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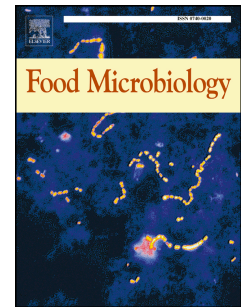
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**A novel role for the glutamate decarboxylase system in *Listeria monocytogenes*;
protection against oxidative stress**

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Abstract

The GAD system is widely present in several types of organisms and is known to play an important role in bacterial acid tolerance. There is only one account of this system playing a role in oxidative stress in bacteria and one in yeasts. Here we show for first time that it affects the oxidative stress resistance of a Gram-positive bacterium, (*L. monocytogenes*, tested in three strains; 10403S, EGD-e, and LO28). We found a statistically significant reduction in survival after H₂O₂ exposure in $\Delta gadD3$ and $\Delta gadD2$ of EGD-e and in $\Delta gadD1$ of LO28. Furthermore, we observed a lag phase prolongation in $\Delta gadD3$ of 10403S and EGD-e and a larger inhibition zone in disk diffusion assay for $\Delta gadD1$ and $\Delta gadD3$ of EGD-e upon H₂O₂ exposure. All GAD genes playing a role in oxidative stress resistance are part of GAD_i system while this occurs partly through catalase activity, while the most potent GAD_e system plays no role. The latter effects could occur through the GABA shunt, but we show here that mutants in succinate semialdehyde dehydrogenase do not show a phenotype suggesting that either effects are through the GABA transaminase or, this pathway is not involved. Our study highlights for first time the role of the GAD system in oxidative stress resistance of a Gram-positive bacterium, which could be used in Food Hurdle Technology to eliminate pathogens such as *L. monocytogenes*, while it gives an insight on the general mechanism.

1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, a life-threatening food borne disease, with a mortality rate reaching the 30% in some outbreaks (Allerberger and Wagner, 2010; Mead et al., 1999). Its success is often attributed to the ability to survive in a large range of adverse conditions, including acidic environments (Cole et al., 1990; McClure et al., 1989). Such feature is especially important for foodborne pathogens, in order to withstand the acidic pH encountered both during food disinfection and during the infectious process, in the human stomach (Feehily and Karatzas, 2013). In order to cope with acid challenge, bacteria employ a variety of strategies. One of best characterized acid resistance mechanisms is the glutamate decarboxylase (GAD) system (Cotter et al., 2001; Feehily and Karatzas, 2013), which is present in all kingdoms of life (Paudyal and Karatzas, 2016), and presents a wide variety of functions in different organisms (Feehily and Karatzas, 2013). Although the system is a major part of the nervous system of animals, and a defence mechanism to stress such as anoxic conditions in plants, in a wide variety of microorganisms including fungi and bacteria such as *L. monocytogenes*, *Escherichia coli*, *Francisella tularensis*, it is a major acid resistance mechanism (Karatzas et al., 2012; Ramond et al., 2014; Smith et al., 1992). Despite the architecture of the system showing variability in different species, generally, in *L. monocytogenes*, the system comprises three glutamate decarboxylase enzymes (GadD1, GadD2 and GadD3), and two glutamate/GABA antiporters (GadT1 and T2; Paul D. Cotter et al., 2005; Karatzas et al., 2012). There is, however, a high variability in the architecture of the GAD system, even between different strains of the same species. For instance, *L. monocytogenes* strains belonging to serotype 4, which is the serotype more often associated with foodborne outbreaks, lack the *gadDIT1* operon (P. D. Cotter et al., 2005). The system works in a cycle that initiates with the import of extracellular glutamate (Glt_c) by the antiporters, which is decarboxylated by the decarboxylase enzymes through replacement of the α -carboxyl group with a proton, resulting in γ -aminobutyric acid (GABA) formation. The GABA produced is subsequently exported back by the antiporter, while simultaneously more glutamate is imported, hence continuing the cycle (O'Byrne and Karatzas, 2008). The consumption of one proton during glutamate

decarboxylation is responsible for an increase of the intracellular pH, attenuating the acid stress (Cotter et al., 2001). Furthermore, the GAD enzymes can utilize intracellular glutamate (Glt_i) in order to increase the intracellular pH (Karatzas et al., 2012). In *L. monocytogenes*, a GAD enzyme, generally the GadD3, is not associated with any antiporter and it is believed to be responsible for processing Glt_i (Karatzas et al., 2012). The GABA produced via the intracellular GAD system (GAD_i) is catabolised to succinate via the GABA shunt pathway, which is a two-step enzymatic pathway catalysed by the enzymes GABA amino-transferase (GABA-AT) and succinate semialdehyde dehydrogenase (SSADH; Zhu et al., 2010). The contribution of GAD_i in acid tolerance is dependent on the strain. In *L. monocytogenes* EGD-e the GAD_i system plays crucial role for acid resistance, since this strain has a defective extracellular GAD (GAD_e) system and is unable to export GABA (Karatzas et al., 2012).

In other organisms, the GAD system is known to play other roles aside from conferring acid resistance. For instance, in plants the production of GABA has been associated with several types of stress such as mechanical, draught, salt, heat and cold stresses (Kinnersley et al., 2000). Aside from stress-related functions, the GAD system is known to play an important role in the mammalian brain where GABA functions as the major inhibitory neuro-transmitter (Petroff, 2002). Interestingly, in several organisms, ranging from *F. tularensis* (Ramond et al., 2014) to *Saccharomyces cerevisiae* (Coleman et al., 2001) and astrocytes (Bellier et al., 2000; Lamigeon et al., 2001), the presence of a functional GAD system has been associated with an adequate response to oxidative stress. The exact mechanism is not well understood, however most studies suggest that a deficient GAD system leads to a reduction of intermediates of the tricarboxylic acid (TCA) cycle and other molecules, such as NADPH and glutathione, which have potent anti-oxidant capacity (Bellier et al., 2000; Coleman et al., 2001; Lamigeon et al., 2001; Ramos et al., 1985; Smirnova and Oktyabrsky, 2017; Vogel et al., 1999).

In *L. monocytogenes*, possibly due to the well-known role for acid resistance, to date, it has not been assessed if the GAD system mediates the response to other types of stress apart from resistance to nisin mediated by GadD1 in LO28 (Begley et al., 2010). This is important since the role of GadD1 in acid resistance is not clear. There is limited evidence involving GAD system

and GABA metabolism in oxidative tolerance. This is especially relevant, since oxidative stress is one of the most common stresses *L. monocytogenes* has to cope with. It occurs not only in the environment, as a consequence of aerobiosis but also during the infectious process, inside the phagolysosome, or during disinfection, as oxidants are often used, and in the processing of foods with technologies such as plasma and ozone (Cabiscot et al., 2000; Imlay, 2003; Jaksch et al., 2004; O'Byrne and Karatzas, 2008; O'Donnell et al., 2012). The utilization of oxidising agents is highly appealing for the food industry since it reduces the microbial activity significantly and without the inconvenient traces of toxic residues and by-products (Jaksch et al., 2004; O'Donnell et al., 2012).

In the current study, we aimed to elucidate the role of different components of the GAD system in three *L. monocytogenes* strains (10403S, EGD-e and LO28) in oxidative stress tolerance. We show, for the first time in *L. monocytogenes*, a secondary role of protection against oxidative stress for the GAD system. This effect is only seen with components of the GAD_i system, which lead us to hypothesise that the GAD_i in *L. monocytogenes* contributes to the maintenance of intracellular antioxidant molecules key for bacterial tolerance to oxidative stress. Our study highlights for first time the important role of the GAD system in oxidative stress in a Gram-positive bacterium. Our observations have great impact on the understanding of this phenomenon in all organisms from all kingdoms of life and most importantly in bacteria. More specifically in *L. monocytogenes* this phenomenon is important for the wider understanding of its complex behaviour when it encounters different types of stress. This work could be considered in Hurdle Technology and thus help us in the easier elimination of this pathogen from foods and food preparation environments.

