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Investigation into the antimicrobial activity of fumarate against *Listeria monocytogenes* and its mode of action under acidic conditions

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Abstract

Organic acids such as fumarate are commonly used as antimicrobials in foods. Apart from the classical mechanism of intracellular dissociation, weak acids are active through important additional mechanisms which are not well-defined. Fumarate, based on its low dissociation constants is expected to have a low antimicrobial activity which is not the case, suggesting additional antimicrobial effects. Previously, fumarate has been shown to inhibit the GAD system of *E. coli* and therefore, we investigated for first time how it affects this system in *Listeria monocytogenes*. We found that fumarate is highly antimicrobial towards *L. monocytogenes* under acidic conditions. We also show that in cell lysates and similarly to *E. coli*, fumarate inhibits the GAD system of *L. monocytogenes*. However, despite the inhibition and in contrast to *E. coli*, *L. monocytogenes* is able to counteract this and achieve a higher extracellular GAD output (measured by GABA export) in the presence of fumarate compared to its absence. The latter is achieved by a dramatic 9.44-fold increase in the transcription of *gadD2* which is the main component of the extracellular GAD system. Interestingly, although maleate, the cis-isomer of fumarate results in a more dramatic 48.5-fold *gadD2* upregulation than that of fumarate, the final GAD_e output is lower suggesting that maleate might be a stronger inhibitor of the GAD system. In contrast, the GAD_e removes more protons in the presence of fumarate than in the presence of HCl at the same pH. All the above suggest that there are additional effects by fumarate which might be associated with the intracellular GAD system (GAD_i) or other acid resistance systems. We assessed the GAD_i output by looking at the intracellular GABA pools which were not affected by fumarate. However, there are multiple pathways (e.g. GABA shunt) that can affect $GABA_i$ pools and we can not conclusively suggest that GAD_i is affected. Furthermore, similarly to maleate, fumarate is able to eliminate *L. monocytogenes* in biofilms under acidic conditions. Overall, fumarate is a good candidate for

L. monocytogenes decontamination and biofilm removal which is not toxic compared to the toxic maleate.

1. Introduction

Foodborne illness is a significant public health problem both in the UK and globally. The World Health Organisation (WHO) estimates that foodborne illness is responsible for 2.2 million deaths annually (Food standards agency, 2011). The majority of this illness in the UK is caused by *Campylobacter* and *Norovirus* whilst most deaths are due to *Listeria monocytogenes* and *Escherichia coli* infections. (Food standards agency, 2011)

Various strategies are employed to eliminate these pathogens in foods aiming to reduce the incidence of foodborne illness. One such strategy is the addition of organic acids which have been used for centuries to prevent the growth of pathogenic and spoilage bacteria (Ricke, 2003). Organic acids are believed to affect microorganisms through diffusion of undissociated molecules across the cell membrane followed by intracellular dissociation and release of protons causing death or growth inhibition (Comes and Beelman, 2002; Lambert and Stratford, 1999; Podolak et al., 1996).

One of the well-known organic acids with antimicrobial activity is fumaric acid, which is a food grade, dicarboxylic acid found widely in nature and active against a number of foodborne pathogens including *E. coli*, *L. monocytogenes* and *Salmonella* sp. (Comes and Beelman, 2002; Kim et al., 2009; Kondo et al., 2006; Miller and Kaspar, 1994; Pérez‐Díaz and McFeeters, 2010; Podolak et al., 1996). In the EU and the US besides as an antimicrobial is also used as an acidulant, and a flavour enhancer (Lee, 2014; Saltmarsh et al., 2013). Fumaric acid is regularly used in various products including baked goods, confectionery, juices and dried powdered foods as well as in animal feed (Lee, 2014). Fumaric acid is considered as one of the relatively strongest among the weak organic acids, which however has low solubility in aqueous solutions

(Arnold et al., 2001; Roa Engel et al., 2013) while its salts are highly soluble (Zhou et al., 2002). Based on its low dissociation constants (pK_{a1} = 3.02 and pK_{a2} = 4.38; Lohbeck et al., 2000; Okuyama and Maskill, 2013; Szalka et al., 2013) it should be expected that fumarate has low antimicrobial activity although this is not the case, if compared to other organic acids including acetic and lactic acid (Podolak et al., 1996). This additional antimicrobial activity of fumarate, beyond what could be explained by the intracellular dissociation theory of weak acids, is normally attributed to unknown factors such as interference with metabolic activities stress mechanisms or other cellular functions. Understanding these additional effects could increase our knowledge and allow us to enhance the antimicrobial activity of these compounds and consequently achieve higher levels of hygiene or develop novel and improved antimicrobial regimes. Furthermore, it is important to understand in detail against which organisms this additional effect occurs and what the mode of action is.

The current study focuses on the foodborne pathogen *L. monocytogenes*, a Gram positive, facultative anaerobic bacterium that is ubiquitous in the environment (Posfay-Barbe and Wald, 2009) causing listeriosis, that mainly affects pregnant women, neonates and immunocompromised individuals (Posfay-Barbe and Wald, 2009). The organism is capable of surviving a wide range of environmental conditions and can grow under refrigeration temperatures affecting ready-to-eat products (Liu et al., 2002; O'Driscoll et al., 1996). It is also able to survive extreme acidic environments such as the stomach or acidic foods through the use key mechanisms of acid resistance of which the main one is the GAD system (Davis et al., 1996; Foster, 2004).

