values in the presence of either peptone, tryptone or casamino acids in DMG were compared to the controls without those compounds and fold changes were calculated.

188

189 **RESULTS**

190

191 *gad genes in 10403S play no role in survival under acidic conditions in DMG*

192 We previously showed that glutamate supplementation of DM had no effect on survival of
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196 We previously showed that glutamate supplementation of DM had no effect on survival of
197 We previously showed that glutamate supplementation of DM had no effect on survival of

198

199 *Discovery of compounds resulting in GAD_e activity and GABA_e export*

200 DMG was supplemented with different compounds or mixes of compounds and following the
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202 DMG was supplemented with different compounds or mixes of compounds and following the
203 growth of *L. monocytogenes* 10403S, GAD_e activity was assessed through GABA_e
204 measurements to identify potential GAD activators (Fig. 1).

205 None of the individual compounds or mixes of compounds tested resulted in major GAD_e
206 activity, with the exception of an array of undefined mixes of amino acids and/or peptides such
207 as casamino acids, tryptone and peptone. The supplementation of DMG with these mixes
208 resulted in significant levels of GABA_e (Fig. 1A). All these experiments were also performed
209 under anoxic conditions (Fig. 1B)
210 and similarly,
211 under these conditions the presence of casamino acids, tryptone and peptone
212 activated the GAD_e system. It seems that casamino acids resulted in higher levels of GABA_e
213 compared to other compounds tested. Under anoxic conditions, some
214 levels of GABA_e were observed in presence of lactic acid (Fig. 1B).

215

216 *Acid resistance of 10403S WT as a result of GAD system activation*

217 Strain

218 10403S WT grown overnight in 6.8% peptone or tryptone or casamino acids was challenged
219 10403S WT grown overnight in 6.8% peptone or tryptone or casamino acids was
220 challenged with HCl at 2.4 pH. We settled doing experiments with one of the three
221 concentrations used above (6.8mM) to allow comparisons between the different mixtures of
222 compounds and because this concentration provided a clear difference with the negative
223 control. In all cases, 10403S WT grown in DMG with peptone, tryptone and casamino acids
224 was more resistant under acidic conditions compared to DMG alone (Fig. 2A).

225 During acid challenge for 40 min, cells of 10403S WT in DMG supplemented with peptone
226 were the most resistant while those in DMG with tryptone or casamino acids were more

227 sensitive, but still more resistant than those in DMG alone. Interestingly, during the course of
228 the experiment, inactivation rate of cells in DMG with peptone or DMG with tryptone increased
229 while that of cells in DMG with casamino acids decreased, at 60 min the latter had higher
230 numbers than those grown in peptone and tryptone (**Fig. 2A**).

231 GABA_e measurements were performed in the presence of 6.8% of each one of these mixtures
232 in pH 4.2. In the presence of these compounds, GABA_e levels increased ($P<0.05$, paired t-test)
233 steadily within the 60 min of the experiment while no GABA was observed in DMG. Tryptone
234 and peptone similarly resulted in the highest levels of GABA_e reaching close to 10 mM after
235 60 min whereas casamino acids resulted in intermediate levels of GABA_e close to 4 mM (**Fig.**
236 **2B**). As expected, the non-supplemented control did not produce GABA_e in response to
237 acidification. Furthermore, upon supplementation with these three mixes of compounds we
238 also observed statistically significant ($P<0.05$, paired t-test) higher levels of GABA_i production
239 in comparison to the DMG control (**Fig. 2C**).

240

241 *The role of GAD genes in GAD_e and GAD_i activation and survival under acidic conditions*

242 Acid survival was assessed in DMG with supplementation of 3.6%, 6.8% and 10% peptone,
243 Acid survival was assessed in DMG with supplementation of 3.6%, 6.8% and 10% peptone,
244 Acid survival was assessed in DMG with supplementation of 3.6%, 6.8% and 10% peptone,
245 Acid survival was assessed in DMG with supplementation of 3.6%, 6.8% and
246 10% peptone, tryptone and casamino acids individually respectively. In the presence of
247 peptone, WT and its three *gad* mutants showed increased resistance compared to DMG alone
248 (**Fig. 3A**). WT, $\Delta gadD1$ and $\Delta gadD3$ had very similar increased levels of GABA_e in presence
249 of peptone (**Fig. 3B**). The $\Delta gadD2$ was the most sensitive strain either in the presence or
250 absence of peptone suggesting that GadD2 is the most important component of the GAD

system for survival (**Fig. 3A**) and this coincides with no detectable levels of GABA_e being found in the $\Delta gadD2$ (**Fig. 3B**) either in presence or absence of peptone.

