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Research Article

Evaluating the Contribution of Acid Resistance Systems and Probing the Different Roles of the Glutamate Decarboxylases of *Listeria monocytogenes* Under Acidic Conditions

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Abstract

Listeria monocytogenes is an important zoonotic foodborne pathogen, which can cause a severe invasive illness to susceptible humans and animals with high mortality. As *L. monocytogenes* is widely distributed in natural environments, the bacterium is easy to contaminate food processing facilities and the products to be ingested by host. But during the transition from a saprophyte to intracellular pathogen, one of the biggest challenge *L. monocytogenes* encounters is the acid stress. To combat the acidic environments, the bacterium developed several acid resistance systems, including acid tolerance response (ATR), F0F1-ATPase, glutamate decarboxylase (GAD), arginine deiminase (ADI) and agmatine deiminase (AgDI). In this study, we comprehensively evaluated the contributions of different acid resistance systems and explored the different roles of the three GAD components under acidic conditions. We found that the GadD2 of GAD system made the largest contribution to the survival of *L. monocytogenes* in artificial gastric juice (AGJ) and acidic brain heart infusion (BHI), which was followed by the global stress regulator SigB, GadD3 of GAD system, AguA1 of AgDI system and ArcA of ADI system. Transcription analysis showed that the mRNA level of the three GADs were consistent with their contribution to acid resistance. Similar results were observed in the other three representative strains EGDe, Lm850658 and M7. We further obtained the purified GADs and their poly-antibodies to demonstrate that the contribution of the three GADs were determined by the protein levels in *L. monocytogenes*. Further studies are needed to focus on the regulation of different expression of the GAD system.

Keywords: *Listeria monocytogenes, Acid resistance, Glutamate decarboxylase, Survival*

Asidik Koşullar Altında *Listeria monocytogenes*'in Glutamat Dekarboksilazlarının Asit Direnç Sistemlerine Katkılarının Değerlendirilmesi ve Farklı Rollerinin Araştırılması

Öz

Listeria monocytogenes, duyarlı insan ve hayvanlarda yüksek ölüm oranı ile seyreden bulaşıcı hastalıklara neden olabilen, önemli bir gıda kaynaklı zoonotik patojendir. *L. monocytogenes* doğal ortamlarda yaygın olarak bulunduğundan, gıda işleme tesislerinin ve konakçı tarafından tüketilen ürünlerin bakteri ile kontaminasyonu kolaydır. Ancak bir saprofitten hücre içi patojene dönüşmesi sırasında, *L. monocytogenes*'in karşılaştığı en büyük güçlüklerden biri asit stresidir. Asidik ortamlarla savaşmak için, bakteri, asit tolerans yanıtı (ATR), F0F1-ATPase, glutamat dekarboksilaz (GAD), arginin deiminaz (ADI) ve agmatin deiminaz (AgDI) dahil olmak üzere çeşitli asit direnç sistemleri geliştirmiştir. Bu çalışmada, farklı asit direnç sistemlerinin katkıları kapsamlı bir şekilde değerlendirildi ve üç GAD bileşeninin asidik koşullar altında farklı rolleri araştırıldı. GAD sistemindeki GadD2'nin, *L. monocytogenes*'in yapay mide sıvısı (AGJ) ve asidik beyin kalp infüzyonunda (BHI) hayatta kalmasına en büyük katkıyı yaptığı ve bunu GAD sisteminden global stres regülatörü SigB, GadD3 ile AgDI sisteminden AguA1 ve ADI sisteminden ArcA'nın izlediği belirlendi. Transkripsiyon analizi, üç GAD'nin mRNA seviyesinin, asit direncine katkıları ile tutarlı olduğunu gösterdi. Benzer sonuçlar, diğer üç temsilci suş olan EGDe, Lm850658 ve M7'de de gözlendi. Ayrıca, üç GAD'nin katkısının, *L. monocytogenes*'teki protein seviyeleri tarafından belirlendiğini göstermek için saflaştırılmış GAD'ler ve bunların poli-antikorlarını elde ettik. GAD sisteminin farklı ekspresyonlarının düzenlenme mekanizmasının anlaşılabilmesi için daha fazla çalışmaya ihtiyaç vardır.

Anahtar sözcükler: *Listeria monocytogenes, Asit direnci, Glutamat dekarboksilaz, Sağkalım*