The GAD system converts glutamate to γ-amino butyric acid (GABA) with the removal of a proton resulting in an increase in the intracellular pH (Cotter et al., 2001; Karatzas et al., 2012). The architecture of the GAD system is highly variable and in *L. monocytogenes* it typically comprises two antiporters, GadT1 and GadT2 and three decarboxylases GadD1, GadD2 and

GadD3. The GadD1T1 operon is typically associated with growth under mild acidic conditions, the GadT2D2 promoting survival under extreme acidic conditions (Cotter et al., 2005) while the GadD3 is the main part of the intracellular GAD system (GAD_i) utilising solely intracellular glutamate to produce intracellular GABA which is catabolised to glutamate by the GABA shunt (Cotter and Hill, 2003; Feehily et al., 2014; Feehily and Karatzas, 2013).

Given that fumarate has previously been described as an inhibitor of the *E. coli* GAD enzyme (Fonda, 1972) we investigate here the antimicrobial activity of fumarate on *L. monocytogenes* under acidic conditions and the possibility that this stems from effects on the GAD system and possibly other aminoacid decarboxylase systems (Grobelny, 1995). Furthermore, we look at the ability of fumarate to remove biofilms of *L. monocytogenes* and investigate further the effects of the cis-isomer of fumarate, maleic acid on the GAD system which has also been previously shown to affect it in *L. monocytogenes* (Paudyal et al., 2018).

2. Materials and methods

2.1 Bacterial strains and growth conditions

All strains (Table 1) were stored in 2 ml cryovials with a 7% dimethyl sulfoxide (DMSO) at -80^{o}C. *L. monocytogenes* 10403S and EGD-e were cultured onto Brain Heart Infusion (BHI) agar (LABM, Lancashire UK) and *L. monocytogenes* LO28 onto Tryptic Soy Broth (Oxoid, UK) supplemented with 5% yeast extract (TSBY; Oxoid, UK) and incubated at 37^{o}C overnight. Three colonies from each plate were transferred, with an inoculation loop, into BHI and TSBY broth respectively in 10 ml Sterilin polystyrene tubes and incubated at 37^{o}C with shaking (150 rpm) for 18 h. These overnight cultures were used to inoculate 20 ml cultures of the corresponding media (1% inoculum) in 250 ml conical flasks which then were subsequently incubated overnight at 37^{o}C with shaking at (150 rpm) for 18 h.

126 **2.2 Calculation of undissociated acids using Henderson-Hasselbalch equation**

127 The percentage of undissociated acid present at pH 3 was determined using the Henderson-
128 Hasselbalch equation as adapted by Wemmenhove et al., (2016) and presented at Table 2

129 $$[\text{Undissociated acid}] = [\text{Total acid}] / 1 + 10^{(\text{pH-pKa})}$$

130

131 **2.3 Determination of minimum inhibitory concentration**

132 A range of concentrations (0, 1, 2, 4, 8, 16 and 32 mg/ml) of selected organic acids (Table 3)
133 were used in BHI inoculated at 1% with overnight culture of *L. monocytogenes* 10403S WT.
134 Cultures were then placed into 96 well plates and had their OD_{620nm} analysed over a 24 h period
135 using a Sunrise plate reader machine (Tecan, Mannedorf, Switzerland) operated by Magellan
136 software (Tecan, Mannedorf, Switzerland) with 20 min intervals between measurements. The
137 plates were kept at 37^{o}C with shaking to assess the MIC.

138

139 **2.4 Survival under acidic conditions**

140 Survival experiments were undertaken with *L. monocytogenes* 10403S WT. Twenty ml
141 cultures were prepared in BHI, using stock cultures prepared as described previously, and
142 grown in 250 ml Erlenmeyer flasks at 37^{o}C with agitation at 150 rpm. Acid challenge took
143 place with the addition of 8.6 mM fumaric acid, and a variety of organic acids (Table 3). Control
144 experiments were performed in the absence of any chemicals in overnight cultures. The pH of
145 the cultures was then adjusted to pH 3.0 using 1 M HCl.
146 One hundred µl samples were obtained prior to the acid challenge and every 20 min for 1 h and
147 placed in 900 µl Maximum Recovery Diluent (MRD; Oxoid Limited, Hampshire UK). Ten-
148 fold serial dilutions were prepared and 10 µl of each dilution were placed onto BHI agar plates
149 using the spot plate method and incubated at 37^{o}C overnight. Subsequently, colonies were

counted to assess the cell concentration in the culture at each time point. All experiments were performed in triplicate.

2.5 Survival in the presence of sodium fumarate

Following initial survival experiments, further survival experiments were performed focusing on the effect of fumaric acid and its salt sodium fumarate on *L. monocytogenes* 10403S WT, and its isogenic mutants Δ*gadD1*, Δ*gadD2*, Δ*gadD3*, on EGD-e WT and its isogenic mutants Δ*gadD1*, Δ*gadD2*, Δ*gadD3* and on LO28 WT with its isogenic mutants Δ*gadD1*, Δ*gadD2*, Δ*gadD1/2*.

Cultures were prepared in BHI or TSBY for LO28, using stock cultures, prepared as described previously and grown in 250 ml Erlenmeyer flasks at 37ºC with agitation at 150 rpm. Due to the significantly differences in strain sensitivity, different concentrations of sodium fumarate were used for 10403S (8.6 mM) and for EGD-e and LO28 (4.3 mM). Control cultures were also prepared containing no additional antimicrobials. Subsequently, all *L. monocytogenes* 10403S cultures had their pH adjusted to 3.0 and *L. monocytogenes* EGD-e and LO28 to pH 3.3.