2. Materials and Methods

2.1 Bacterial strains and growth

L. monocytogenes WT strains of EGD-e, 10403S and LO28 and their isogenic mutants in different genes of the GAD system (Table 1) were used in this study. Stock cultures were stored at -80°C in 15% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset, United Kingdom).

Prior to experiments, stock cultures were streaked onto brain heart infusion (BHI) agar (LAB M, Lancashire, United Kingdom) and incubated at 37°C overnight.

A single colony was transferred to 3 ml of either sterile BHI broth (LAB M) for EGD-e and 10403S or tryptone soy broth (Lab M) supplemented with 0.6% (w/v) of yeast extract (TSBY) for LO28 and incubated at 37°C with shaking (160 rpm). Bacteria were allowed to grow until stationary phase and used to inoculate the experimental culture with a 1% inoculum (v/v). These cultures were prepared in 250 ml conical flasks containing 20 ml of the same medium as used for the inoculum and incubated overnight at 37°C with shaking (120 rpm).

2.2. Growth in the presence of sub-lethal doses of H_2O_2

L. monocytogenes was grown overnight in the appropriate broth containing a sub-lethal concentration of H_2O_2 (0.01%). The cultures were incubated at 37°C and the optical density at 620 nm was measured every 20 minutes in a Sunrise plate reader (Tecan, Austria). The Baranyi and Roberts model was used to estimate the growth parameters (length of lag phase, growth rate and maximum OD; Baranyi et al., 1996; Baranyi and Roberts, 1994), taking advantage of the embedded analysis tool of Microsoft Excel 2013, *Solver*, as previously described (Walsh and Diamond, 1995).

2.3. Hydrogen Peroxide Disk Diffusion Assay

Overnight liquid cultures grown either in BHI (EGD-e and 10403S) or TSBY (LO28) were diluted to an OD_{600nm} of 0.2, and 100 µl was spread onto Mueller-Hinton agar (MHA; Oxoid, Basingstoke, United Kingdom). Then, 10 µl of 30% (v/v) H_2O_2 was pipetted onto Whatman 3MM paper disks (0.7-cm diameter), and these disks were placed on top of the agar and incubated for 18 h at 37°C. The zones of inhibition (in mm) denoting H_2O_2 sensitivity were measured in three dimensions, and the mean values and standard deviations were calculated. All experiments were performed on three independent biological replicates.

2.4. Survival against H_2O_2

Stationary phase aerobic cultures, grown as described for 18 h, were challenged with 4% (10403S and LO28) or 4.5% (EGD-e) of H_2O_2 . The survival pattern was assessed by plating serial dilutions in either BHI or TSBY agar before and after (20, 40 and 60 min) the H_2O_2 challenge. The agar plates were incubated at 37°C for 24 h and colony forming units (CFU) were enumerated to assess the concentration of bacteria in each time point.

2.5. Catalase activity assay

The catalase activity was assessed using the methodology described by Iwase et al. (2013), with minor modifications. Briefly, 10 mg of bacterial culture pellet was re-suspended in 100 µl phosphate buffer (K_2HPO_4/KH_2PO_4 ; pH 7) and transferred to a test-tube containing 100 µl of 1% (v/v) Triton X-100 (Sigma-Aldrich, Dorset, UK). One hundred µl of H_2O_2 were then added to each test tube. The formation of oxygen bubbles, as a result of the enzymatic degradation of H_2O_2 , was visualised in the form of foam. After 5 min the height of the foam was measured and photographic images were taken as well. All experiments were performed on six independent biological replicates.

2.6. Statistical analysis

In all cases, experiments were run at least in triplicate, and results were assessed with a paired Student T -test. A p value lower than 0.05 was considered statistically significant.

3. Results

3.1. Survival against hydrogen peroxide

L. monocytogenes strains (10403S, EGD-e and LO28) WT and the respective deletion mutants in the different genes of the GAD system, were challenged with H_2O_2 . In *L. monocytogenes* 10403S, the WT strain showed approximately 3 log reduction of CFU/ml after being challenged with H_2O_2 (Fig. 1A) which did not show any statistically significant difference compared to any

of its isogenic mutants ($\Delta gadD1$, $\Delta gadD2$, $\Delta gadD3$, $\Delta lmrG_02013$) despite $\Delta gadD3$ showing a 4.6 log reduction of CFU/ml.

In *L. monocytogenes* EGD-e, all GAD mutants showed lower survival than the WT against an oxidative stress (Fig. 1B) but only $\Delta gadD2$ and $\Delta gadD3$ showed statistical significance. Sixty min after being challenged with H_2O_2 , the WT strain showed 0.5 log reduction of CFU/ml, while the GAD mutants $\Delta gadD1$, $\Delta gadD2$, $\Delta gadD3$ and $\Delta lmo0913$ showed a reduction of 2.4, 3.5, 4.8 and 2 log CFU/ml respectively. However, despite the major differences relatively to the WT, only these for $\Delta gadD2$ and $\Delta gadD3$ were statistically significant.

In *L. monocytogenes* LO28, only the $\Delta gadD1$ showed a significant reduction of 5.67 log CFU/ml ($p=0.01$) after exposure to H_2O_2 in comparison to a reduction of 1.05 log CFU/ml for the WT (Fig. 1C). Interestingly, both $\Delta gadD2$ and the double mutant that lacks both glutamate decarboxylases, $\Delta gadD1/D2$, showed negligible difference relatively to the WT.

3.2. Hydrogen Peroxide Disk Diffusion Assay

Disk diffusion assays were performed in all three WT strains and their respective isogenic mutants missing GAD system genes. *L. monocytogenes* EGD-e $\Delta gadD1$ and $\Delta gadD3$ showed larger inhibition zones relatively to the WT ($p=0.04$ and $p=0.001$ respectively; Fig. 2). All the remaining GAD mutants, both in 10403S and LO28, showed no significantly different growth inhibition in comparison with the WT.

3.3. Catalase Assay

The catalase activity was assessed in *L. monocytogenes* 10403S, EGD-e and LO28 WT and their respective isogenic mutants missing GAD system genes (Fig. 3), using a visual approach. Briefly, the degradation of H_2O_2 by catalase produces water and molecular oxygen that is released and forms a foam. It has been demonstrated previously that the height of the foam is directly proportional to the catalase activity (Iwase et al., 2013). The activity of catalase in EGD-e $\Delta gadD3$ was significantly reduced comparatively to the WT ($p=0.01$). No statistically significant difference in catalase activity was found in the GAD mutants in *L. monocytogenes*

10403S and LO28 or in the remaining mutants in EGD-e. However, in LO28 the $\Delta gadD2$ and $\Delta gadD1/2$ seemed to have slightly higher catalase activity than the WT.

3.4. Effect of H_2O_2 on growth

The effect H_2O_2 on the growth of *L. monocytogenes* WT and the respective GAD mutants was assessed by inoculating the bacterial cultures in the presence of 0.01% of H_2O_2 and monitoring the absorbance at 620 nm (OD_{620}).

As expected, growth in the presence of H_2O_2 caused a significant extension in the lag phase of all WT strains and mutants in study (comparison with and without H_2O_2 ; Table 2). The maximum growth (max OD_{620}) was only significantly decreased in LO28 WT and the corresponding $\Delta gadD2$ (Table 3).

In the absence of H_2O_2 , none of the mutants showed a significant difference in the growth parameters in comparison to the WT. However, in the presence of H_2O_2 some of the GAD mutants showed alterations of the growth parameters, in comparison with the parental WT strain. In both 10403S (Fig. 4) and EGD-e (Fig. 5), the $\Delta gadD3$ had a significant delay in the lag phase (Table 2), while in LO28 (Fig. 6) the $\Delta gadD1$ and $\Delta gadD1/2$ showed a significant decrease of the max OD_{620} (Table 3).

4. Discussion

Oxidative stress resistance is very important for survival of *L. monocytogenes* in foods, food preparation environments and finally in its ability to cause disease. Various disinfectants, ozone treatments, cold plasma or sonication are used or investigated to eliminate microbes and pathogens such as *L. monocytogenes* through the application of oxidative stress in foods or food preparation environments (Cabiscol et al., 2000; Imlay, 2003; Jaksch et al., 2004; O'Byrne and Karatzas, 2008; O'Donnell et al., 2012). Understanding the behaviour of this pathogen in oxidative environments is important for our ability to design effective treatments, especially as

some of these novel techniques such as cold plasma and sonication have not yet found their way in the food industry.