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INTRODUCTION

Listeria monocytogenes is a facultative anaerobic, gram-positive bacterium that is ubiquitous in natural environment as a saprophyte. In field environment, *L. monocytogenes* is thought to live off decaying plant material. Following ingestion by susceptible humans or animals, *L. monocytogenes* is capable of making the transition into a pathogen [1]. As an important zoonotic foodborne pathogen, *L. monocytogenes* could cause a severe invasive illness with high mortality in immunocompromised individuals [2,3]. Food-borne infection is the most common pathway of both epidemic and sporadic listeriosis, with 99% of human cases caused by consumption of contaminated food products [4]. Upon entering into the host gastrointestinal tract, *L. monocytogenes* adhere and invade various types of cells, including the phagocytic cells by the internalins InlA and InlB as well as Lap and InlP [5]. Following theentry into cell, *L. monocytogenes* must escape from host cell vacuoles via the pore-forming cytolysin listeriolysin O (LLO) and two phospholipases PI-PLC and PC-PLC quickly [6]. If not, the bacteria can be killed by the acidic endosome and digested by enzymes from the fused lysosome (phagolysosome). Entry into the cytosol, *L. monocytogenes* uses cytosolic nutrient to proliferate, then the bacteria spread to the neighboring cells by usurping actin polymerization as motile force by the bacterial surface protein ActA and with the help of InlC to relieve the cortical tension [7]. Then *L. monocytogenes* need to escape from the double membrane vacuoles to finish cell-to-cell spread with the help of LLO, PC-PLC and PI-PLC once again [5]. So it is not difficult to find that during the infection process, one of the biggest challenge *L. monocytogenes* encounters is acid stresses from stomach and phagolysosomes [8].

Listeria monocytogenes contains several enzyme systems including F_0F_1-ATPase, ADI, AgDI, GAD and acid tolerance response, to maintain intracellular pH homeostasis in acidic environments [9]. Under acid stress, F_0F_1-ATPase system uses ATP hydrolysis to produce proton motive force to pump cytoplasmic protons, while the ADI and AgDI use arginine and agmatine to produce ammonia to neutralize the cytoplasmic protons, respectively [10,11]. The glutamate decarboxylase (GAD) system, which consumes intracellular protons by converting glutamate to γ-aminobutyrate [12], also plays a role in acid resistance of *L. monocytogenes* to protect them in low pH foods. Moreover, pre-exposure of *L. monocytogenes* to mild acid could induce acid tolerance response (ATR) that improves the survival rate under fatal acid stress. As a global transcriptional regulator, SigB has been reported to positively regulate the ATR to help *L. monocytogenes* to deal with acid stress [13].

Although all the acid resistance systems were individually demonstrated to play important roles in acid stress and pathogenicity of *L. monocytogenes*, to date, no comprehensive assessment was conducted on these acid resistance systems, and the relative roles of these systems remain unclear. Moreover, *L. monocytogenes* contains several copies for some acid resistance systems. For example, *L. monocytogenes* 10403S has two AgDI genes *(aguA1* and *aguA2)*, and both of them were upregulated in response to acid stress, but only AguA1 contributed to acid resistance and pathogenicity of the bacteria [11]. For the GAD system, most of the *L. monocytogenes* strains (lineages I and II) contain three GADs [14], but the contributions of different GADs remain unclear. In this study, we tried to evaluate the contributions of different acid resistance systems and to clarify the different roles of the three GAD components under the acidic condition.

MATERIAL and METHODS

Bacterial Strains, Plasmids and Culture Conditions

Listeria monocytogenes 10403S, EGDe, Lm850658 and M7 were used as the wild-type strains. *Escherichia coli* DH5α was employed as the host strain for plasmids pET30a and pKSV7. *E. coli* Rosetta was used as expression host. *L. monocytogenes* and *E. coli* were cultured in brain heart infusion (BHI, Oxoid, Basingstoke, U.K.) and Luria-Bertani medium (LB, Oxoid), respectively, at 37°C. Stock solutions of ampicillin (50 mg/mL), kanamycin (50 mg/mL) and chloramphenicol (10 mg/mL; Sangong Biotech Co., Ltd, Shanghai, China) were added to the media, when appropriate, at the required concentrations.

Survival in AGJ or Acidic BHI Broth

Survival assay in artificial gastric juice (AGJ) or acidic BHI broth was conducted as in previous research [15]. *L. monocytogenes* wild-type and mutant strains were grown overnight at 37°C in BHI broth at pH 7.0 with shaking. The cultures were collected by centrifugation at 3000 g at 4°C for 10 min, washed and resuspended in phosphate buffered saline (PBS, 10 mM, pH 7.4) with the $OD_{600\ nm}$ adjusted to 1.0. Then 50 μL bacterial suspension was mixed in 950 μL AGJ (8.3 g proteose peptone, 3.5 g D-glucose, 2.05 g NaCl, 0.6 g KH_2PO_4, 0.11 g $CaCl_2$, 0.37 g KCl, 0.05 g bile salt, 0.1 g lysozyme and 13.3 mg pepsin dissolved in 1 liter distilled water with pH adjusted to 2.5 with HCl, filter sterilized) or BHI broth with indicated pH values (filter sterilized). After 1 h of incubation at 37°C, the mixtures were serially diluted and plated on BHI agar plates. The plates were incubated at 37°C for 24 h and viable bacteria were counted. Survival rate was calculated as percentage of survived bacteria after incubating in the acidic conditions for 1 h relative to the incubated bacteria. Data was reported as the mean ± SD of three independent experiments, each performed in triplicate.