In the presence of tryptone, WT and all its *gad* mutants showed similar increased resistance compared to those ones without tryptone (**Fig. 4A**). This coincides with increased levels of GABA_e, however $\Delta gadD2$ did not produce GABA_e compared to other strains even in presence of tryptone (**Fig. 4B**). If we follow the trend, we find that $\Delta gadD2$ with and without tryptone is the most sensitive during survival suggesting that GadD2 is important under acidic conditions.

The $\Delta gadD2$ was the most sensitive strain whereas WT, $\Delta gadD1$, $\Delta gadD3$ were the most resistant in the presence of casamino acids. Although the presence of casamino

10403S WT *gad* gene transcription

Overall transcription of all GAD decarboxylases was low at basal conditions in DMG with

Overall

transcription of all GAD decarboxylases was low at basal conditions in DMG with *gadD3*

The presence of these mixtures resulted in *gadD2* having the highest transcription levels, followed by *gadD3* while *gadD1* had the lowest transcription. Remarkably, the supplementation with these three mixtures did not result in a significant change in the transcription of *gadD1* (**Fig. 6A**) and *gadD3* (**Fig. 6C**) but only in that of *gadD2*. From all three mixtures, peptone had the strongest effect on *gadD2* transcription followed by casamino acids and tryptone (**Fig. 6B**).

DISCUSSION

276 The GAD system plays an important role in *L. monocytogenes* under acidic conditions and it
277 functions by converting extracellular glutamate to GABA, a reaction that removes protons from
278 the intracellular milieu and helps to maintain pH homeostasis (Karatzas et al., 2010; O’Byrne
279 and Karatzas 2008; Waterman and Small 2003). Although work has increased our knowledge
280 on the function of the GAD system in this organism and other Gram-positive bacteria, the
281 effects of environmental conditions and signals on its expression and function are still poorly
282 understood. The only known environmental conditions known to result in increased
283 transcription of the GAD system in this organism are growth in rich media under stationary
284 phase (Cotter et al., 2001a; Cotter et al., 2001b; Penfound et al., 1999), [anoxic](#)
285 conditions (Feehily et al., 2013; Jydegaard-Axelsen et al., 2004) and gastric fluid (Cotter et al.,
286 2001a). No other conditions are currently known to activate the system and even the first
287 condition is vague as the specific compounds, other than glutamate, that lead to activation in
288 the rich media are unknown.

289 In our study, we used the chemically defined medium (Amezaga et al., 1995), which was
290 previously shown to prevent functioning of the GAD_e system in this organism, even upon
291 supplementation with glutamate under acidic conditions (Karatzas et al., 2010). This suggests
292 that unknown compounds are essential for GAD activity in *L. monocytogenes* (Karatzas et al.,
293 2010) and we attempt to identify these compounds in this study. We used this DMG medium
294 as a basis for our screening experiments since *L. monocytogenes* is unable to export GABA in
295 this medium. Supplementation of this medium with various compounds could give an
296 indication of which compounds are required for GAD system upregulation and/or function.

297 Since we also planned to see which part of the GAD system is affected by the supplemented
298 compound(s) we initially assessed if the removal of each one of the decarboxylases in 10403S
299 had an effect on survival in DMG. No difference was observed (**Fig. 1S**) confirming that each
300 of the GAD system components is completely inactive in this medium. Subsequently, a variety

of different compounds was tested for their ability to activate the GAD system of *L. monocytogenes*. None of the individual compounds resulted in increased GAD activity however, supplementation of DMG while all three crude mixtures of aminoacids and/or peptides namely, tryptone, peptone and casamino acids resulted in significant GAD_e activity (Fig. 1A). As anoxic conditions have been shown to activate expression of the GAD system in rich media (Jydegaard-Axelsen et al., 2004) we repeated the above experiments under anoxic conditions. Similar results were obtained in oxic conditions however, levels of GABA looked higher (Fig. 1B). These mixes also activated the GAD_i system (Fig. 1C) and together with the increased GAD_e activity, the overall increased GAD activity resulted in increased acid resistance (Fig. 1A). It should be stated that this increased resistance might also be the result of other mechanisms however, most probably it is majorly the result of GAD system activation since this is the most dominant acid resistance mechanism.