One hundred μl samples were taken immediately prior to the acid challenge and every 20 or 5 min thereafter for 10403S or EGD-e and LO28 respectively. Samples were subsequently added in 900 μl MRD (Oxoid Limited, Hampshire UK) to prepare decimal serial dilutions and 10 μl of each dilution was plated onto BHI agar or TSBY agar respectively and incubated at 37ºC overnight. Following incubation, colonies were counted to assess the cell concentration at every time point.

2.6 GABAse assay

GABase assay was used to determine the concentrations of intracellular ($GABA_i$) and extracellular ($GABA_e$) GABA in 10403S and LO28 WT as described by O'Byrne et al., (2011). *E. coli* K-12 samples were assayed following the same methodology with the modification of the initial culture being grown in Lysogeny broth (LB LAB M, Lancashire, UK) supplemented with 10 mM monosodium glutamate (MSG; Steinheim, Germany), $GABA_e$ was quantified according to Tsukatani et al. (2005) as modified by Karatzas (2010).

2.7 GAD activity in protein lysates

WT 10403S cultures were prepared in 20 ml BHI in 250 ml Erlenmeyer as described previously by Paudyal et al (2018), while for LO28 and EGD-e 40 ml cultures were used. All cultures were then transferred to 50 ml falcon tubes (VWR, Leighton Buzzard UK) with 10 µg/ml chloramphenicol (Sigma-Aldrich, Steinheim, Germany). The samples were then centrifuged at 12,000 x g for 15 min and washed with a buffer solution, as described previously (Abrams et al., 2008; Boura et al., 2016). Suspensions were then incubated at 37°C in an orbital shaker at 150 rpm (Gallenkamp, Germany).

A 2 ml cryovial (Sarstedt, Germany) was filled with 0.07g acid washed glass beads (< 106 µm diameter Sigma-Aldrich, Steinheim, Germany) together with 1 ml cell suspension. The sample was then agitated using a Mini-Beadbeater (Biospec, Bartesville, USA), thrice for 1 min, followed by 1 min on ice. DNAse I (Thermo Fisher Scientific, California, USA) was then added up to 0.1% in the cell lysates and were then incubated at 37°C in an orbital shaker at 150 rpm for 30 min. One ml sample was then transferred to an Eppendorff tube and centrifuged at 5,000 x g for 15 min. The supernatant was then transferred to an Eppendorf tube and the pellet discarded. Subsequently, 100 µl of the supernatant was added to a pyridine hydrochloride buffer (Fonda, 1972) supplemented with 30 mM MSG and with or without 20 mM sodium fumarate. All samples were then adjusted to a pH of 4.5. The GABase assay was then used to assess

GABA levels. It had previously been established using standard concentrations of GABA, that the presence of sodium fumarate does not affect the accuracy of this assay.

2.8 Amino acid analysis by GC-MS

As the activity of the GABase enzyme could be affected by the presence of other molecules, GABA concentrations were also assessed in the supernatant or the bacterial lysates with the use of gas chromatography mass spectrometry (Elmore et al., 2005). The method also assessed the concentration of a wide range of aminoacids. Intra- and extra-cellular samples taken from *L. monocytogenes* 10403S were assessed in the presence and absence of sodium fumarate. Previous work by Paudyal et al. 2018 has shown that in similar conditions to those described here, GABA levels quantified by GC-MS and GABase were always within ± 5%.

2.9 Real-time PCR determination of GAD gene expression

The transcription of the *gad* genes in the presence and absence of sodium fumarate was assessed in *L. monocytogenes* 10403S WT (*gadD1*, *gadD2* and *gadD3*) using real time reverse transcription-PCR (RT-PCR) as previously described by Karatzas et al., (2010). The transcription of the antiporter-encoding genes (*gadT1* and *gadT2*) was not examined as it has previously been demonstrated that it is similar to the corresponding glutamate decarboxylases (*gadD1* and *gadD2*) belonging to the same corresponding operon (Karatzas et al., 2012). Overnight cultures of *L. monocytogenes* 10403S WT grown for 24 h until stationary phase in BHI were treated with 10 mM of either sodium fumarate or maleic acid for 40 min. Samples were taken and prepared as previously described by Karatzas et al (2010). Relative expression of the data was calculated as a ratio between expression of each of the target genes and the expression of the 16S rRNA which was used as the reference gene for each cDNA sample. Calculations were carried out following the advanced relative quantification settings of the

Light Cycler 480 SW 1.5.1 software programme, with PCR efficiencies of the primer pairs gadD1F-gadD1R, gadD2F-gadD2R, gadD3F-gadD3R and 16SF-16SR being 2.12, 2.09, 2.03 and 2.27 respectively (Karatzas et al., 2010).