Transcriptional Analysis

Overnight cultures of *L. monocytogenes* strains were inoculated into fresh BHI broth and grown to exponential phase ($OD_{600\ nm}$=0.25) or stationary phase ($OD_{600\ nm}$=0.6) at

37°C. One milliliter of each culture was treated with pH 4.5 BHI for an hour and then pelleted by centrifugation at 4°C. Total RNA was extracted using the Trizol reagent according to the manufacturer's instruction (Sangong Biotech Co., Ltd) and cDNA was synthesized with reverse transcriptase (TOYOBO Biotech Co., Ltd, Shanghai, China). Quantitative real-time PCR (qRT-PCR) was performed in 20 µL reaction mixtures containing SYBR green qPCR mix (TOYOBO (SHANGHAI) Biotech Co., Ltd) to detect the transcriptional levels of indicated genes on the iCycler iQ5 real-time PCR system (Bio-Rad, Hercules, California, U.S.A.) with specific primer pairs listed in *Table 1*. The housekeeping gene *gyrB* was selected as an internal control for normalization as previous research [16].

Construction of Deletion and Complementation Mutants

A homologous recombination strategy was used to construct the deletion mutants of *L. monocytogenes* 10403S according to the previous research [17] using the primer pairs listed in *Table 2*. The homologous fragments of overlapping PCR were purified and ligated to pMD18-T (TaKaRa, Beijing). After sequencing, the inserted fragments were digested with the indicated restriction enzymes, ligated to the temperature-sensitive shuttle vector pKSV7 and transformed into DH5α. Plasmids containing the inserted fragments were subsequently extracted and electroporated into *L. monocytogenes* competent cells. Transformants were grown at a non-permissive temperature (41°C) on BHI agar containing chloramphenicol (10 µg/mL) to promote chromosomal integration. The recombinants were passed in succession in BHI without antibiotic at a permissive temperature (30°C) to enable plasmid excision and curing. The deletion mutants were identified by PCR and confirmed by sequencing.

For the complementation strains, the encoding sequences of *gadD1*, *gadD2* and *gadD3* were amplified from *L. monocytogenes* EGDe with the indicated primer pairs listed in *Table 2*. After restriction digestion with appropriate enzymes, the PCR fragment was cloned into pIMK2 following the P_{Help} promoter. The recombinant plasmids were then electroporated into *L. monocytogenes* EGDe competent cells. The transformants were plated on BHI agar containing kanamycin (50 µg/mL) and positive clones were picked up and identified by PCR.

Prokaryotic Expression and Purification of GadD1, GadD2 and GadD3

GadD1, GadD2 and GadD3 were expressed as fusion proteins with His-tag using the expression vector pET30a (Invitrogen, U.S.A.) as previously shown [18]. The full-length *gadD1*, *gadD2* and *gadD3* were amplified with primer pairs listed in *Table 3*. The amplified fragments were cloned into

Table 1. *Primers used for q-PCR*

Primers	Sequences (5′-3′)	Size (bp)
gadD1-fwd	AGAATATCCACAGACAGCAAAG	142
gadD1-rev	CATAGCCATTCCACCAAGCAT	
gadT1-fwd	CGTTCTCGGTATTACAATTCCT	150
gadT1-rev	GCAAGCATGAAGATAACAAGAG	
gadT2-fwd	CCCTGTACCACTTATTATGGTT	116
gadT2-rev	CTACAGTTAAGGAAATTGCGGT	
gadD2-fwd	CCTTTGGAAAGATGAAAGCTAC	128
gadD2-rev	TGTAGTATTGACCGATGATGTG	
gadD3-fwd	ACCAATAATTTGGCTCGCACTA	144
gadD3-rev	TTAGTTTATCCGGGTGTTGGTT	
gyrB-fwd	AGACGCTATTGATGCCGATGA	91
gyrB-rev	GTATTGCGCGTTGTCTTCGA	

Table 2. *Primers used for deletion and complement mutants construction*

Primers	Sequences (5′-3′)	Size(bp)
gadD1-a	AATAAGCTTACTACACAGGTTTACAAGCA	515
gadD1-b	ACTCTCCCATTTTTCATAAATTCCTCCA	
gadD1-c	GAAAAATGGGAGAGTGATAAAATTTCTAG	524
gadD1-d	GCTGAATTCTTTTAATTGAAGTAACGTCA	
gadD1-e	AACCAACAGAAACATCGCTTCGTAT	
gadD2-a	ATAGCATGCCACTTATTATGGTTCAAG	536
gadD2-b	GATTTTTTCCTCCTATAATTTGTCTTGATT	
gadD2-c	TAGGAGGAAAAAATCTTCACACATTAA	545
gadD2-d	ATAGAATTCGGACTTATTCCGAGTAATG	
gadD2-e	GCAGCACTTTGTTACTTTTTGAAGAAG	
gadD3-a	GCAGGATCCAGCTTCTACTCTAACATGGTTCACG	567
gadD3-b	TTATAGTGAAGACGACAAGCGAACTTGGATGGT GAGTCCGA	
gadD3-c	TTCGCTTGTCGTCTTCACTATAAAGC	605
gadD3-d	AACGGTACCCGAGCGTGTCTATCTCACTATTCAT	
gadD3-e	GAAATTGTCGATTCCGGTGATGACT	
gadD1-CF	CGGGATCCTATGTTTAAAACAAATGTTGAACAAA	1406
gadD1-CR	GGGGTACCTTAATGAGTAAAGCCATGTGT	
gadD2-CF	CGGGATCCCATGTTATATAGTAAAGAAAATAA	1412
gadD2-CR	GGGGTACCTTAATGTGTGAAGCCGTGGA	
gadD3-CF	CGGGATCCGATGCTTTATAGTGAAGACGACA	1421
gadD3-CR	GGGGTACCTTAGTGCGTAAATCCGTATGAA	