It could be suggested that the above observed GAD system activation and increased acid resistance is the result of osmotic effects. To clarify this, we could compare the osmotic pressure in DMG supplemented with 0.5 M NaCl with DMG supplemented with the amino acid and peptide mixes. However, it is difficult to accurately calculate the levels of osmotic pressure in the DMG supplemented with the amino acid and peptide mixes since they are crude and not defined. However, it is known from chemical analyses of these mixes that their average molecular weight is above 250 Daltons (>250 g/mol). Based on this value a 2, 3.6, 6.8 and 10% solution is equivalent to 0.08, 0.14, 0.27, 0.40 M respectively. Therefore, since 0.5 M NaCl has absolutely no effect on GABA export and given that the molarity of all these mixtures is significantly lower than 0.5 M, we could conclude that the effects observed were not related to osmotic pressure.

325 Interestingly, all three activators identified here were crude mixtures of amino acids and
326 peptides. Casamino acids are a mixture of free amino acids and some very small peptides
327 resulting from the acid hydrolysis of casein (Mueller and Johnson, 1941) whereas tryptone is
328 an assortment of peptides formed by the digestion of casein by the protease trypsin (Fraser and
329 Powell, 1950). Peptone is derived from animal milk or meat digested by proteolysis. It contains
330 peptides, salts, vitamins and many other biological compounds (Payne, 1976). Peptone and
331 tryptone are rich in tryptophan whereas casamino acids lack tryptophan because of acid
332 treatment during casein hydrolysis.

333 As seen in **Fig. 2B** and by comparing the overall effect shown in **Fig. 3B, 4B and 5B**,
334 supplementation with peptone and tryptone resulted in similarly high levels of GAD_e activity.
335 Furthermore, the effect of casamino acids on GAD_e activity seemed to be weaker compared to
336 the effect of the above compounds (Fig. 2B) although the effect on GABA_i was similar to
337 tryptone and peptone (**Fig. 2C**). We speculate that some higher peptides contained in these
338 compounds but not in casamino acids might be activating the GAD_e system. However, it
339 should be stated that under **anoxic** conditions casamino acids seem to have a higher
340 effect on GAD_e activity than the other two compounds (**Fig. 1**). Although supplementation of
341 DMG with these compounds increases GABA_e, GABA_i levels and acid resistance, the overall
342 GABA_e levels achieved by each of these mixes of compounds did not completely correspond
343 to the acid resistance. For example, although supplementation with peptone and tryptone
344 resulted in similar GABA_e levels (**Fig. 2B**), peptone conferred a higher acid resistance than
345 tryptone (**Fig. 2A**). Furthermore, although supplementation with casamino acids provided a
346 much weaker GAD_e activity compared to tryptone and peptone (**Fig. 2B**), it conferred the
347 highest acid resistance (**Fig. 2A**). As stated before, other acid resistance and survival
348 mechanisms might be affected by the supplementation by these compounds. However, it should
349 be noted that the general trend in GAD_e activity corresponded with the trends in survival. It