2.10 Biofilm removal by sodium fumarate

Biofilm formation was assessed using *L. monocytogenes* 10403S WT, EGD-e WT and LO28 WT overnight cultures grown in their corresponding BHI or TSBY agar as described previously and then inoculated to 1% in a 2 ml of BHI broth or TSBY broth. The broth was mixed thoroughly and placed in a 24-flat-bottom-well Corning Costar cell culture plate and sealed using petrifilm. Following incubation at 37°C for 48h, the culture was removed and the wells washed thrice with sterile water. Subsequently a fourth treatment was applied using 2.5 ml of either water, 100 ppm free chlorine from calcium hyperchlorite, HCl (pH 2.4), HCl (pH 2.4) with 25 mM sodium fumarate, AM (an organic acid disinfectant) at pH 2.4 and AM at pH 2.4 with 25 mM of sodium fumarate. The biofilm was exposed to these solutions in the well for 5 min and then the supernatants were discarded and wells were rinsed with 2.5 ml deionized water. Subsequently, 500 µl MRD was placed in the well and the bottom of the well was scraped using a 200 µl pipette tip for 30 s in a pattern covering the whole well bottom. This was repeated 4 times to provide a total volume of 2 ml which was serially diluted 10-fold and then 10 µl was plated onto BHI or TSBY agar and incubated at 37°C for 24 h and then growth was assessed (Ramírez et al., 2015). The impact of the treatments on the biofilm was assessed using the following calculation (Hamilton, 2003; Heersink J., 2003; Ramírez et al., 2015).

Density = (Average count/Volume plated) * Dilution * Volume of MRD scraped into *(1/well surface area).

2.11 Statistical analysis

In all cases all experiments were run in triplicate unless stated otherwise. Subsequently results were assessed using paired Student t-tests. A P value below 0.05 indicated a statistically significant result accompanied by an asterisk.

3 Results

3.1 Calculation of the percentage of undissociated acid

The pKa of sodium fumarate is low compared to the other acids tested (maleic acid, tartaric acid and oxaloacetic acid; Table 2). This suggest that fumaric acid has a lower level of undissociated acid (51.7 %) and therefore, lower antimicrobial activity.

3.2 Growth in the presence of selected acids

The MICs of a variety of organic acids on *L. monocytogenes* 10403S WT were assessed (Table 3). However, tartaric acid seemed to be the most bacteriostatic as it had the lowest MIC (14.9 mM). Sodium fumarate as a salt did not inhibit *L. monocytogenes* under the conditions of the current experiment.

3.3 Acid survival of *L. monocytogenes* 10403S, LO28 and EGD-e in the presence of different organic acids.

Under acidic conditions (pH 3), 8.6 mM sodium fumarate showed a significant bactericidal effect on survival of the 10403S (Fig. 1A). Similar results were obtained with both EGD-e and LO28 (Fig. 1B and 1C) assessed at pH 3.3 as more acid-sensitive than 10403S (Karatzas et al., 2012). Based on previous work, it was expected that EGD-e might be the most sensitive strain, however it displayed a similar response with LO28 to sodium fumarate (Fig. 1B and 1C; Karatzas et al., 2012). Furthermore, we tested survival of 10403S against all compounds mentioned in Table 1, and fumaric acid, sodium fumarate and maleic acid were the most

bactericidal at 8.6 mM and pH 3.3 with all other compounds hardly conferring any inactivation (data not shown).

3.4 Survival of *L. monocytogenes* 10403S LO28 and EGD-e and their isogenic mutants under acidic conditions in the presence and absence of sodium fumarate.

Once it was determined that sodium fumarate conferred the highest bactericidal activity, the role of the GAD genes in the presence of sodium fumarate was assessed. In all cases, the presence of sodium fumarate resulted in significant increase in the log reduction in all WT strains and mutants (Fig. 2).

In 10403S the absence of sodium fumarate at pH 3 with HCl, minor log reductions in survival occurred with Δ*gadD2* being the most sensitive. In the presence of 8.6 mM sodium fumarate (pH 3) a significantly higher log reduction occurred for all strains while a similar trend occurred, with all mutants except Δ*gadD2*, behaving similarly to the WT (10403S WT, Δ*gadD1* and Δ*gadD3* showed 2.29, 1.99, and 2.56 log reduction of CFU/ml respectively; Fig. 2A). Δ*gadD2* was the most sensitive strain and impacted more by the presence of sodium fumarate (5.21 log reduction of CFU/ml).

In LO28 the effect of sodium fumarate showed a similar trend to 10403S, although this strain was more sensitive and the effect was significantly more pronounced. Also, in this case the addition of 4.3 mM sodium fumarate significantly affected survival at pH 3.3 and *gadD2* was also in this case the main determinant either with HCl alone or with sodium fumarate (Fig. 2B). In the presence of 4.3 mM sodium fumarate (pH 3.3), LO28 WT and Δ*gadD1*, showed a 4.80 and 5.11 log reduction CFU/ml respectively while that of Δ*gadD2* and Δ*gadD1/2* was higher than the maximum of 6 logs that could be determined with this protocol (Fig. 2B).

In EGD-e the major difference compared to the other two strains was that removal of *gadD2* did not result in increased sensitivity. In the presence of 4.3 mM sodium fumarate (pH 3.3),

EGD-e WT, Δ*gadD1*, Δ*gadD2* and Δ*gadD3* showed 4.62, 2.65, 2.60 and 5.07 log reduction CFU/ml respectively (Fig. 2C). In the presence of sodium fumarate, Δ*gadD3* was the most sensitive strain, while Δ*gadD1* and Δ*gadD2* appeared significantly more resistant than the WT probably due to the activation of another acid resistance mechanism. This trend had also been observed in the presence of maleic acid the GAD_i system may play a survival role in the presence of sodium fumarate (Paudyal et al., 2018).