Furthermore, oxidative stress resistance plays a role in the pathogenesis of intracellular pathogens as oxidative stress occurs in the phagocytic lysosome. However, up to now, there is no evidence showing that in *L. monocytogenes*. In contrast, our work suggests the opposite, as hypersensitive to oxidative stress *L. monocytogenes* $\Delta sigB$ mutants, have similar intracellular proliferation to their corresponding WT (Boura et al., 2016). In the latter work we also demonstrated that SigB, the main stress gene regulator in *L. monocytogenes*, affects oxidative stress resistance in a different fashion to other stresses (Boura et al., 2016). Although upregulation of *sigB* enhances general resistance to multiple stresses, it results in hypersensitivity to oxidative stress and this occurs through lower catalase activity. It is not known how SigB affects catalase (Boura et al., 2016). It is possible that SigB negatively affects oxidative stress resistance through regulation of another mechanism, which in turn affects catalase and possibly other oxidative stress mechanisms. The *L. monocytogenes* GAD system is under the control of SigB (Wemekamp-Kamphuis et al., 2004) while in the Gram-negative bacterium, *Francisella tularensis* (Ramond et al., 2014), the yeast *Saccharomyces cerevisiae* (Coleman et al., 2001) and the animal cells astrocytes (Bellier et al., 2000; Lamigeon et al., 2001) it has been implicated in oxidative stress resistance. We thought of the possibility that the GAD system could mediate the effect of SigB to catalase and oxidative stress in *L. monocytogenes*. However, this theory is problematic because in *L. monocytogenes*, SigB upregulates the GAD system (Wemekamp-Kamphuis et al., 2004), and based on the results obtained in these three aforementioned organisms, it should be expected that SigB-mediated GAD system upregulation results in the opposite effect (enhancement of oxidative stress resistance) from what is actually observed (hypersensitivity to oxidative stress). In addition, none of the above organisms except *L. monocytogenes* possesses a *sigB* gene, while the GAD system effects on oxidative stress have been only observed in three organisms so far and only once in a bacterium which is Gram-negative.

Despite the above, we decided to assess for first time the role of the *Listeria monocytogenes* GAD system in oxidative stress. We looked first at the role of the different GAD system genes in oxidative stress tolerance, in three common reference *L. monocytogenes* strains namely, 10403S, LO28, EGD-e which reflect the major differences between different strains of *L. monocytogenes* in terms of GAD system activity which significantly affects acid resistance. *L. monocytogenes* 10403S possesses one of the most active GAD systems resulting in one of the most acid resistant phenotypes described in the literature, LO28 has a normal GAD activity and acid resistance, while EGD-e has a defective GAD_e system unable to export GABA and it is one of the most acid sensitive strains described in the literature (Karatzas et al., 2012).

The GAD system is the major determinant of acid resistance in *L. monocytogenes* and many other organisms (Cotter et al., 2001; Ryan et al., 2008) with mutants in GAD genes showing severe impairment in acid resistance (Paudyal et al., 2018). The GAD system comprises two distinctive subsystems, which are defined by the original location of glutamate utilised by the GAD system. Our group has previously described the extracellular GAD system (GAD_e), which is the one investigated in vast majority of publications, utilising extracellular glutamate imported by the GAD glutamate/GABA antiporter and the intracellular GAD system (GAD_i), which utilises glutamate transported by glutamate transporter(s) or that produced by metabolic pathways (Karatzas et al. 2012).

In the present study we show for first time that the GAD system affects oxidative stress in a Gram-positive bacterium (*L. monocytogenes*) and this is the second account in any bacterium. We found that *gadD3* deletion resulted in a decrease in survival against oxidative stress in EGD-e (Fig. 1B) and growth inhibition (lag phase extension) in both EGD-e and 10403S (Table 2) and larger inhibition zone in EGD-e (Fig. 2). Unfortunately, it was impossible for other workers and for us to obtain a Δ *gadD3* mutant in the LO28 background (Cotter et al. 2005). Results clearly highlight the role of GadD3 in oxidative cell defence. GadD3 only contributes to the GAD_i system (Karatzas et al., 2012) but not to the GAD_e system as it is not associated with any antiporter and it does not affect the GABA export. The GAD_i plays an important role in acid tolerance and in EGD-e it is the only functional GAD system component, while it

contributes in acid resistance in other strains that use the GAD_e (e.g. 10403S; Karatzas et al., 2012).

Another GAD gene that affected oxidative stress resistance was *gadD1*. Deletion of this gene resulted in lower survival in LO28 (Fig. 1C) and larger inhibition zone in EGD-e (Fig. 2) underpinning its role in oxidative stress defence. It has been suggested previously that GadD1 plays a role in growth under mildly acidic conditions, as strains that belong to serotype 4 lack *gadD1* and grow poorly at pH 5.1 (Cotter et al., 2005). This however, might not be solely the result of the lack of *gadD1* but the result of other genetic differences in this serotype. In addition, following various attempts we have never detected GABA being exported by GadD1T1, while a mutant lacking *gadD1* has a comparable survival to its corresponding WT under acidic conditions (Karatzas et al., 2012; Cotter et al., 2005). All these suggest that the role of *gadD1* in acid resistance is debatable however, it has a clear role in tolerance of LO28 against nisin, which has been suggested to stem from its contribution to ATP production as its absence resulted in 60% reduction of ATP pools (Begley et al., 2010). ATP levels in *L. monocytogenes* are critical for survival against nisin (Bonnet et al., 2006). Interestingly, a study in astrocytes found that GAD-expressing cells not only produced more ATP (Bellier et al., 2000) but also had increased ability to cope with oxidative stress (Lamigeon et al., 2001). We expected that also the double LO28 mutant lacking both *gadD1* and *gadD2* ($\Delta gadD1/D2$) should demonstrate a similar phenotype since $\Delta gadD2$ showed no phenotype to $\Delta gadD1$ in this background. However, this was not the case with $\Delta gadD1/D2$ which showed a similar phenotype to WT and $\Delta gadD2$ (Fig. 1C). It has been commented previously for acid resistance that $\Delta gadD1/D2$ could result in an unknown response which could counteract the double deletion (Feehily et al., 2014) and possibly explain the lack of phenotype in this mutant.

We also assessed the role of *gadD2* gene in oxidative stress and we found that its deletion affected survival in EGD-e (Fig. 1B). GadD2 is the main component of the GAD_e system, which however, is inactive in EGD-e resulting in a highly acid sensitive phenotype in this strain (Karatzas et al. 2012). It is not known if the absence in GAD_e activity in EGD-e stems from a

defect in the decarboxylase GadD2 or the antiporter GadT2. Since our results show a phenotype for $\Delta gadD2$, this could suggest that GadD2 is active in EGD-e utilising only intracellular glutamate as part of the GAD_i system and a possible defect in the GadT2 antiporter prevents glutamate/GABA antiport activity. Although GadD2 is highly active especially in 10403S and in LO28 we did not see any phenotype, which might be related with the fact that these strains are not highly resistant to oxidative stress and changes in oxidative stress resistant mechanisms might not bring major changes in the phenotypes. This might also explain why all GAD mutants in EGD-e, which is the most resistant strain to oxidative stress (Boura et al., 2016), showed lower survival than the WT, with only in $\Delta gadD3$ and $\Delta gadD2$ however, showing statistical significance. Furthermore, our results show that the GAD genes that might play a role in survival against H₂O₂ in one strain, might be different from the ones playing a role in growth in broth or agar in the presence of H₂O₂. This might be due to the fact that survival assays assessed oxidative defences at a different growth stage from disk diffusion and growth experiments, while the latter two were also performed in different media phases (liquid or solid agar) resulting in different responses (Boura et al., 2016).

Furthermore, it should be mentioned that all phenotypes seem to be associated with glutamate decarboxylases working on intracellular glutamate and therefore, contributing only to the GAD_i but not to the GAD_e system.