could be seen that upon supplementation with casamino acids, deployment of both GAD_e and acid resistance was delayed as both curves seem to have an upward trend during the course of the experiment (**Fig. 2A, B**). In contrast, supplementation with tryptone and peptone resulted in a rapid deployment of GAD_e (~70% of GABA_e is produced within the first 20 min) which corresponded well with a higher acid resistance in the initial stages (**Fig. 2A, B**). As the experiment progressed, GAD_e activity of cultures supplemented with tryptone or peptone seemed to reduce, which correlated well with a reduction in acid resistance at later stages (**Fig. 2A, B**). The above suggests that the time of the GAD_e deployment is critical for survival. Furthermore, it should be stated that acid survival at 60 min (Fig.3A) correlated well with the GABA_i levels (**Fig. 2C**), with casamino acids having the highest effect followed by peptone and tryptone. However, GABA_i levels are not only the result of GAD_i activity but are also affected by GABA catabolism through the GABA shunt (Feehily et al.,2013). It should be stated though that, high levels of GABA_i are possibly associated with higher GAD_i activity as GABA catabolism might not be affected significantly, but this is an area that requires further work. Overall it could be said that peptone and tryptone resulted in higher GAD_e activity, while casamino acids most probably resulted in higher GAD_i activity. Similarly, in previous work casamino acids enhanced prolonged survival of *Streptococcus lactis* by supplying amino acids and minimizing break down of essential components needed for survival (Thomas and Batt, 1968). However, we are not aware if this strain possessed a GAD system. Another study showed that various *E. coli* strains and several other bacteria had less acidic shock in Tryptone Soy Broth when supplemented with casamino acids, however there was decreased survival in presence of glutamate alone (Park and Diez-Gonzalez, 2004). These authors have suggested that additional amino acids might enhance the glutamate-dependent acid resistance, which agrees with what we demonstrate here. Recent work has also demonstrated that the presence of amino acids enhanced the ability of *L. monocytogenes* to

grow under mild acidic conditions although no explanation was offered for this (Muchaamba et al., 2019).

Furthermore, we investigated the effect of 6.8% tryptone 3.6% peptone and 10% casamino acids on strains carrying deletions of the GAD decarboxylase genes. We used different concentrations of these compounds in order to obtain measurable levels of inactivation during these survival experiments. The results showed that the $\Delta gadD2$ was the most sensitive either in the presence or absence of peptone or tryptone (Fig. 3A, 4A). Similarly, in presence of casamino acids $\Delta gadD2$ was the most sensitive (Fig. 5A). Even in presence of either of those compounds $\Delta gadD2$ exported less GABA_e than the other mutants and the WT (Fig. 3B, 4B, 5B). The results suggest that GadD2 is the GAD component most affected by supplementation. This is expected as GadD2 is the main component of the GAD_e system that is responsible for GABA export (Cotter et al., 2001a; Cotter et al., 2005) and highly important for survival under acidic conditions (Cotter et al., 2001a). WT, $\Delta gadD1$ and $\Delta gadD2$ were able to export GABA_e in the presence of peptone, tryptone, or casamino acids which also suggests that these compounds might activate the GAD system. This might be the reason for the ability of the strains to export GABA_e.

Subsequently, to identify how these compounds activate the GAD system, we performed RT-qPCR to quantify the transcription of *gadD1*, *gadD2* and *gadD3* that encode the three different decarboxylases of the GAD system. We looked at transcription during stationary phase as it is well-known that the GAD system is mainly expressed at this stage of growth. Furthermore, we have shown previously that *L. monocytogenes* does not respond to acidic conditions by significant increase in the transcription of the GAD system genes *gadD1*, *gadD2* and *gadD3* (Karatzas et al., 2010; Karatzas et al., 2012). It seems that the GAD system needs to be deployed prior to acidification and therefore we just assessed transcription at stationary phase and not following an acid drop. Furthermore, we did not quantify the transcription of

400 antiporters *gadT1* or *gadT2* as it has been previously confirmed that they follow the
401 transcription of their corresponding GAD decarboxylases (*gadD1* and *gadD2*) with which they
402 belong to the same operon (Karatzas et al., 2012).

403 Previously it has been shown that GadD2 is the most important part of the GAD system under
404 acidic stress (Cotter et al., 2001a; Cotter et al., 2005). In 10403S WT (**Fig. 6B**) peptone had
405 the strongest effect on *gadD2* transcription followed by casamino acids and tryptone (**Fig.**
406 **6B**). Although previously GadD3 was identified as a part of the GAD_i system, it did not seem
407 to be upregulated by any of the compounds used here and it did not seem to affect survival in
408 any of the experiments. It might be possible that GadD3 plays a role under different conditions
409 or activated by other environmental conditions.