3.5 Extracellular GABA of *L. monocytogenes* 10403S, LO28 and *E. coli* K-12.

To assess the possible influence of sodium fumarate on the activity of the GAD system an examination of the effect of this compound on the levels of $GABA_e$ was undertaken while the cells were in stationary phase**.** In *L. monocytogenes* 10403S WT the presence of sodium fumarate resulted in significant higher $GABA_e$ levels from (4.11 mM GABA) compared to its absence at pH 4.2 (2.01 mM GABA; 2.04-fold higher GABA levels $P < 0.05$; paired T-test, Fig. 3A). Similarly, higher levels were obtained with all isogenic mutants except Δ*gadD2* (data not shown). Similarly, LO28 WT also showed higher $GABA_e$ levels in the presence of sodium fumarate (2.89 mM $GABA_e$) compared to 1.24 mM $GABA_e$ in its absence (2.33-fold higher $GABA_e$ levels Fig. 3B) although this result was not statistically significant. Also, its isogenic mutants followed the same pattern (data not shown). These experiments were not performed with EGD-e as it is defective in extracellular GABA production.

Finally, when a similar experiment was undertaken using *E. coli* K-12 WT (pH 4) in contrast to the above, $GABA_e$ levels were significantly lower (4.5 mM $GABA_e$) in the presence of sodium fumarate compared to its absence (9.2mM $GABA_e$; 2.01-fold lower levels, $P < 0.05$; paired T-test; Fig. 3C). $GABA_i$ was also examined however, no significant difference in any of the strains tested was observed in the presence of sodium fumarate.

3.6 10403S WT GAD activity in protein lysates

The GAD activity of protein lysates was assessed by monitoring GABA production in the presence of MSG (Sigma-Aldrich, Steinheim, Germany). The results indicate that, sodium fumarate inhibited the GAD system activity in 10403S WT, resulting in reduced levels of GABA from 3.4 mM to 2.7 mM (0.79-fold reduced levels; $P < 0.05$; Fig. 4). A similar protocol was attempted with EGD-e and LO28 however, GABA levels were below the detection limit of the GABase assay and despite protocol alterations in the pH, the buffer used, higher glutamate supplementation or increasing the volume of culture utilised no improvement occurred (Fig. 5).

3.7 Real-time PCR determination of GAD gene expression.

Real time quantitative Polymerase Chain Reaction (RT-qPCR) was used to quantify the transcription of the *L. monocytogenes* 10403S WT GAD system genes in the presence of sodium fumarate and its cis-isomer maleic which has previously been shown to inhibit the listerial GAD system (Paudyal et al., 2018). Transcription of *gadD1* was very low and not affected by the presence of sodium fumarate or maleic acid (Fig. 6A). In contrast, both sodium fumarate and maleic acid resulted in a significant upregulation ($P < 0.05$) of the main component of the GAD_e system, *gadD2* by 9.44- and 48.51-fold respectively (Fig. 6B). The latter gene also showed the highest expression compared to the other two decarboxylases. Regarding *gadD3,* expression was not affected by the presence of sodium fumarate although that of maleic acid showed to result in an increase of 22.33-fold which however, was not statistically significant (Fig. 6C).

3.8 Biofilm formation

348 The survival of *L. monocytogenes* biofilms was assessed after the application of various
349 antimicrobial treatments including 100 ppm chlorine and an acidic disinfectant (AM).
350 When *L. monocytogenes* 10403S was assessed, all treatments did not affect the survival in the
351 biofilm with the exception of those with 25 mM sodium fumarate, either alone at pH 2.4 (1M
352 HCl; 1.49 log reduction of CFU/cm^2) or in combination with AM at the same pH (1.98 log
353 reduction of CFU/cm^2; Fig. 7A).
354 In *L. monocytogenes* EGD-e three treatments achieved a statistically significant reduction of
355 CFU/ml. Those were the same ones that affected 10403S and the AM disinfectant alone (2.35
356 log reduction of CFU/cm^2; Fig. 7B). Sodium fumarate alone at pH 2.4 resulted in a significant
357 3.72 log reduction of CFU/cm^2 while in combination with AM resulted in a 4.7 log reduction
358 of CFU/cm^2.
359 In *L. monocytogenes* LO28 all treatments resulted in a significant reduction in biofilm survival.
360 However, this was due to a lower variability between the replicates and overall the results were
361 similar to EGD-e with the exception of the AM treatment which seemed to be highly effective
362 against this strain. In this case also the two treatments with sodium fumarate were the most
363 effective along with 100 ppm chlorine (2.96 log reduction of CFU/cm^2). Sodium fumarate alone
364 at pH 2.4 resulted in a significant 2.67 log reduction of CFU/cm^2 while in combination with
365 AM resulted in a 3.40 log reduction of CFU/cm^2. The AM disinfectant treatment at pH 2.4
366 resulted in a 2.23 log reduction of CFU/cm^2 while pH 2.4 alone resulted in 1.13 log reduction
367 of CFU/cm^2 (Fig. 7C).
368
369 **4. Discussion**
370 The antimicrobial effects of organic acids are mainly explained by the theory of passive
371 diffusion of undissociated molecules and their intracellular dissociation (Foster, 2004) which
372 also dictates that organic acids that dissociate more (higher Ka) are less antimicrobial, while