The GAD_i system results in the production of intracellular GABA which is metabolised through the GABA shunt (Fig. 7). The latter pathway contributes to the maintenance of the intracellular pools of NADPH and other anti-oxidant molecules (Bellier et al., 2000; Feehily et al., 2013; Lamigeon et al., 2001; Ramond et al., 2014) which could be involved in the oxidative stress resistance and maybe explain the observed phenotypes. In *Francisella tularensis*, oxidative stress resistance is important for replication in the cytosol of infected cells and the GadC antiporter is crucial for this process and the escape from the phagosome compartment (Ramond et al., 2014). The authors suggest that a decrease of glutamate acquisition results in a reduction of tricarboxylic acid (TCA) cycle intermediates with potent anti-oxidant effect, such as oxoglutarate and NADPH, produced by the conversion of glutamate into oxoglutarate (Ramond

et al., 2014). Reduced NADPH pools could also result in the reduction of glutathione, a potent antioxidant tripeptide with a well-known role in oxidative stress tolerance, since NADPH functions as a co-factor of the glutathione reductase (Smirnova and Oktyabrsky, 2017; Vogel et al., 1999). A similar hypothesis was postulated to explain the increased resistance of GAD-expressing astrocyte cells (Lamigeon et al., 2001) which was the result of a 10-fold increase in the levels of released glutathione (Lamigeon et al., 2001), and of increased NADPH and ATP levels (Bellier et al., 2000). Furthermore, a study in *Saccharomyces cerevisiae*, showed that the presence of a functional GAD system and GABA shunt pathway, are essential for oxidative stress resistance (Coleman et al., 2001) which is expected as GABA shunt contributes in NADPH pools (Ramos et al., 1985).

To investigate the above hypothesis that the GABA shunt plays a role in oxidative stress and can explain the above phenotypes in *L. monocytogenes*, we employed mutants lacking succinate semialdehyde dehydrogenase (SSDH), the second step of the GABA shunt, in two different backgrounds; EGD-e ($\Delta lmo0913$) and 10403S (Δlmr_{g_02013}). Deletion of SSDH resulted in no phenotype in 10403S and a slight effect in the EGD-e background (2 log cycles of CFU/ml further reduction compared to WT) without any statistical significance. We have previously shown that Lmo0913 and Lmr_g_02013 are the only SSDH proteins in EGD-e and 10403S *L. monocytogenes* backgrounds respectively, as both mutants were defective in SSDH activity (Feehily et al., 2013). Given that the SSDH reaction results in NADPH production that contributes in oxidative stress resistance, the absence of a strong phenotype from both SSDH mutants is surprising (Fig. 7). Furthermore, since this oxidative stress resistance is directly associated with the GAD_i which directly feeds GABA into the GABA shunt it should be expected that its disruption would result in a phenotype which is not the case. We can not exclude the possibility that GABA shunt plays a role in oxidative stress resistance, but this might happen through the GABA transaminase, the first step of the GABA shunt pathway, which affects the TCA cycle by removing α -ketoglutaric acid that is converted to glutamate (Fig. 7). Furthermore, it should be considered that the GABA shunt in *L. monocytogenes* and various other bacteria (e.g. several lactic acid bacteria) plays an important role as it bypasses

two missing steps of the TCA cycle while other organisms have a complete TCA cycle (Fig. 7). Further work is required to identify the actual link between the GAD system - GABA shunt and oxidative stress possibly focusing on GABA transaminase.

We further looked at the mechanism resulting in these phenotypes by investigating the catalase activity of these mutants. We found a statistically significant decrease in catalase activity of $\Delta gadD3$ in EGD-e, which correlated with the lower survival of this strain under oxidative stress (Fig. 3). However, the same was not observed in LO28 $\Delta gadD1$, which also showed a marked reduction in survival. Despite catalase being the main pathway of H_2O_2 degradation, there are other antioxidant molecules, such as NADPH and NADH pools, ascorbic acid etc. that help to maintain an intracellular reducing environment (Cabiscol et al., 2000).

It should also be mentioned that experiments in this study were performed in native pH (6.4 for *L. monocytogenes* in stationary phase), where the GAD system is not in its maximum activity level, which occurs between pH 4 and 5 (Karatzas et al., 2012). It is possible that at lower pH these phenotypes might be more enhanced. However, we did not use these conditions to avoid the application of an additional stress to the bacteria, as it is known that the mutants of the GAD system are sensitive to acidic conditions, and this additional stress could have a significant impact in understanding their role in oxidative stress response (Paudyal et al., 2018).

It should be mentioned that this work could have major implications for applications of oxidative stress in the food industry and hurdle technology. Modern hurdle technology design could be based on knowledge of the molecular bacterial mechanisms to predict bacterial behaviour, instead of randomly looking at combinations of stresses. We have exemplified this in our previous work where we demonstrated that the use of a GAD system inhibitor such as maleic acid, could render *L. monocytogenes* cells highly sensitive to acidic stress and eliminate them in disinfection regimes (Paudyal et al., 2018). Our work here shows that the GAD system, the main acid resistance mechanism in *L. monocytogenes*, protects against oxidative stress, suggesting that downregulation or inhibition through a signal could make cells highly sensitive to oxidative stress processes and further work should demonstrate that in various oxidative stress food applications. In addition, further work also needs to look if this involvement of

the GAD system in oxidative stress is a common characteristic among other bacteria and other organisms and identify the molecular mechanisms involved in this phenomenon further to the ones described in this manuscript.

5. Conclusions

Overall, our results suggest that the GAD_i in *L. monocytogenes* plays a role in survival and growth under oxidative stress. This is concluded since, all GAD genes affecting oxidative stress resistance (*gadD3* and *gadD2* in EGD-e, *gadD1* in LO28) or tolerance during growth (*gadD3* in 10403S, *gadD1* and *gadD3* in EGD-e) are all part of the GAD_i system. Furthermore, in an attempt to assess if these effects are through the GABA shunt we show that, if this is the case, it rather occurs through the GABA-AT step and not through SSDH step as disruption of the latter step did not result in a phenotype linked with oxidative stress resistance.

The targeted inhibition of the GAD system, as a way to make *L. monocytogenes* more sensitive to acid stress has been recently exploited as a promising tool to eliminate this pathogen (Paudyal et al., 2018; Paudyal and Karatzas, 2016) and similar steps could be taken in hurdle technology using oxidative stress. Furthermore, additional work should be carried out in other microorganisms that possess the GAD system, to identify if GAD system affects oxidative stress resistance.

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Legends to the Figures

Fig. 1. Survival of *Listeria monocytogenes* 10403S (A), EGD-e (B) and LO28 (C) WT and

respective GAD mutants, after being challenged with 4 % (A and C) or 4.5 % (B) H₂O₂. DL

denotes the detection limit of the assay. The error bars represent standard deviations of triplicate

observations (biological replicates) and asterisks denote statistically significance relatively to

the WT.

Fig. 2. Catalase activity of *Listeria monocytogenes* 10403S, EGD-e and LO28 WT and

respective GAD mutants. Asterisks denote statistically significance and the error bars represent

standard deviations.

Fig. 3. Growth inhibition zone (diameter, mm) of *L. monocytogenes* 10403S, EGD-e and LO28 WT and respective isogenic mutants of the GAD, in diffusion assay using 30% H₂O₂. Asterisks denote statistically significance and the error bars represent standard deviations.

Fig. 4. Effect of sub-lethal concentration of H₂O₂ on *L. monocytogenes* 10403S WT (A), $\Delta gadD1$ (B), $\Delta gadD2$ (C), $\Delta gadD3$ (D), $\Delta lmrG_02013$ (E) growth (squared symbols, red line). As a control 10403s cells grown with no H₂O₂ were used (round symbols, black lines). Symbols represent individual data points of three independent experiments and lines the respective fit curve.