Sequences with underline were restriction enzyme sites

Table 3. *Primers used for expression of the glutamate decarboxylases*

Primers	Sequences (5′-3′)	Size (bp)
gadD1-exp-fwd	GGAGGTACCATGTTTAAAACAAATGTTGAACAAA	1407
gadD1-exp -rev	CCAGGATCCTTAATGAGTAAAGCCATGTGT	
gadD2-exp-fwd	GAAGGTACCATGTTATATAGTAAAGAAAATAAAGA	1413
gadD2-exp -rev	GCCGGATCCTTAATGTGTGAAGCCGTG	
gadD3-exp-fwd	GGAGGTACCATGCTTTATAGTGAAGACGACA	1422
gadD3-exp -rev	TCTGGATCCTTAGTGCGTAAATCCGTATGAA	

Sequences with underline were restriction enzyme sites

the pET30a after restriction digestion. The recombinant plasmids were transformed to expression host *E. coli* Rosetta competent cells. Positive clones were confirmed by sequencing and then grown in 200 mL of LB medium supplemented with 50 μg/mL kanamycin at 37°C until $OD_{600\ nm}$ of the cultures reached 0.6-0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added into the medium at a final concentration of 0.4 mM to induce expression of GadD1, GadD2 and GadD3 for 12 h at 15°C. Then IPTG-induced cell pellets were collected, resuspended in 50 mM PBS (pH 7.4), and disrupted with 100 cycles of sonication at 300W for 5 sec with intermittent cooling on ice for 10 sec (25 min in total). After centrifugation at 12.000 g for 20 min, the supernatant samples were collected and loaded onto a 2-mL prepacked nickel-chelated agarose gel column (Weishi-Bohui Chromtotech Co., Ltd, Beijing, China). The columns were washed with 50 mM PBS containing 500 mM NaCl and 30 mM imidazole, and the bound proteins were eluted with a linear gradient of 25-500 mM imidazole prepared in the same buffer. Expression and purification of the recombinant proteins were analyzed on a 10% SDS-PAGE gel followed by Coomassie Brilliant Blue staining.

Polyclonal Antibodies Preparation

The purified recombinant protein was used for raising polyclonal antibodies in New Zealand white rabbits according to the previous study [19]. Rabbits were first immunized with 500 μg protein emulsified by the equal volume of Freund's complete adjuvant (Sigma, St. Louis, U.S.A.) through subcutaneous injection. After two weeks, the rabbit was boosted subcutaneously three times with 250 μg protein emulsified by incomplete Freund's adjuvant (Sigma, St. Louis, U.S.A.) at two-week intervals. Rabbits were bled 10 days after the last immunization and sera were isolated from the whole blood to collect polyclonal antibodies. Animal experiments were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

Western Blot Analysis

Listeria monocytogenes wild type and mutant strains were cultured with BHI broth to stationary phase at 37°C, then treated with pH 4.5 BHI broth for an hour. Then bacteria were harvested by centrifugation and pellets were lysed with lysis buffer and then homogenized with a refiner. The supernatant of cell lysis was isolated by centrifugation and analyzed by 10% SDS-PAGE. GadD1, GadD2 and GadD3 were blotted and probed with respective polyclonal antibodies produced in this study. Glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as loading control. Then HRP conjugated goat-anti-rabbit IgG (Sangong Biotech Co., Ltd) was used as the second antibody to probe GadD1, GadD2, GadD3 and GAPDH. The abundance of indicated proteins was evaluated with software Quantity One (Version 4.6.6, Bio-Rad, U.S.A.) to calculate the gray level of specific bands.

Statistical Analysis

All data comparisons were analyzed using the two-tailed homoscedastic Student's T-test. In all cases, differences with $P<0.05$ were considered as statistically significant. The GraphPad Prism 5 (Version 5, GraphPad, U.S.A.) software was used to produce the graphs.