373 those that dissociate less (lower Ka) more antimicrobial. However, there are major deviations
374 to this rule (Ricke, 2003) suggesting the existence of additional effects which are highly
375 important for our understanding of the mode of action of various organic acids and the
376 behaviour of microorganisms in foods and many other environments.
377 The present work focuses on fumaric acid which is widely used in foods while it is present in
378 all foods, all living organisms and various environments as it is a key component of the TCA
379 cycle. It is one of the relatively strongest among the weak organic acids and based on its low
380 pKa of 3.02 it should have low antimicrobial activity as it dissociates more (Table 2).
381 Therefore, as predicted, the MIC of fumaric acid was among the highest (34 mM) with only
382 oxaloacetic acid (60.5 mM) and sodium fumarate having higher MICs (Table 3).
383 However, in contrast to the above fumarate showed high bactericidal activity against all three
384 strains of *L. monocytogenes* used (Fig. 1A, B & C). This is a clear deviation from the behaviour
385 that would be predicted by the intracellular dissociation theory of weak acids suggesting
386 additional antimicrobial effects which however, only occur at highly acidic conditions. This
387 high antimicrobial activity of fumarate has been noted previously (Chikthimmah et al., 2003;
388 Comes and Beelman, 2002; Podolak et al., 1996; Kondo et al., 2006) and our aim was to
389 identify the complementary mode of action of fumarate which does not stem from the theory
390 of intracellularly dissociation of organic acids.
391 Fumarate is an inhibitor of the *E. coli* GAD system (Fonda, 1972) and based on this, an obvious
392 hypothesis to explain the above effects, is the inhibition of the *L. monocytogenes* GAD system.
393 To investigate this hypothesis and in parallel identify which component of the GAD system
394 might be affected, deletion mutants in GAD decarboxylase genes for all three strains of *L.*
395 *monocytogenes* were used. In 10403S, removal of *gadD1* and *gadD3* resulted in similar
396 population reduction when compared to the WT in presence of sodium fumarate. However,
397 removal of *gadD2* caused the greatest death both in the presence and in the absence of sodium

fumarate as expected since most strains rely on the GadD2 for the operation of the dominant GAD_e system (Fig. 2A; Karatzas, Brennan et al. 2010). Similarly, to 10403S, in LO28 the removal of the key *gadD2* significantly reduced survival under acidic conditions (Fig. 2B). However, EGD-e does not possess a GAD_e system associated with GadD2, and only utilises the GAD_i which is mediated by GadD3 (Feehily et al., 2014; Karatzas et al., 2012). In this strain, Δ*gadD3* was the most sensitive either in the presence or absence of sodium fumarate, without statistical significance though, whereas *gadD1* and *gadD2* removal significantly increased resistance but only in the presence of sodium fumarate (Fig. 2C). Overall inactivation was much higher in the presence of sodium fumarate and more exaggerated for the more sensitive mutants that showed higher inactivation in the absence of sodium fumarate.

Furthermore, it should be noted that removal of *gadD1* from all strains did not significantly influence their ability to survive acidic conditions. This gene has previously been linked to increased growth under mild acidic conditions based on observations with WT strains that do not possess it (e.g. serotype 4b strains; Cotter et al., 2005). However, mutants in this operon have never been shown to possess an acid sensitive phenotype, or evidence showing GadD1T1 exporting GABA neither in the literature or in our experiments, raising questions over its function as a glutamate decarboxylase. Overall, GadD2 and GadD3, depending on the strain, were significant for survival against sodium fumarate under acid conditions (Fig. 2A, B & C).

Sodium fumarate resulted in significant increase in $GABA_e$ exported by *L. monocytogenes* 10403S (2.04-fold increase) at pH 4.2 ($P < 0.05$; Fig. 3A). Interestingly, this increased GAD_e output did not confer increased survival under acidic conditions (Fig. 2A). This is highly interesting as each GABA molecule exported, removes one intracellular proton and based on the above, GAD_e removes twice more protons in the presence than in the absence of fumarate, but instead of conferring higher resistance the opposite occurs (Fig. 2A & B). A similar trend was observed with LO28 although it was not statistically significant ($P > 0.05$; Fig. 3B). It

should be stated that as we have shown previously, maleic acid, the cis-isomer of fumaric acid works in an opposite fashion than fumarate, as it reduces GAD_e output (Paudyal et al., 2018). However, in contrast to *L. monocytogenes*, when we challenged *E. coli* K12, sodium fumarate resulted in a significant -2.01-fold decrease in $GABA_e$ export ($P < 0.05$; Fig. 3C). The latter was expected as fumarate is an inhibitor of *E. coli* GAD enzyme (Fonda, 1972) and it demonstrates a different GAD system behaviour between these two organisms. Further work should investigate if this is a different feature between Gram-positive and Gram-negative bacteria.

To further assess the effect of sodium fumarate on the GAD system, we measured $GABA_i$ levels, and found no significant effect of sodium fumarate. At first glance, this might suggest no effect of sodium fumarate however, $GABA_i$ levels are also affected by its metabolic flux through the GABA shunt pathway and therefore the above results are not conclusive.

Surprisingly, we observed that sodium fumarate significantly inhibited GAD activity as measured through GABA levels in *L. monocytogenes* 10403S cell lysates ($P < 0.05$; Fig. 4). This coincides with its role as GAD inhibitor in *E. coli* (Fonda, 1972) and in plants (Ohno and Okunuki, 1962). We further investigated these inhibitory effects of sodium fumarate in lysates of LO28 and EGD-e but unfortunately, we were not able to get measurable GABA levels (Fig. 5) even in the absence of sodium fumarate, despite various protocol modifications (usage of higher cell numbers, higher levels of glutamate, different buffer pH values). This might be related to lower GAD activity or a different optimal pH of the GAD enzymes in these strains.