Fig. 5. Effect of sub-lethal concentration of H₂O₂ on *L. monocytogenes* EGD-e WT (A), $\Delta gadD1$ (B), $\Delta gadD2$ (C), $\Delta gadD3$ (D), $\Delta lmo0913$ (E) growth (squared symbols, red line). As a control EGD-e cells grown with no H₂O₂ were used (round symbols, black lines). Symbols represent individual data points of three independent experiments and lines the respective fit curve.

Fig. 6. Effect of sub-lethal concentration of H₂O₂ on *L. monocytogenes* LO28 WT (A), $\Delta gadD1$ (B), $\Delta gadD2$ (C), $\Delta gadD1/D2$ (D) growth (squared symbols, red line). As a control LO28 cells grown with no H₂O₂ were used (round symbols, black lines). Symbols represent individual data points of three independent experiments and lines the respective fit curve.

Fig. 7. Overview of chemical reactions carried out by the extracellular and intracellular GAD system (GAD_e and GAD_i respectively) the GABA shunt and the TCA cycle. GABA-AT is GABA aminotransferase and SSDH is succinate semialdehyde dehydrogenase.

Tables

Table 1. Strains used in this study

Strains	Relevant properties	Reference/ Source
10403S	Serotype ½ a, wild type	(Karatzas et al., 2010)
10403S $\Delta gadD1$	10403S with an in-frame deletion of <i>gadD1</i>	(Feehily et al., 2014)
10403S $\Delta gadD2$	10403S with an in-frame deletion of <i>gadD2</i>	(Feehily et al., 2014)
10403S $\Delta gadD3$	10403S with an in-frame deletion of <i>gadD3</i>	(Feehily et al., 2014)
10403S Δlmr_{02013}	10403S with an in-frame deletion of <i>lmr_{02013}</i>	(Feehily, 2014)
EGD-e	Serotype ½ a, wild type	(Feehily, 2014; Murray et al., 1926)
EGD-e $\Delta gadD1$	EGD-e with an in-frame deletion of <i>gadD1</i>	(Feehily, 2014)
EGD-e $\Delta gadD2$	EGD-e with an in-frame deletion of <i>gadD2</i>	(Feehily, 2014)
EGD-e $\Delta gadD3$	EGD-e with an in-frame deletion of <i>gadD3</i>	(Feehily, 2014)
EGD-e $\Delta lmo0913$	EGD-e with an in-frame deletion of <i>lmo0913</i>	(Feehily et al., 2013)
LO28	Serotype ½ c, wild type	(Cotter et al., 2001)
LO28 $\Delta gadD1$	LO28 with an in-frame deletion of <i>gadD1</i>	(Cotter et al., 2001)
LO28 $\Delta gadD2$	LO28 with an in-frame deletion of <i>gadD2</i>	(Cotter et al., 2001)
LO28 $\Delta gadD1/D2$	LO28 with an in-frame deletion of <i>gadD1</i> and <i>gadD2</i>	(Cotter et al., 2001)

* All above genes encode glutamate decarboxylases except for *lmo0913* and *lmr_{02013}* which encode a succinate semialdehyde dehydrogenase in EGD-e and 10403S strains respectively.

Table 2. Duration of lag phase (in hours) of *L. monocytogenes* strains grown in the presence (+) or absence (-) of H₂O₂.

Lag phase	10403S		EGD-e		LO28	
	H ₂ O ₂ (+)	H ₂ O ₂ (-)	H ₂ O ₂ (+)	H ₂ O ₂ (-)	H ₂ O ₂ (+)	H ₂ O ₂ (-)
WT	5.11 (±0.52)	2.66 (†) (±0.20)	4.49 (±0.11)	2.92 (†) (± 0.01)	6.60 (±0.32)	3.71 (†) (±0.95)
<i>ΔgadD1</i>	5.83 (±0.81)	2.92 (†) (±0.02)	3.93 (±0.34)	2.86 (†) (±0.05)	5.26 (±0.37)	3.83 (†) (±0.14)
<i>ΔgadD2</i>	6.77 (±1.09)	3.01 (†) (±0.04)	3.78 (±0.36)	2.85 (†) (±0.01)	6.22 (±0.45)	3.66 (†) (±1.06)
<i>ΔgadD3</i>	6.63 (*) (±0.78)	2.97 (†) (±0.002)	5.26 (*) (±0.23)	3.09 (†) (±0.05)		
<i>Δlmo0913</i> or <i>Δlmrg_02013</i>	6.59 (±0.82)	2.90 (†) (±0.01)	5.61 (±0.79)	3.13 (†) (±0.07)		
<i>ΔgadD1/D2</i>					5.99 (±0.64)	4.19 (†) (±0.31)

(*) denotes statistically significant difference relatively to the WT strain, under the same condition.

(†) denotes statistically significance between (+) and (-) H₂O₂ in each strain.

Table 3. Maximum optical density (620 nm) of *L. monocytogenes* strains grown in the presence (+) or absence (-) of H₂O₂.

Max growth	10403S		EGD-e		LO28	
	H ₂ O ₂ (+)	H ₂ O ₂ (-)	H ₂ O ₂ (+)	H ₂ O ₂ (-)	H ₂ O ₂ (+)	H ₂ O ₂ (-)
WT	0.625 (±0.045)	0.676 (0.057)	0.680 (±0.015)	0.705 (±0.013)	0.563 (±0.068)	0.723 (†) (±0.040)
<i>ΔgadD1</i>	0.636 (±0.030)	0.623 (±0.005)	0.661 (±0.023)	0.675 (±0.012)	0.81 (*) (±0.023)	0.822 (±0.01)
<i>ΔgadD2</i>	0.691 (±0.075)	0.621 (±0.025)	0.652 (±0.018)	0.661 (±0.008)	0.588 (±0.040)	0.737 (†) (±0.040)
<i>ΔgadD3</i>	0.660 (±0.08)	0.633 (±0.02)	0.643 (±0.02)	0.666 (±0.005)		
<i>Δlmo0913</i> or <i>Δlmrg_02013</i>	0.642 (±0.040)	0.600 (±0.024)	0.711 (±0.005)	0.702 (±0.02)		
<i>ΔgadD1/D2</i>					0.797 (*) (±0.01)	0.797 (±0.003)

(*) denotes statistically significant difference relatively to the WT strain, under the same condition.

(†) denotes statistical significance between (+) and (-) H₂O₂ in each strain.