RESULTS

To evaluate the contribution of different acid resistance systems of *L. monocytogenes*, we knocked out the acid resistance-associated enzymes genes in the background of the reference strain 10403S and then compared the survival rate of the mutants with the reference strain. Our data showed that mutant strain ΔgadD2 exhibited the lowest survival rate (0.005%) in AGJ, which was followed by strains Δ*sigB*, ΔgadD3, ΔaguA1, ΔarcA, ΔgadD1 and ΔaguA2 *(Fig. 1A)*. Among these mutants, only Δ*aguA2* and Δ*gadD1* did not show a significant difference on the survival rate of *L. monocytogenes* in the acidic condition. A similar result was also observed in pH 2.5 acidic BHI broth *(Fig. 1B)*. These data indicated that the contribution of the acid resistance-associated enzymes was quite different. In *L. monocytogenes* 10403S, GadD2 made the largest contribution to acid resistance, which was followed by SigB, GadD3, AguA1 and ArcA.

To elucidate the different roles of the components of GAD system in acid resistance, we analyzed the transcriptional

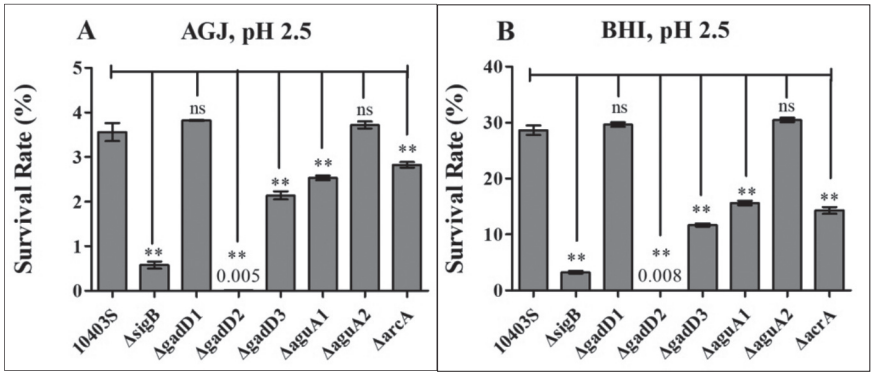


Fig 1. Survival of *L. monocytogenes* wild type 10403S and different mutant strains for an hour in pH 2.5 artificial gastric juice (AGJ) (A) or pH 2.5 brain heart infusion (BHI) broth (B). Experiments were conducted at least three times and values were expressed as mean ± SD. ** and ns indicate a statistically significant difference ($P<0.01$) and no significant difference between indicated strains, respectively

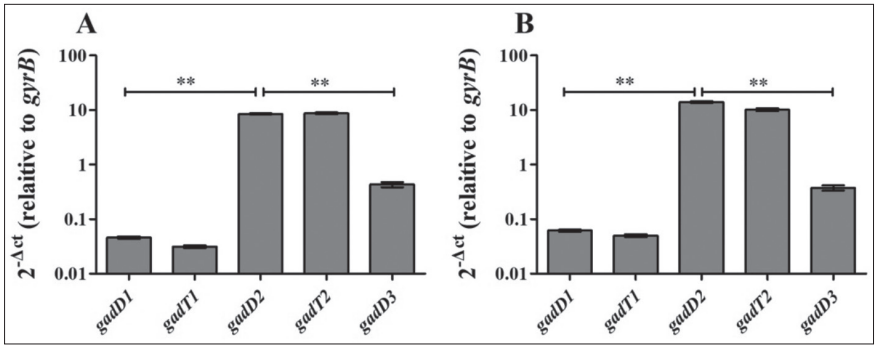


Fig 2. Relative transcriptional level of GAD system of *L. monocytogenes* wild type 10403S treated with pH 4.5 BHI for an hour at exponential phase (A) and stationary phase (B). Values were expressed as mean ± SD and ** indicate a statistically significant difference (P<0.01) between indicated strains