Further we looked at the effect of fumarate on transcription of GAD genes *gadD1*, *gadD2* and *gadD3* including maleic acid which is a cis-isomer of fumarate and we have previously shown that reduces GAD output and activity in *L. monocytogenes* enhancing its acid sensitivity (Paudyal et al., 2018). RT-qPCR showed no effect of fumarate or maleate on *gadD1* and *gadD3* (Fig. 6A and C) however, *gadD2*, the key component of GAD_e system in *L. monocytogenes* 10403S WT (Cotter et al., 2001; Cotter et al., 2005) was upregulated by sodium fumarate and

even more by sodium maleate ($P<0.05$, paired t-test, Fig. 6 B). The above suggest that *L. monocytogenes* tries to counteract the inhibitory effects of fumarate and maleate on GAD activity by increasing *gadD2* transcription and the final result of these opposing actions in the case of fumarate is increased GAD_e system output which however, does not enhance acid resistance. However, in the case of maleate, higher increase in *gadD2* transcription is unable to increase GAD_e system output (main difference with fumarate) but similarly to fumarate, the acid resistance is reduced.

The explanation for the antilisterial effects of fumarate might lie in the effects on the GAD_i system (Feehily and Karatzas, 2013), or other possible effects on other acid resistance systems or on cell metabolism that in its turn could affect acid resistance. For example, fumarate is highly antimicrobial against organisms such as *Salmonella* (Kondo et al., 2006) that lack GAD system suggesting these additional effects (Park et al., 1996). To assess this, we first looked at the aminoacid profile in presence or absence of fumarate and the only difference found was the increased $GABA_e$ levels in presence of fumarate confirming the GABase results. This suggests that other aminoacid decarboxylase systems are possibly not affected and the above effects of fumarate are on GAD_i system or possibly another non-amino acid decarboxylase system.

Furthermore, the ability of sodium fumarate to act on cells in a biofilm was examined. It has previously been shown that maleic acid can act on biofilms of *L. monocytogenes* (Paudyal et al., 2018) and *E. faecalis* (Ferrer-Luque et al., 2010). Due to these properties it has been suggested that maleic acid could be an effective alternative to the more toxic EDTA commonly used to remove biofilms from the oral cavity and dental equipment (Ballal et al., 2009; Ferrer-Luque et al., 2010). However, fumarate has no toxicity and therefore further work could investigate other potential applications. Our results showed the striking ability of sodium fumarate (25 mM) to eliminate cells of three different strains of *L. monocytogenes* in a biofilm, which was significantly higher than that of hypochlorite and a commonly used organic acid

disinfectant AM at pH 2.4 (Fig. 7). Furthermore, the addition of fumarate together with the AM disinfectant increased significantly the ability of the disinfectant to eliminate cells in biofilm. Our results also show that the more acid resistant strain 10403S survived the treatments better than the other two acid sensitive strains (EGD-e and LO28), underpinning the important role of acid resistance and GAD system in survival in a biofilm. Furthermore, we also found that LO28 was highly sensitive to chlorine. This coincides with previous reports suggesting a high variation in resistance to chlorine-based sanitisers among different strains (Brackett, 1987; Jacquet and Reynaud, 1994) and that mixed culture strains of *L. monocytogenes* are better able to resist chlorine treatments (Vaid et al., 2010). Our results suggest that fumarate has a great potential for removal of biofilms of *L. monocytogenes* while it is also nontoxic.

Conclusions

Overall, we investigated the effect of fumarate on *L. monocytogenes* under acidic conditions showing that although it is a GAD_e inhibitor, the bacterium is able to counteract this with increased transcription, being able to increase its overall GAD_e output, which however does not translate into increased acid resistance. We also show that there is a difference between fumarate and maleate as although the first increases GAD output, the latter reduces it, but both significantly enhance death under acidic conditions. The antimicrobial activity of fumarate might be related to reduced GAD_i or other systems. Further work is required to elucidate the full extent of the antimicrobial activity of fumarate on *L. monocytogenes* and other organisms. Such work will allow us to successfully eliminate this pathogen in food and food preparation environments but also explain its behaviour in environments where fumarate is present.

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Legends to the figures

Fig. 1. Survival of *L. monocytogenes* (A) 10403S WT in the presence (black circles) and absence (black triangles) of 8.6 mM sodium fumarate adjusted to pH 3 using 1 M HCl (B) EGD-e WT and (C) LO28 WT in the presence (black circles) and absence (black triangles) of 4.3 mM sodium fumarate at pH 3.3 using 1 M HCl. Asterisks represent statistically significant result ($P < 0.05$ paired student T-test) while D.L denotes detection limit of the experimental setup.

Fig. 2. Survival of *L. monocytogene*s and its GAD mutants in the **(A)** 10403S background in the presence and absence of 8.6 mM of sodium fumarate adjusted to pH 3 for 60 min, in the **(B)** LO28 and **(C)** EGD-e background in the presence and absence of 4.3 mM of sodium fumarate adjusted to pH 3.3 for 15 min. Adjustment of pH was done using 1 M HCl. Asterisks represent statistically significant result as assessed with paired student T-test ($P < 0.05$) and M.L. denotes the maximum log reduction could be recorded with the current protocol.