410 Up to now there is no previous work showing environmental conditions or signals that affect
411 expression of the GAD system in *L. monocytogenes* and most other microorganisms with the
412 exception of *E. coli*. Our research contributes to our understanding of the activation of the GAD
413 system and the acid resistance in this organism. We show that casamino acids, peptone and
414 tryptone are major GAD system activators resulting in upregulation of the transcription of
415 *gadD2*, which is the most important component of the GAD system. This research would be
416 important to scientists working on the acid resistance of *L. monocytogenes* and other organisms.
417 It can also help us predict the behaviour of *L. monocytogenes* in acidic foods and contribute in
418 the development of strategies to manipulate the acid resistance of *L. monocytogenes* and
419 possibly other pathogens by restricting the availability of these activators. This could lead to
420 the elimination of these pathogens from food and therefore contribute in the reduction of
421 foodborne infections.

422

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432 REFERENCES

433

434 Amezcaga, M.-R., Davidson, I., McLaggan, D., Verheul, A., Abee, T., Booth, I.R., 1995. The
435 role of peptide metabolism in the growth of *Listeria monocytogenes*. Microbiol. 141,41–
436 49. doi.org/10.1099/00221287-141-1-41.

437 Capitani, G., Biase, D De., Aurizi, C., Gut, H., Bossa, F., Gru, MG., Ch, È., 2003. Crystal
438 structure and functional analysis of *Escherichia coli* glutamate decarboxylase. EMBO
439 J. 22, 4027-4037. doi:10.1093/emboj/cdg403.

440 Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V.,
441 Eiglmeier, K., Gas, S., Barry, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D.,
442 Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin,
443 N., Holroyd, S., Hornsb,y T., Jagels, K., Krogh, a., McLean, J., Moule, S., Murphy, L.,
444 Oliver, K., Osborne, J., Quail, M a., Rajandream, M a., Rogers, J., Rutter, S., Seeger,
445 K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., Barrell,
446 B.G., 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete
447 genome sequence. Nature. 393,537–544. doi:10.1038/31159.

448 Cotter, P.D., Gahan, C.G., Hill C., 2001a. A glutamate decarboxylase system protects *Listeria*

449 *monocytogenes* in gastric fluid. Mol. Microbiol 40, 465–475. doi.org/10.1046/j.1365-
450 2958.2001.02398.x.

451 Cotter, P.D., O'Reilly, K., Hill, C., 2001b. Role of the glutamate decarboxylase acid resistance
452 system in the survival of *Listeria monocytogenes* LO28 in low pH foods. J. Food. Prot.
453 64,1362–1368. doi: 10.4315/0362-028x-64.9.1362.

454 Cotter, P.D., Ryan, S., Gahan, C.G.M., Hill, C., 2005. Presence of GadD1 glutamate
455 decarboxylase in selected *Listeria monocytogenes* strains is associated with an ability to
456 grow at low pH. Appl. Environ. Microbiol. 71,2832-2839. doi: 10.1128/AEM.
457 71.6.2832-2839.

458 Damiano, M. A., Bastianelli, D., Al Dahouk, S., Köhler, S., Cloeckert, A., De Biase, D.,
459 Occhialini, A. 2014. Glutamate decarboxylase-dependent acid resistance in *Brucella*
460 spp.: distribution and contribution to fitness under extreme acid conditions. Appl.
461 Environ. Microbiol. 81(2):578-86. <https://doi.org/10.1128/AEM.02928-14>

462 European Food Safety Authority and European Centre for Disease Prevention and Control
463 (EFSA and ECDC). 2018. The European Union
464 summary report on trends and sources of zoonoses, zoonotic agents and food-borne
465 outbreaks in 2017
466 . EFSA Journal, 16
467 (12). doi.org/10.2903/j.efsa.2018.5500.

468 Feehily, C., Finnerty, A., Casey, P.G., Hill, C., Gahan, C.G.M., O'Byrne, C.P., Karatzas, K.-
469 A.G., 2014. Divergent evolution of the activity and regulation of the glutamate
470 decarboxylase systems in *Listeria monocytogenes* EGD-e and 10403S: Roles in
471 virulence and acid tolerance. PloS one 9,e112649. doi:10.1371/journal.pone.0112649.