Fig. 1

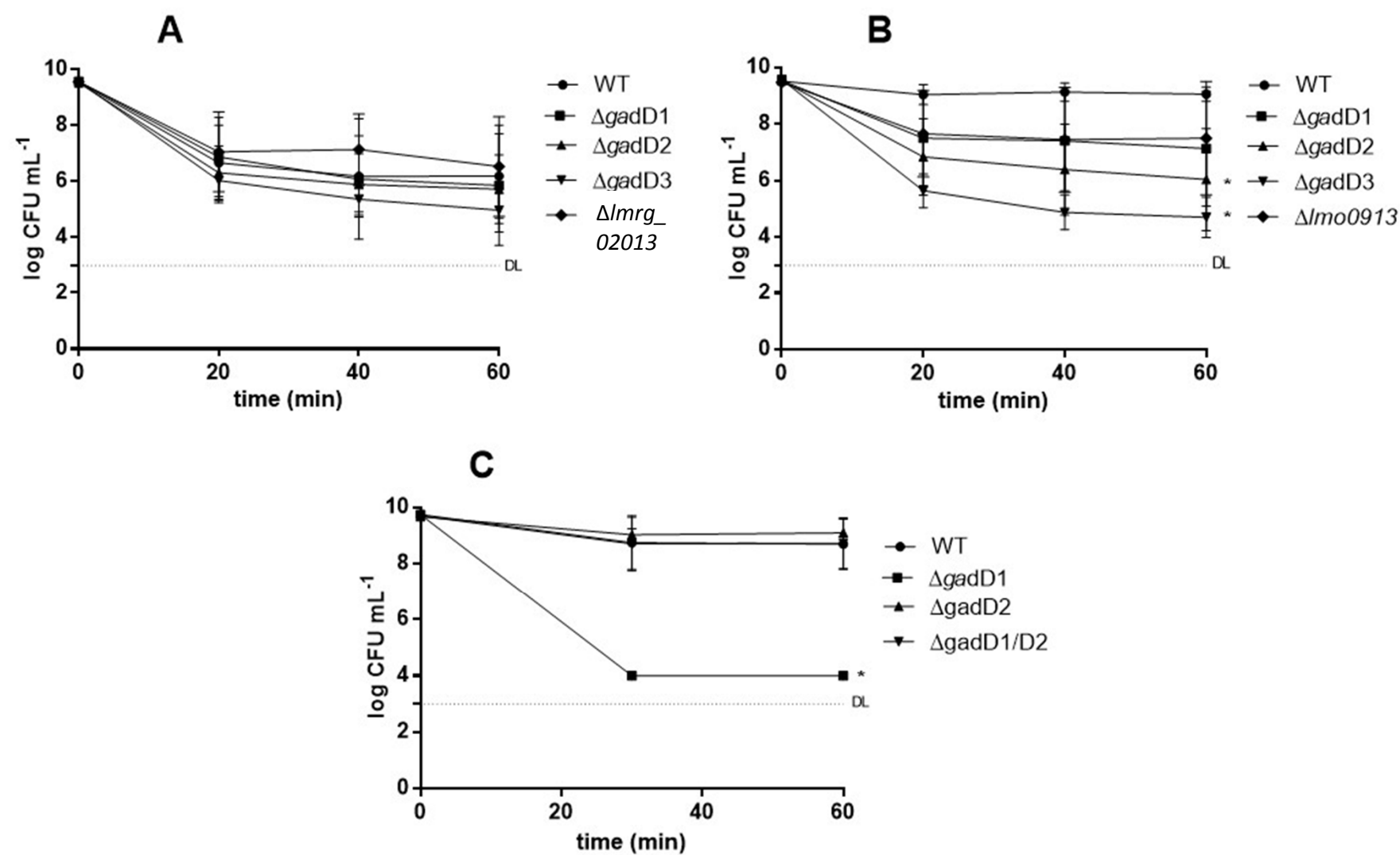


Fig. 2

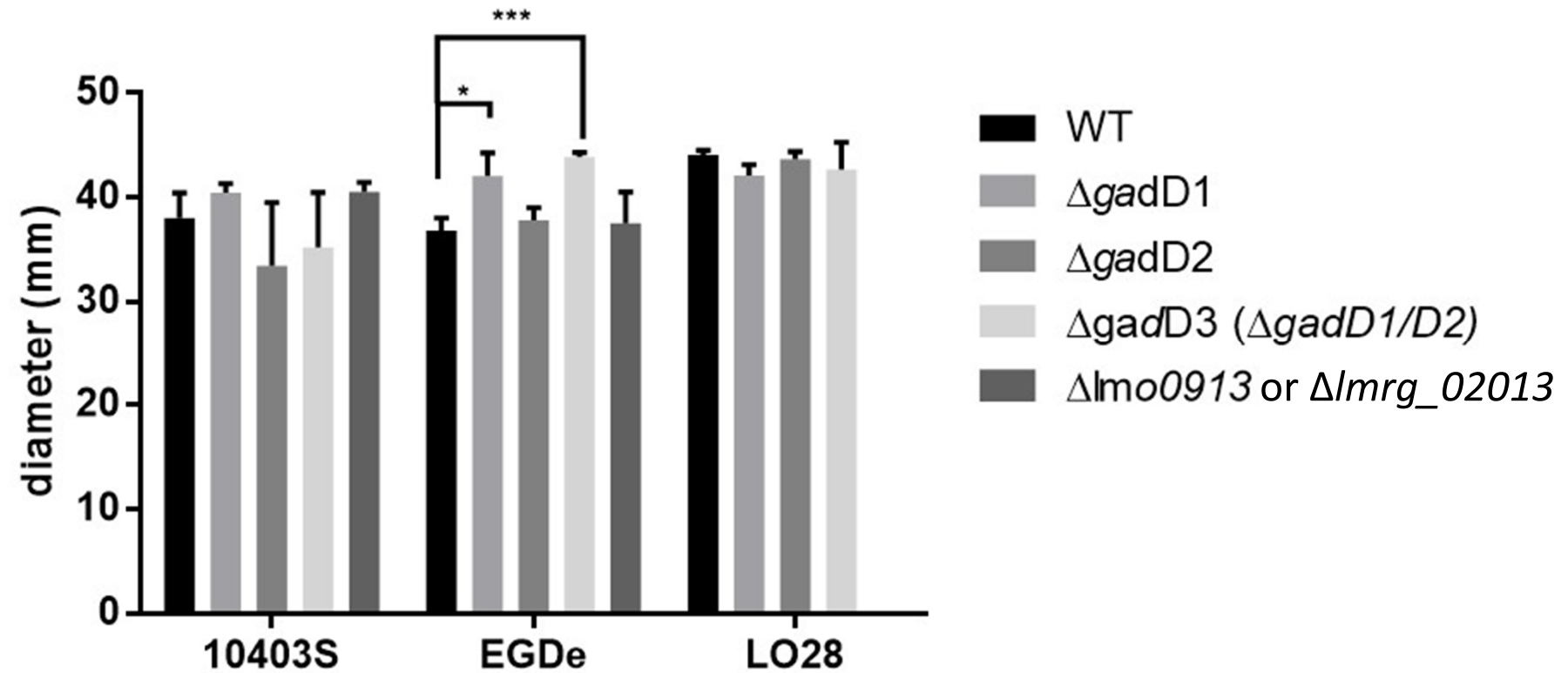


Fig. 3

