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

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ABSTRACT

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

1. Introduction

Listeriosis caused by *Listeria monocytogenes* is the leading cause of death due to a food borne illness in the UK (Mook et al., 2011) and as such is a serious problem of Public Health affecting the Food Industry. *L. monocytogenes* is a facultative anaerobic bacterium that can be isolated from soil, water, animal feed, faeces and tissues from various invertebrates and vertebrate animals including humans (Cooper and Walker, 1998). This bacterium has the ability to proliferate in a wide range of temperatures even below zero (Hudson et al., 1994) while it can persist (Fagerlund et al., 2016; Holch et al., 2013) and it is difficult to control in food processing environments (Salyers and Whitt, 2002). Normally, sodium hypochlorite or benzalkonium chloride are used while to a lesser extend acidic disinfectants are also used (Barker and Park, 2001; Zhang and Farber, 1996). However, a major factor affecting the popularity of a specific class of disinfectants is their antimicrobial activity and here we present a concept that could significantly increase the antimicrobial activity of the class of acidic disinfectants. This novel approach that could be used to eliminate *L. monocytogenes* involves specifically inhibiting acid resistance mechanisms in combination with acidic conditions. One such cellular target is the glutamate decarboxylase (GAD) system which is the most important acid resistance system in *L. monocytogenes* (Cotter et al., 2001a) that comprises three decarboxylases (GadD1, GadD2 and GadD3) and two antiporters, (GadT1 and GadT2; Cotter et al., 2005). The antiporters import extracellular glutamate, which is converted to GABA and CO_2, with a subsequent removal of protons, pH increase and export of GABA in exchange of another glutamate molecule (Paudyal and Karatzas, 2016). Furthermore, the decarboxylases can remove protons through processing intracellular glutamate through the intracellular GAD system (GAD_i; Karatzas et al., 2012). Therefore, affecting the activity of the GAD system could enhance the sensitivity to acid treatments, resulting in successful elimination from food processing environments and food contributing to

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the reduction of foodborne disease.

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

2. Materials and methods

2.1. Bacterial strains and growth conditions

All strains (Table 1) were stored in cryovials with 7% DMSO at −80 °C. Stock cultures from −80 °C were passed onto Brain Heart Infusion (BHI) agar (LABM, Lancashire UK) and incubated at 37 °C overnight. Three colonies from each plate were transferred with a loop in 3 ml of sterile BHI (LAB M, Lancashire UK) and incubated overnight at 37 °C with shaking (140 rpm). Subsequently, the overnight cultures were used to inoculate 20 ml of sterile BHI medium (1% inocula) in 250 ml conical flasks and incubated overnight (~18 h) at 37 °C with shaking (140 rpm). These overnight cultures were used for all acid challenges and assays described below.

2.2. Determination of minimum inhibitory concentrations (MICs)

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

Table 1
Strains used in this study.

Strain	Description	Source reference
LO28	Serotype 1/2c, wild type	6
EGD-e	Serotype 1⁄2a, wild type	34
ΔgadD1	EGD-e isogenic *gadD1* mutant	31
ΔgadD2	EGD-e isogenic *gadD2* mutant	31
ΔgadD3	EGD-e isogenic *gadD3* mutant	31
10403S	Serotype 1⁄2a, wild type	17
ΔgadD1	10403S isogenic *gadD1* mutant	31
ΔgadD2	10403S isogenic *gadD2* mutant	31
ΔgadD3	10403S isogenic *gadD3* mutant	31

2.3. Survival under acidic conditions

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

2.4. GABase assays

GABase assay was used to determine the concentrations of intracellular GABA ($GABA_i$) in 10403S and EGD-e and extracellular GABA ($GABA_e$) in 10403S and LO28. $GABA_i$ was quantified as described by O'Byrne et al. (2011) while $GABA_e$ was quantified according to the method of Tsukatani et al. (2005) as modified by Karatzas et al. (2010). The GABAse reaction was monitored by the measurement of 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).

2.5. GAD activity in protein lysates

Cultures of 10403S and EGD-e were grown in BHI broth overnight and they were transferred in 50 ml centrifuge tubes supplemented with 10 μg/ml chloramphenicol to prevent any further protein translation and were centrifuged at 12,000 × g for 15 min. Cell pellets were washed with sonication buffer as described previously (Abram et al., 2008; Boura et al., 2016) and final cell suspensions were incubated for 30 min with shaking at 37 °C. An Eppendorf tube was then filled with acid-washed glass beads (106 μm diameter; Sigma-Aldrich, Steinheim, Germany) and 1 ml of cell suspension was transferred to it. Samples were disrupted thrice by a Mini-Beadbeater (Biospec, Bartesville, USA) for 1 min and cooled for 1 min on ice. Then 0.1% DNAse1 (Sigma-Aldrich, Saint Louis, USA) was added to the cell lysate, incubated at 37 °C for 30 min with shaking and 1 ml was transferred into Eppendorf tubes and centrifuged at 5000 × g for 15 min. The supernatant was then transferred to sterile Eppendorf tubes and 40 μl of this was mixed with 450 μl of pyridine hydrochloride buffer (P-HCl; Fonda, 1972) adjusted at pH 4.5 and supplemented with 30 mM glutamate with or without 8.6 mM maleic acid. Subsequently, GABA levels were measured through GABase assays as described above. Previously, with the use of standard concentrations of GABA it was shown that maleic acid does not inhibit the activity of GABase.

2.6. Determination of GABA by GC-MS

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