472 Feehily, C., O'Byrne, C.P., Karatzas, K.-A.G., 2013. Functional γ -aminobutyrate shunt in
473 *Listeria monocytogenes*: role in acid tolerance and succinate biosynthesis. Appl.

474 Environ. Microbiol. 79,74–80. doi:10.1128/AEM.02184-12.
 475 Foster, JW., 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. Nature Rev.
 476 Microbiol. 2,898–907. doi: 10.1038/nrmicro1021.
 477 Hersh, B.M., Farooq, F.T., Barstad, D.N., Blankenhorn, D.L., Hersh, BM., Farooq, F.T.,
 478 Slonczewski, J.L., 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*
 479 . J Bacteriol. 178,3978-81. doi: 10.1128/jb.178.13.3978-3981.
 480 Jydegaard-Axelsen, A.-M., Høiby, P.E., Holmstrøm, K., Russell, N., Knøchel, S., 2004. CO2-
 481 and anaerobiosis-induced changes in physiology and gene expression of different
 482 *Listeria monocytogenes* strains. Appl. Environ. Microbiol. 70,4111–4117. doi:
 483 10.1128/AEM.70.7.4111-4117.2004.
 484 Karatzas, K.-AG., Brennan, O., Heavin, S., Morrissey, J., O’Byrne, C.P., 2010. Intracellular
 485 accumulation of high levels of gamma-aminobutyrate by *Listeria monocytogenes*
 486 10403S in response to low pH: uncoupling of gamma-aminobutyrate synthesis from
 487 efflux in a chemically defined medium. Appl. Environ. Microbiol. 76,3529–37.
 488 doi:10.1128/AEM.03063-09.
 489 Karatzas, K.-A.G., Suur, L., O’Byrne, C.P., 2012. Characterization of the intracellular
 490 glutamate decarboxylase system: analysis of its function, transcription, and role in the
 491 acid resistance of various strains of *Listeria monocytogenes*. Appl. Environ. Microbiol.
 492 78,3571–9. doi:10.1128/AEM.00227-12.
 493 Muchaamba, F., Eshwar, A.K., Stevens, M.J.A., von Ah, U., Tasara T., 2019. Variable carbon
 494 source utilization, stress resistance, and virulence profiles among *Listeria*
 495 *monocytogenes* strains responsible for listeriosis outbreaks in Switzerland. Frontiers
 496 Microbiol. 10, 957. doi:10.3389/fmicb.2019.00957.
 497 Mueller, J.H., Johnson, E. R., 1941. Acid hydrolysates of casein to replace peptone in the
 498 preparation of bacteriological media. J. Immunol. 40,33–38.

499 Fraser, D., Powell, E., 1950. The kinetics of trypsin digestion. J. Biol. Chem. 187,803-820.

500 O'Byrne, C.P., Karatzas, K.A.G., 2008. Chapter 5. The Role of Sigma B (σ B) in the stress
501 adaptations of *Listeria monocytogenes*: Overlaps between stress adaptation and
502 virulence. Adv. Appl. Microbiol. 65,115–140. doi: 10.1016/S0065-2164(08)00605-9

503 O'Byrne, C.P., Feehily, C., Ham, R. and Karatzas, K.A.G., 2011. A modified rapid enzymatic
504 microtiter plate assay for the quantification of intracellular γ -aminobutyric acid and
505 succinate semialdehyde in bacterial cells. J. Microbiol. Meth., 84 (1), 137-139. doi:
506 <https://doi.org/10.1016/j.mimet.2010.10.017>.

507 Park, G.W., Diez-Gonzalez, F., 2004. A novel glutamate-dependent acid resistance among
508 strains belonging to the proteae tribe of enterobacteriaceae. FEMS Microbiol. Lett.
509 237,303-9. doi: 10.1016/j.femsle.2004.06.050.

510 Payne, J.W., 1976. Peptides and Micro-Organisms. Adv. Microb. Physiol.13,55-113.

511 Penfound, T.A., Elliott, J.F., Foster, J.W., Smith, D., 1999. Control of acid resistance in
512 *Escherichia coli*. J Bacteriol . 181,3525-35.