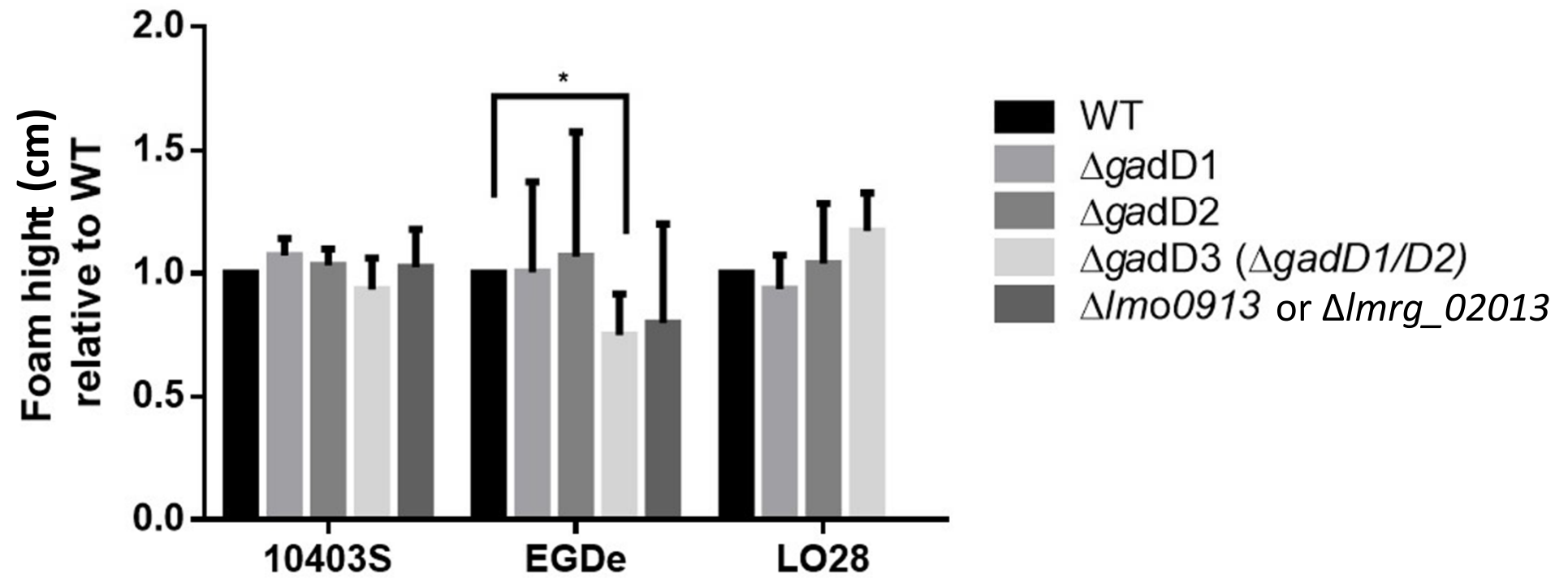
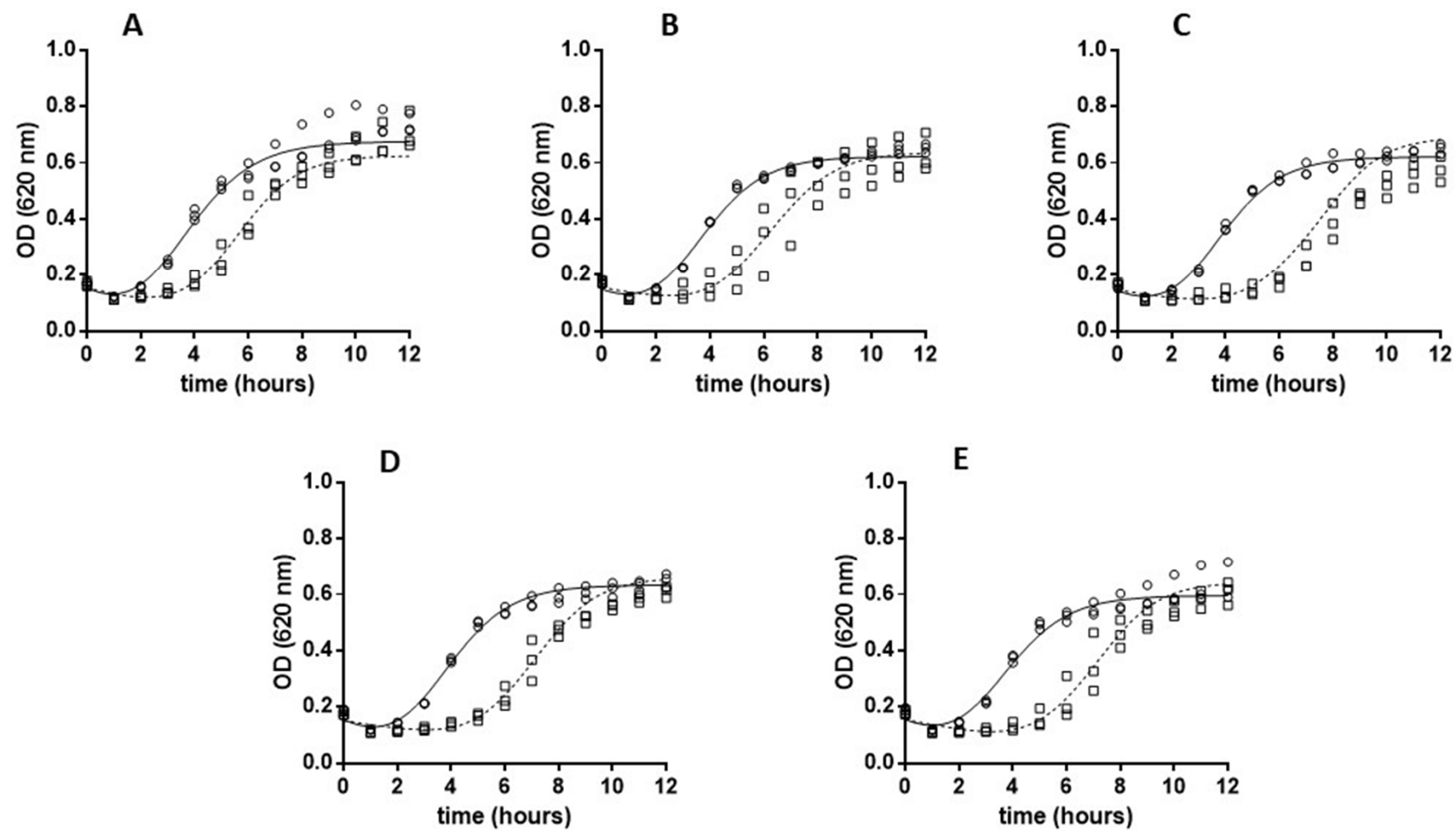
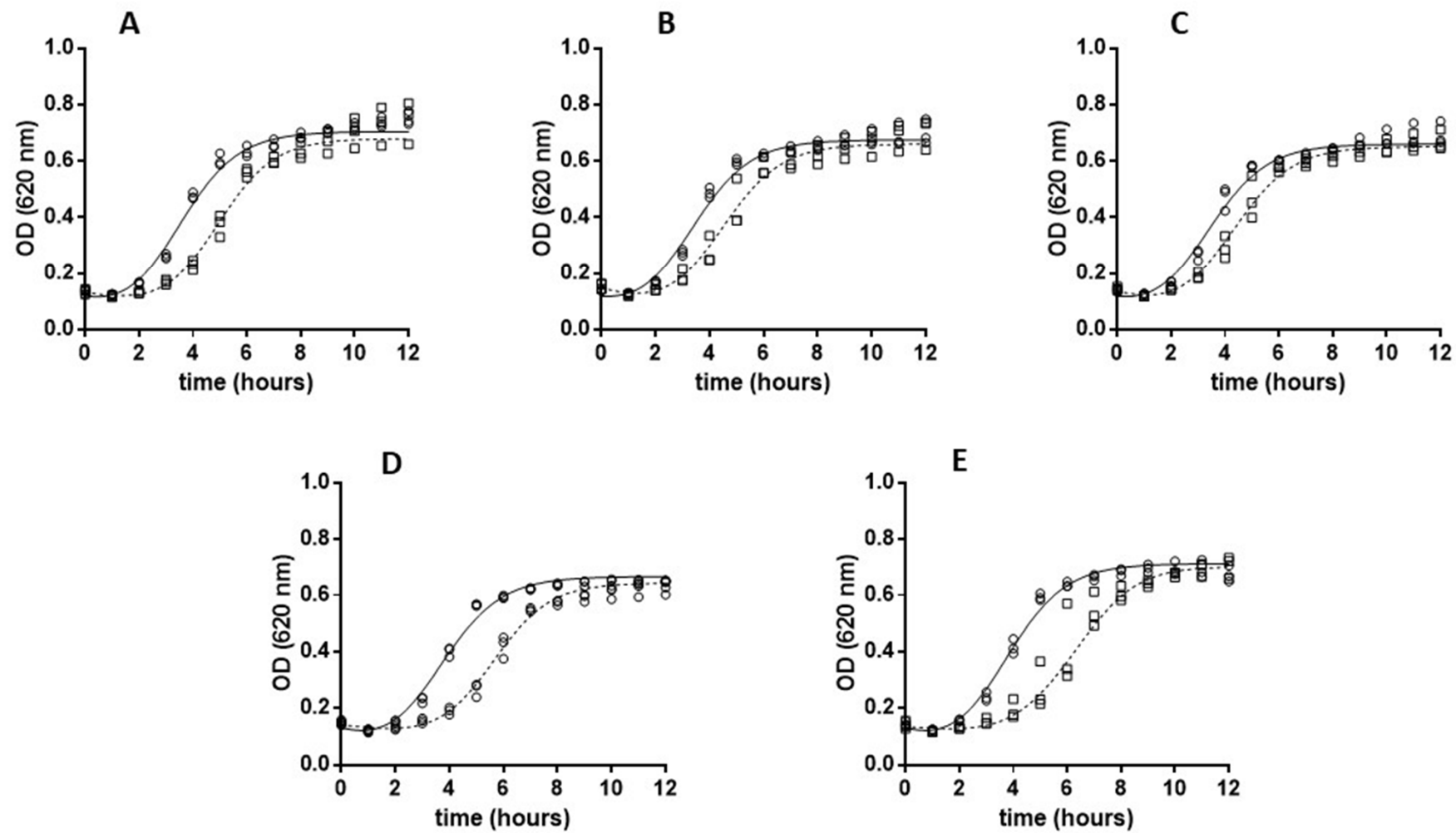