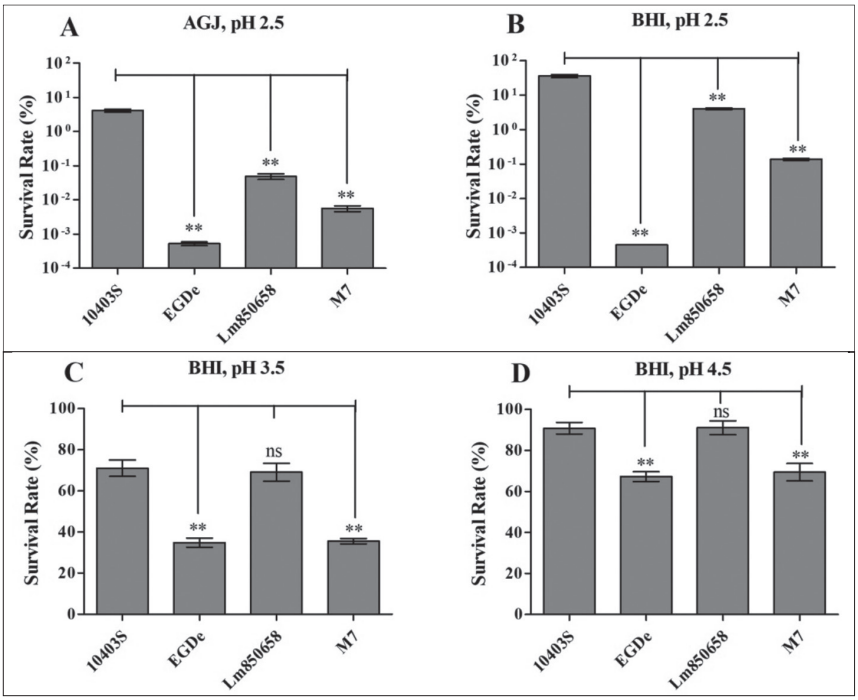


Fig 3. Survival of *L. monocytogenes* strains under different acidic conditions. Bacteria were treated with artificial gastric juice (AGJ) (A) or brain heart infusion (BHI) with pH 2.5 (B), pH 3.5 (C) and pH 4.5 (D) for an hour respectively. Experiments were repeated three times and values were expressed as mean ± SD. ** and ns indicate a statistically significant difference (P<0.01) and no significant difference between indicated strains, respectively

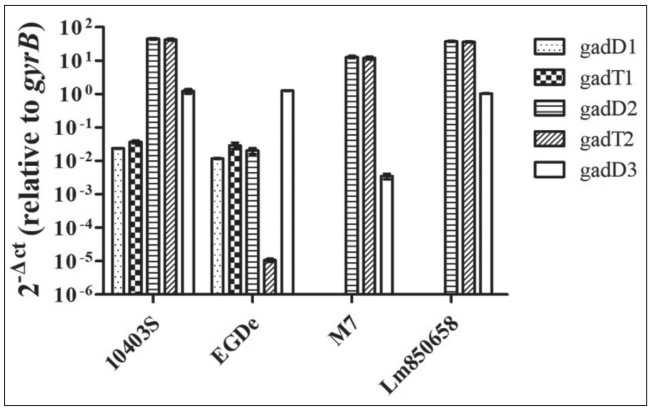


Fig 4. Relative transcriptional level of GAD system of the four *L. monocytogenes* wild type strains treated with pH 4.5 BHI for an hour at the stationary phase

level of the GAD system under acidic conditions. Our data showed that the transcriptional level of *gadT2/gadD2* was significantly higher than that of *gadD3* and *gadD1/gadT1* both at exponential and stationary phases in pH 4.5 BHI broth *(Fig. 2A,B)*. Moreover, the mRNA level of *gadD3* was ten-fold more than that of *gadD1/gadT1*. These results suggest that the contribution to acid resistance of different GAD components was correlative to their transcriptional level.

To confirm this hypothesis, we analyzed the survival rate of four representative strains, including 10403S, EGDe, Lm850658 and M7, under acidic conditions and the transcriptional level of their GAD system. Our data showed that strain 10403S exhibited the highest survival rate in AGJ for 1 h, which was followed by Lm850658, M7 and EGDe *(Fig. 3A)*. A similar result was also observed in pH 2.5 BHI broth *(Fig. 3B)*. When the pH increased to 3.5 or pH 4.5 in BHI, the survival rate of the four strains were increased, and the survival rate of 10403S and Lm850658 remained significantly higher than that of M7 and EGDe *(Fig. 3C,D)*. This survival rate was consistent with the transcriptional level, as the transcriptional level of *gadT2/gadD2* in EGDe and M7 were significantly lower than that of 10403S and Lm850658 in pH 4.5 BHI *(Fig. 4)*. The *gadD1/gadT1* mRNA level of EGDe was equal to that of 10403S, and *gadD1/gadT1* genes were deficient in lineage III strains