513 Shelp, B.J., Bown, A.W., Mclean, M.D., 1999. Metabolism and functions of gamma-
514 aminobutyric acid. Trends Plant Sci. 4, 446-452. doi.org/10.1016/S1360-
515 1385(99)01486-7.

516 Su, M.S., Schlicht, S., Gänzle, M.G., 2011. Contribution of glutamate decarboxylase in
517 *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation.
518 Microb. Cell Fact. 10,S8. doi:10.1186/1475-2859-10-S1-S8.

519 Thomas, T.D., Batt, RD., 1968. Survival of *Streptococcus lactis* in starvation conditions J. gen.
520 Microbiol. 50,367-382. doi: 10.1099/00221287-50-3-367.

521 Tsukatani, T., Higuchi, T., Matsumoto, K., 2005. Enzyme-based microtiter plate assay for γ -
522 aminobutyric acid: application to the screening of γ -aminobutyric acid-producing lactic
523 acid bacteria. Anal. Chim. Acta, 540, 293-297

Waterman, S.R., Small, P.L.C., 2003. The glutamate-dependent acid resistance system of *Escherichia coli* and *Shigella flexneri* is inhibited in vitro by L-trans-pyrrolidine-2,4-dicarboxylic acid. FEMS Microbiol. Lett. 224,119–125. doi:10.1016/S0378-1097(03)00427-0.

TABLES

Table 1. Strains used in this study

Strain	Description	Nature of mutation	Source reference
10403S	Serotype 1/2a, wild type		(Karatzas et al.,2010)
<i>AgadD1</i>	10403S isogenic <i>gadD1</i> mutant	In-frame deletion	(Feehily et al., 2014)
<i>AgadD2</i>	10403S isogenic <i>gadD2</i> mutant	In-frame deletion	(Feehily et al., 2014)
<i>AgadD3</i>	10403S isogenic <i>gadD3</i> mutant	In-frame deletion	(Feehily et al., 2014)

LEGEND TO FIGURES

Fig. 1. Survival after acid challenge stationary phase cultures of 10403S-WT and its isogenic *gad* mutants in DMG. Cultures were grown at 37°C with shaking until stationary phase in DMG

Fig. 1. Cells of 10403S WT grown until stationary phase in DMG in the presence of 0.5 mg/ml of sodium chloride, 0.5 mg/ml maleic acid, 0.5 mg/ml succinic acid, 0.5 mg/ml acetic acid, 0.5 mg/ml lactic acid, 0.1% sodium deoxycholate, 5% oxgall, 5% bile salts, 2%, 10% casamino acids (C.A.), 3.6% peptone and 6.8% tryptone. Following growth, the pH was adjusted to pH 4 the extracellular GABA (GABA_e) was measured under (A) oxic and (B) anoxic conditions in DMG. Bars represent an average of measurements performed in triplicate, and error bars represent the standard deviation. D.L. denotes the detection limit of the GABase method.

Fig. 2. (A) Cells of 10403S WT grown under oxic conditions in DMG alone (grey circles) or in DMG with 6.8% tryptone (grey diamonds), peptone (black squares), casamino acids (C.A.; black triangles) grown under oxic conditions until stationary phase (~24 h). Subsequently, cultures were acid challenged at pH 2.4 with the addition of 1M HCl. Monitoring GABA_e (B) was conducted for 0, 20, 40 and 60 min and GABA_i (C) for 60 min. GABA_e and GABA_i were assessed following adjustment of the pH of overnight cultures of 10403S WT at pH 4.2 grown until stationary phase in DMG and DMG with 6.8% tryptone, peptone and casamino acids (C.A.). Markers represent an average of measurements performed in triplicate, and error bars represent the standard deviation. D.L. denotes detection limit. Asterisk represents statistical significant difference with the control cultures grown in DMG (P<0.05).