Fig4



104035

Fig5



EGD-e

Fig. 6

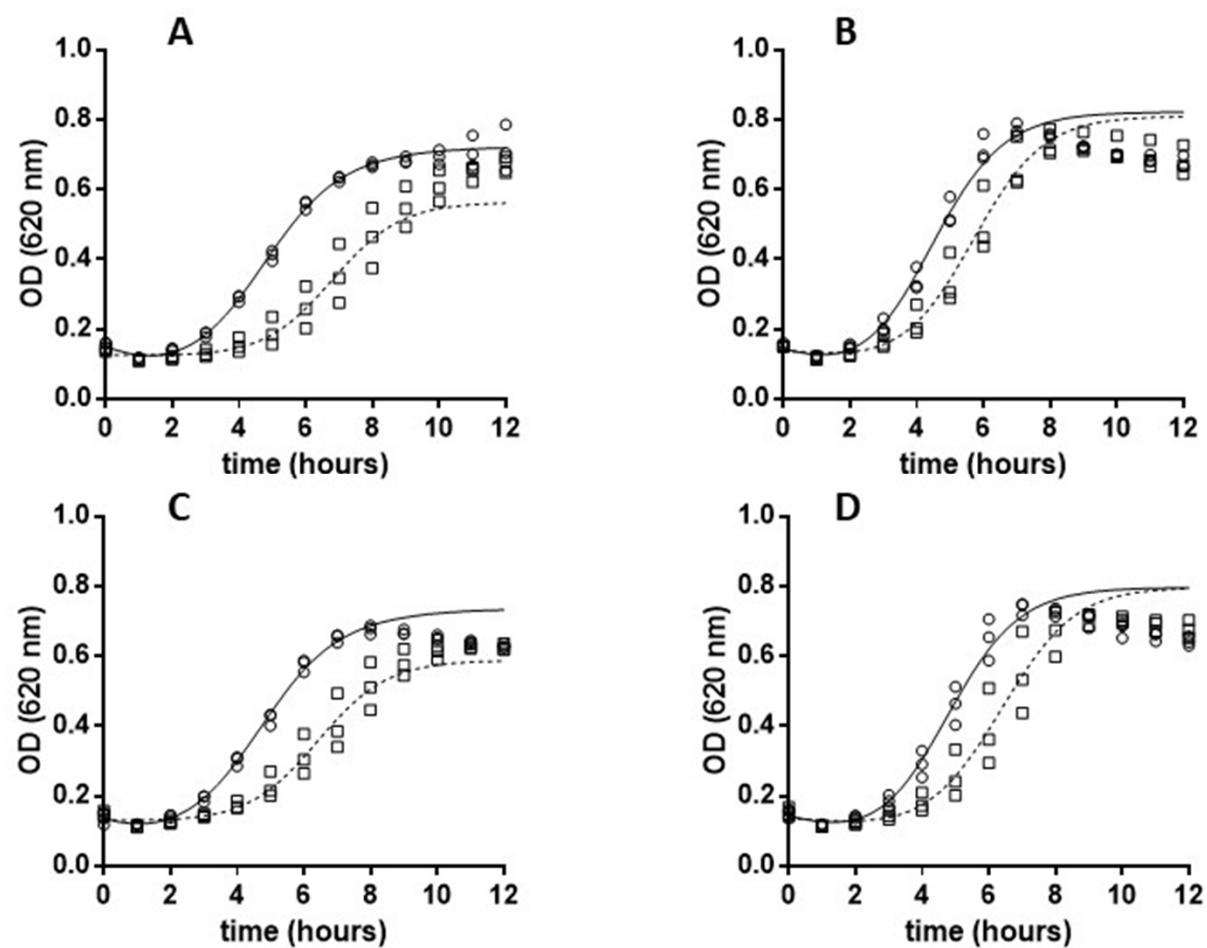
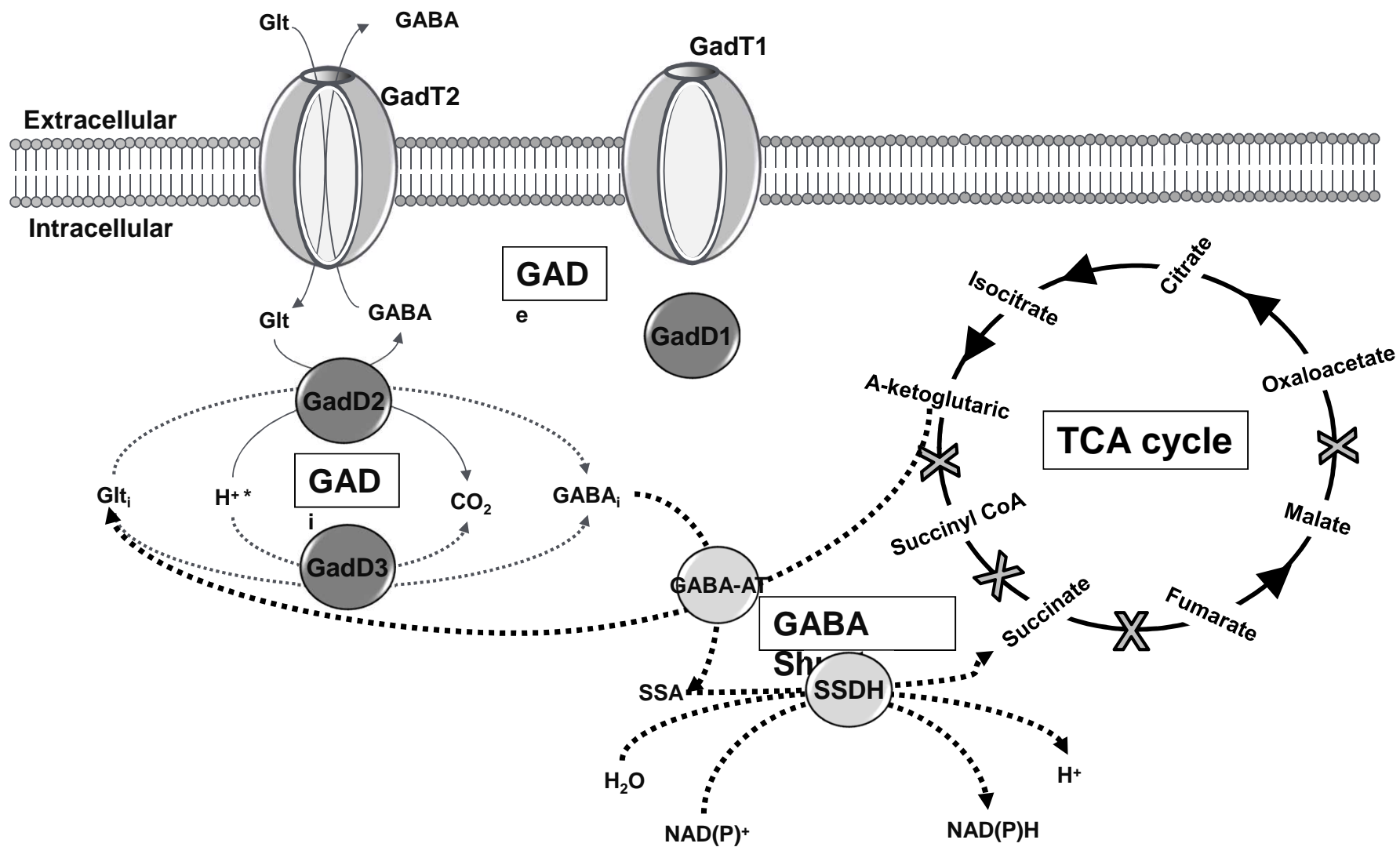


Fig.7



Highlights

- The GAD system affects oxidative stress resistance in a Gram-positive bacterium (*L. monocytogenes*)
- The GAD_i and mainly GadD3 affects oxidative stress resistance in *L. monocytogenes*
- The GAD_e system does not affect oxidative stress resistance in *L. monocytogenes*
- GadD1 in LO28 has a novel role in oxidative stress resistance
- SSDH of GABA shunt does not affect oxidative stress in *L. monocytogenes*.

Declarations of interest: none