Fig. 3 $GABA_e$ levels of overnight cultures grown to stationary phase (~18 h at 37°C) with shaking in the presence or absence of 10 mM sodium fumarate (SF) for **(A)** *L. monocytogenes* 10403 WT at pH 4.2, **(B)** *L. monocytogenes* LO28 WT at pH 4.2 and **(C)** *E. coli* K-12 WT at pH 4. pH was adjusted with the addition of 1 M HCl. Asterisk represents statistically significant result. $P < 0.05$ paired student T-test.

Fig. 4. GAD activity in cell lysates of *L. monocytogenes* 10403S WT cells grown overnight until stationary phase (~18h) at 37°C with agitation (150 rpm) in the presence or absence of 20 mM sodium fumarate at pH 4.2. Lysates were prepared and then levels of GAD activity were assessed using GC-MS. Asterisk represents statistically significant result. P <0.05 paired student T-test.

Fig. 5 GAD activity in cell lysates of *L. monocytogenes* 10403S WT, EGD-e WT and LO28 WT cells grown overnight until stationary phase (~18h) at 37°C with agitation (150 rpm). Lysates were produced and then levels of GAD activity were assessed using the GABase enzymatic assay. Asterisks represents statistically significant result. P <0.05 paired student T-test.

Fig. 6 Expression of **(A)** *gadD1* **(B)** *gadD2* and **(C)** *gadD3* gene in *L. monocytogenes* 10403S WT in the absence or presence of 10 mM sodium fumarate or 10 mM maleic acid. Relative expression of each gene was calculated by comparing expression relative to 16S rRNA gene in each strain. Numbers above the bars represent fold difference in relative expression compared to control. Markers represent an average of triplicate measurements and error bars represent standard deviations.Asterisks * denote statistical significant difference compared to the control (P <0.05 paired student T-test).

Fig. 7 Survival of cells in biofilms of *L. monocytogenes* **(A)** 10403S WT **(B)** *L.* EGD-e WT and **(C)** LO28 WT following no treatment (water) or treatment with an acidic disinfectant (AM), AM together with 25 mM sodium fumarate (SF), HCl and HCl together with 25 mM SF. All treatments were at pH 2.4. Asterisks represent statistically significant difference between no treatment and a treatment (P <0.05; paired student T-test).

Tables

Table 1. list of strains used in these experiments

Strains	Relevant properties	Source
L. monocytogenes 10403S	Serotype 1⁄2a, WT	Karatzas et al., 2010
L. monocytogenes 10403S Δ*gadD1*	10403S with *gadD1* deleted	Karatzas et al., 2010
L. monocytogenes 10403S Δ*gadD2*	10403S with *gadD2* deleted	Karatzas et al., 2010
L. monocytogenes 10403S Δ*gadD3*	10403S with *gadD3* deleted	Karatzas et al., 2010
L. monocytogenes EGD-e	Serotype 1⁄2a, WT	Feehily et al., 2013
L. monocytogenes EGD-e Δ*gadD1*	EGD-e with *gadD1* deleted	Feehily et al., 2013
L. monocytogenes EGD-e Δ*gadD2*	EGD-e with *gadD2* deleted	Feehily et al., 2013
L. monocytogenes EGD-e Δ*gadD3*	EGD-e with *gadD3* deleted	Feehily et al., 2013
L. monocytogenes LO28	Serotype 1/2c, WT	Cotter et al., 2001
L. monocytogenes LO28 Δ*gadD1*	LO28 with *gadD1* deleted	Cotter et al., 2001
L. monocytogenes LO28 Δ*gadD2*	LO28 with *gadD2* deleted	Cotter et al., 2001
L. monocytogenes LO28 Δ*gadD1/2*	LO28 with *gad D1/2* deleted	Cotter et al., 2001

E. coli K-12	WT	KEIO collection	

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773 **Table 2. Percentage of undissociated acids at pH 3.**

Compound	pKa_1 1	pKa_2 2	pKa_3 3	%undissociated /total acid (pKa_1)	%undissociated /total acid (pKa_2)	%undissociated/ total acid (pKa_3)
Maleic acid	1.9	6.07		7.35	99.91	
Fumaric acid	3.03	4.44		51.72	96.49	
Sodium fumarate	3.55			78.01		
Pimlic acid	4.71	5.58		98.08	99.73	
Valeric acid	4.82			98.50		
Adipic acid	4.43	5.41		96.41	99.61	
Glutaric acid	4.34	5.22		95.62	99.40	
Malic acid	3.4	5.44		71.52	99.63	
Citric acid	3.13	4.76	6.39	57.42	98.29	99.95
Tartaric acid	2.98	4.34		48.84	95.62	
Oxaloacetic acid	2.22	3.89		14.23	88.58	

Alpha ketoglutaric acid	3.08	54.59
Valeric acid	4.82	98.50
Levulinic acid	4.59	97.49

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Table 3. MICs of compounds tested

Potential inhibitor	MIC
Maleic acid	34.4 mM
Fumaric acid	34.4 mM
Sodium fumarate	Above solubility threshold 0.22 mg/ml
Glutaric acid	30mM
Pimelic acid	24.9 mM
Adipic acid	27.3 mM
Malic acid	14.9 mM
Citric acid	20.8 mM
Tartaric acid	26.6 mM
Oxaloacetic acid	60.5 mM
α-Ketoglutaric acid	27.3 mM
Valeric acid	13 mM
Levulinic acid	60 mM

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