Fig. 3. (A) Cells of 10403S WT (white boxes) and its *gad* mutants $\Delta gadD1$ (white triangles), $\Delta gadD2$ (white circles) and $\Delta gadD3$ (white diamonds) grown in DMG alone or in DMG with 3.6% peptone, with WT (black boxes) and its *gad* mutants $\Delta gadD1$ (black triangles), $\Delta gadD2$ (black circles) and $\Delta gadD3$ (black diamonds) grown under oxic conditions until

stationary phase (~24 h). Subsequently, cultures were acid challenged at pH 2.5 with the addition of 1M HCl. Monitoring of GABA_e (B) was conducted for 0, 20, 40 and 60 min and were assessed following adjustment of the pH of overnight cultures of 10403S WT and its *gad* mutants at pH 4.2 grown until stationary phase in DMG and in DMG with 3.6% peptone. Markers represent an average of measurements performed in triplicate, and error bars represent the standard deviation. D.L denotes detection limit.

Fig. 4. (A) Cells of 10403S WT (white boxes) and its *gad* mutants $\Delta gadD1$ (white triangles), $\Delta gadD2$ (white circles) and $\Delta gadD3$ (white diamonds) grown in DMG alone or in DMG with 6.8% tryptone, with WT (black boxes) and its *gad* mutants $\Delta gadD1$ (black triangles), $\Delta gadD2$ (black circles) and $\Delta gadD3$ (black diamonds) grown under oxic conditions until stationary phase (~24 h). Subsequently, cultures were acid challenged at pH 2.5 with the addition of 1M HCl. Monitoring of GABA_e (B) was conducted for 0, 20, 40 and 60 min and were assessed following adjustment of the pH of overnight cultures of 10403S WT and its *gad* mutants at pH 4.2 grown until stationary phase in DMG and in DMG with 6.8% tryptone. Markers represent an average of measurements performed in triplicate, and error bars represent the standard deviation. D.L denotes detection limit.

Fig. 5. (A) Cells of 10403S WT (white boxes) and its *gad* mutants $\Delta gadD1$ (white triangles), $\Delta gadD2$ (white circles) and $\Delta gadD3$ (white diamonds) grown in DMG alone or in DMG with 10% casamino acids (C.A.), with WT (black boxes) and its *gad* mutants $\Delta gadD1$ (black triangles), $\Delta gadD2$ (black circles) and $\Delta gadD3$ (black diamonds) grown under oxic conditions until stationary phase (~24 h). Subsequently, cultures were acid challenged at pH 2.5 with the addition of 1M HCl. Monitoring of GABA_e (B) was conducted for 0, 20, 40 and 60 min and were assessed following adjustment of the pH of overnight cultures of 10403S WT

594 and its *gad* mutants at pH 4.2 grown until stationary phase in DMG and in DMG with 10%
595 casaminoacids (C.A.). Markers represent an average of measurements performed in triplicate,
596 and error bars represent the standard deviation. D.L denotes detection limit.

597

598 **Fig. 6.** Real-time PCR determination of transcription of *gadD1*, *gadD2* and *gadD3*. Relative
599 expression of each gene was calculated by comparing expression relative to that of 16S rRNA
600 in 10403S WT cultures grown overnight until stationary phase in DMG or DMG supplemented
601 with 6.8% tryptone, peptone or casamino acids (C.A.).
602 Markers represent an average of measurements performed in triplicate, and error bars represent
603 Markers represent an average of measurements performed in triplicate, and error bars represent
604 Markers represent an average of
605 measurements performed in triplicate, and error bars represent standard deviations. Asterisks
606 represent statistically significant difference in the expression of each gene in the supplemented
607 media compared to that in DMG ($P < 0.05$). Numbers above the bars indicate fold difference of
608 the relative expression for each gene in DMG supplemented with either tryptone, peptone or
609 casaminoacids (C.A.) compared to its expression in DMG without supplementation.

610

611

612 Supplementary data

613 LEGEND TO FIGURES

614

615 Fig. 1. Survival after acid challenge stationary phase cultures of 10403S WT and its isogenic
616 *gad* mutants in DMG. Cultures were grown at 37°C with shaking until stationary phase in DMG
617 and subsequently, the medium was adjusted to pH 2.4 with the addition of HCl and survival
618 was assessed after 60 min through preparation of decimal dilutions and plating. Error bars

619 represent standard deviation from triplicate observations.

620