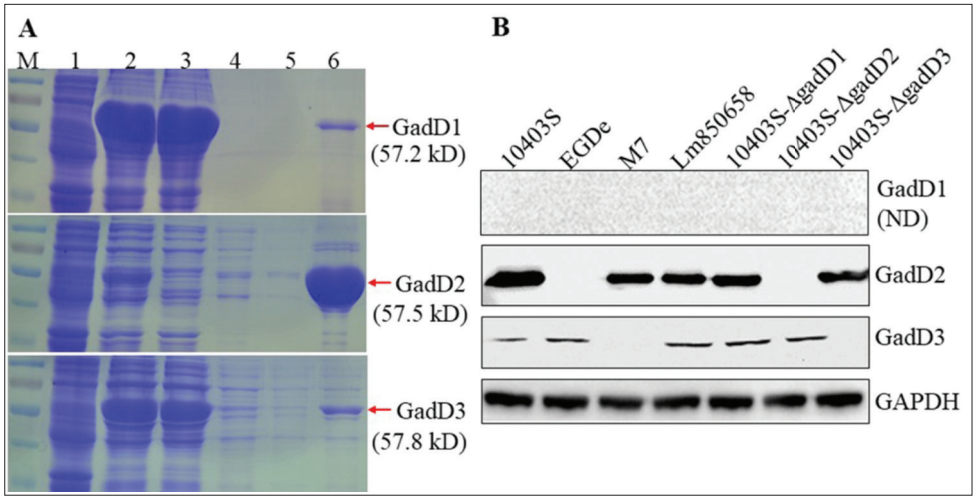
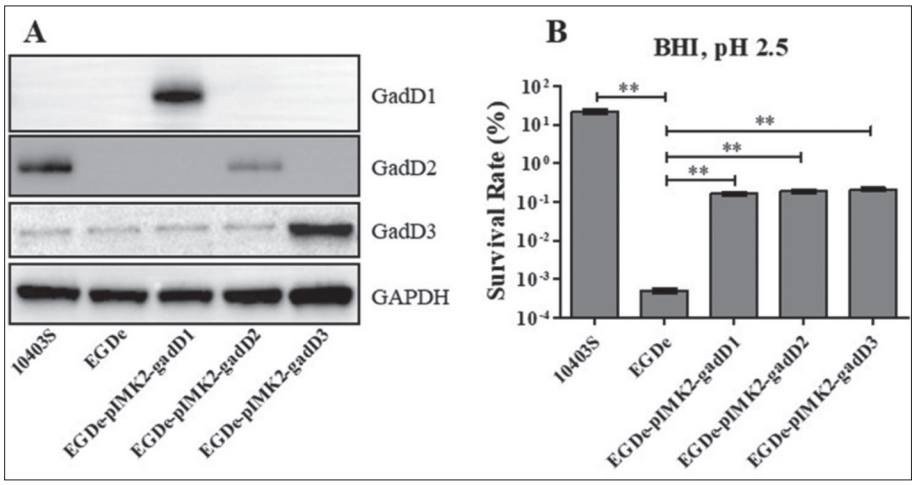


Fig 5. Affinity purification of GadD1, GadD2 and GadD3 (A) and expression of GadD1, GadD2 and GadD3 in the four wild type strains and the three mutant strains (B). M, Pre-stained Marker; lane 1, Control; lane 2 and 3, post-column sample; lane 4 and 5, wash with 4 mL 50 mM imidazole; lane 6, elution with 1 mL 400 mM imidazole; ND, not detected

Fig 6. Complementation of the GadD components rescued the survival of *L. monocytogenes* EGDe under acidic stress. Overexpression of GadDs (A) and survival of the indicated *L. monocytogenes* strains in pH 2.5 BHI broth for an hour (B). Values were expressed as mean ± SD and ** indicate a statistically significant difference (P<0.01) between indicated strains



Lm850658 and M7. The mRNA level of *gadD3* of 10403S, EGDe and Lm850658 were at the same level in the acidic condition, which was significantly higher than that of M7 *(Fig. 4)*.

To confirm whether the role of GAD system was determined by their expression, we purified prokaryotic expressed GadD1, GadD2 and GadD3 *(Fig. 5A)*. Poly-antibodies were obtained from immunized rabbit with the indicated purified proteins. Then the protein level of the three components in the acidic condition was detected by Western blot. The results showed that GadD1 was detected neither in wild type strain nor in the mutants *(Fig. 5B)*. GadD2 was not detected in *gadD2* mutant and EGDe, but highly expressed in 10403S and Lm850658 *(Fig. 5B)*. GadD3 was not detected in *gadD3* mutant and M7 *(Fig. 5B)*, which was similar to the tendency of its mRNA level *(Fig. 4)*. To confirm the hypothesis that the role of GAD in acid resistance was determined by their expression level, we overexpressed GadD1, GadD2 and GadD3 in EGDe to determine their function in acid resistance individually. Survival assay in pH 2.5 BHI broth showed that the overexpression any of the three GADs significantly improves the acid resistance of EGDe *(Fig. 6)*.

DISCUSSION

Listeria monocytogenes is a bacterium that lives in natural environments as a saprophyte but is capable of making the transition into a pathogen following its ingestion by susceptible humans or animals [1]. As an important zoonotic foodborne pathogen, this bacterium has the ability to adapt to a variety of environmental conditions [20]. Acidic environments such as silage, fermented foods, stomach and phagolysosomes, are the most common conditions that the bacterium encounters. *L. monocytogenes* contains several enzyme systems including F_0F_1-ATPase, ADI, AgDI and GAD to cope with these unfavorable conditions. Although all of the acid resistance systems had been demonstrated to play important roles in acid resistance in *L. monocytogenes* [9,21-24], it's unclear which system plays the major role in acid resistance of *L. monocytogenes*. Here we evaluated the effects of these systems on the survival of *L. monocytogenes* under acidic conditions. Our data showed that GadD2 of GAD system made the largest contribution to *L. monocytogenes* 10403S survival in different acidic conditions, which was followed by SigB, GadD3, AguA1, and ArcA. Since the constitution of these systems was complicated, it is difficult to knock out the whole system to

determine its contribution. In this study, we only knocked out the key enzymes of the acid resistance systems, which might be inadequate to evaluate the whole function of an acid resistance system, but we knocked out the whole AgDI system encoding region *(lmo0036-lmo0042)* at once. No significant difference was observed about the survival rate of strains Δ*aguA1(Δlmo0038)* and ΔAgDI *(Δlmo0036-lmo0042)* in acidic broth (data not shown). F_0F_1-ATPase system is essential for *L. monocytogenes*, in which mutantion will cause a lethal effect to the bacteria [25]. we didn't evaluate its contribution to acid resistance of *L. monocytogenes*. Datta et al.[25] and Cotter et al.[26] treated *L. monocytogenes* LS2 and LO28 at the exponential phase with N, N'-Dicyclohexylcarbodiimide (DCCD), an F_0F_1-ATPase inhibitor, which resulted in significantly reducing survival rate of the bacteria under the lethally acidic condition [25,26].

To better deal with acid stress, *L. monocytogenes* might employ several systems simultaneously. Moreover, the AgDI and GAD systems encode multiple isoenzymes, as two AgDI *(aguA1* and *aguA2)* and three GADs *(gadD1*, *gadD2* and *gadD3*) genes are encoded in most of *L. monocytogenes* [14,27]. The roles of these isoenzymes might make different contributions to the acid resistance of *L. monocytogenes*. As our previous study showed that *aguA1* and *aguA2* were both significantly up-regulated in response to acid conditions, only AguA1 but not AguA2 contributed to survival and growth under acidic environments and was involved in the pathogenicity of *L. monocytogenes* 10403S, in which Glycine 157 determined the activity of AguA1 and AguA2 [11]. In this study we found that the three GADs in *L. monocytogenes* 10403S also made different contribution (GadD2 > GadD3 > GadD1) to the survival of bacteria in acid stress. We further demonstrated that the function of the three GADs were determined by the expression level instead of their enzyme activity, which were different from AugA1 and AguA2 [11]. As our results showed that *L. monocytogenes* EGDe with little GadD1 and GadD2 was quite sensitive to acid stress, while overexpressed GadD1, GadD2 or GadD3 in EGDe with the *pHelp* promoter of plasmid pIMK2 significantly improved its survival rate in pH 2.5 BHI broth *(Fig. 3, 4, 6)*. Previous studies showed the difference of GAD system in *L. monocytogenes* and divided them into two groups, the outside GAD system (GAD_o, including GadD1/GadT1 and GadT2/GadD2) and the inside GAD system (GAD_i, GadD3) [23], and GAD_o played the major role in LO28 and 10403S, while acid resistance of EGDe was dependent on GAD_i [22,28]. These studies found the different roles of GAD system in various strains, but did not clarified the determinants that involved in the contribution in acid resistance of the GAD components. In this study, we demonstrated that the contribution of the three GADs was determined by their expression level for the first time.

The pH values and substrates that could be used by the bacterium to combat with low pH were various in different conditions [29], which might promote the bacterium to choose suitable acid resistance systems to cope with specific acidic environment. Whether these acid resistance systems perform the same in different conditions need further investigate. For instance, acid resistance in the phagosome might be complicated. On one hand, the bacteria need to initiate acid resistance system to cope with the acidic phagosome, on the other hand, the activation of LLO, which mediate *L. monocytogenes* escape from phagosome, need acidic compartment [30]. But food products contain glutamates that tend to benefit for the GAD system to deploy acid resistance [8]. Moreover, the molecular mechanisms involved in the different expression remained unclear. Kazmierczak et al.[31] found that *gadD3* was positivly regulated by SigB, and Cotter et al.[32] also found that *gadD1*/*gadT1* partially regulated by SigB. Bowman et al.[33] found that *gadT2*/*gadD2* operon was constitutively expression in *L. monocytogenes* LO28 by proteomic analysis. We found that the expression of *gadT2*/*gadD2* in the four representative strains were not in response to the acidic treatment, but the sequence of this operon (including the promoter region) was quite conserved between 10403S and EGDe or between Lm850658 and M7 (data not shown). It is suggested that different expression of *gadT2*/*gadD2* in these strains might be regulated in an undiscovered manner. Taken together, we demonstrated that the different contribution to acid resistance of GAD components were determined by the expression levels. The mechanisms that mediate the expression difference of GAD system need further investigation.

Statement of Author Contributions

CF and XWF designed and conducted experiments, analyzed data, and they were contributed equally to the work. CF, XWF, XYC, XYL, CW, YFG, WHF and YYY were involved in study design and data collection. YYY supervised the study and critically read the manuscript.

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Compliance With Ethical Standards

Disclosure of potential conflicts of interest: All authors declare no conflict of interest.

Research involving Human Participants and/or Animals: This article does not contain any studies with human participants. Rabbits used for antibodies preparation were approved by the Laboratory Animal Management Committee of Yangtze University (Approval No. 20161212).

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