

Production of Bacterial Ghosts from Gram-Positive Pathogen *Listeria monocytogenes*

Xueyou Wu,¹ Xingrong Ju,^{1,2} Lihui Du,² Jian Yuan,² Lifeng Wang,² Rong He,² and Zhengxing Chen¹

Abstract

Introduction: The Gram-positive bacterium *Listeria monocytogenes* is a ubiquitous intracellular pathogen, which has been implicated within the past decade as the causative organism in several outbreaks of foodborne disease. Bacterial ghosts (BGs) are nonliving and vacant cell envelopes of bacteria generated by releasing the bacterial cytoplasm through a channel in the cell envelope. This study attempted to produce Gram-positive pathogenic *L. monocytogenes* ghosts (LMGs) with simple chemicals.

Materials and Methods: The generation of LMGs was based on minimum inhibition concentrations of 1.10 mg/mL NaOH, 0.0675 mg/mL SDS, and 0.035% (v/v) H₂O₂. The potential efficacy of LMGs as vaccines and their ability to induce protective immune responses against virulent *L. monocytogenes* challenge through multipoint subcutaneous injection were also evaluated.

Results: The detected activity and viability of LMGs showed that nonliving LMGs could be induced. The detected LMG DNA and protein, as well as its morphological features, indicated that the produced LMGs were empty cells with the correct morphological structure. The subcutaneous vaccination of LMGs through multipoint injection conferred effective protection, with an antibody titer that reached 10⁴ U.

Conclusions: These findings strongly suggested that this chemical method can be used to produce LMGs and could be useful in future vaccine development against this foodborne pathogen.

Keywords: *Listeria monocytogenes*, bacterial ghost, Gram-positive bacterium, protective immunity

Introduction

THE GRAM-POSITIVE BACTERIUM *Listeria monocytogenes* is a ubiquitous intracellular pathogen, which has been implicated within the past decade as the causative organism in several outbreaks of foodborne disease (Gray and Killinger, 1966; Farber and Peterkin, 1991; Gandhi and Chikindas, 2007). However, managing its infection remains a challenge (Farber and Peterkin, 1991; Portnoy *et al.*, 1992; Pamer, 2004; Alvarez *et al.*, 2015; Lebreton *et al.*, 2015). Thus, an efficient vaccine against this pathogen may play an important role in the prevention of listeriosis.

Bacterial ghosts (BGs) are nonliving empty cell envelopes of bacteria generated by releasing the bacterial cytoplasm through a channel in the cell envelope, which was previously created through the controlled production of the bacteriophage phi X174 lysis protein E (Witte *et al.*, 1992; Mader *et al.*, 1997; Eko *et al.*, 1999). This method was recognized as the potential technology to develop efficient vaccines against foodborne pathogens (Eko *et al.*, 1999; Lubitz *et al.*, 1999; Ebensen *et al.*, 2004; Wang and Lu, 2009).

The superior conservation of antigenic determinants on the surface of genetically inactivated BGs makes them attractive immunogenic inactivated vaccine candidates (Ebensen *et al.*, 2004). Meanwhile, ghosts possess all the immunostimulatory surface properties of the original host strain, but none of the infectious threats associated with live vaccines (Eko *et al.*, 1999; Lubitz *et al.*, 1999). Unwanted leaky expression of gene E in the absence of appropriate induction temperature reduced biomass production of the host bacterium, consequently leading to a lower yield of BG (Lubitz *et al.*, 1999). The application of BGs as vaccines is limited because of their low lysis efficiency and production, as well as the presence of pathogenic islands and/or antibiotic resistance genes within ghost preparations (Paukner *et al.*, 2005; Zhang and Zhaoshan, 2006).

BGs received increasing interest in recent years for their promising medicinal and pharmaceutical applications. Most of the previously reported BGs were Gram-negative bacteria (Jechlinger *et al.*, 1999; Panthel *et al.*, 2003; Mayr *et al.*, 2005; Peng *et al.*, 2011; Chen *et al.*, 2014). However, more Gram-positive foodborne pathogenic bacteria exist, but we cannot

¹School of Food Science and Technology, Jiangnan University, Wuxi, China.

²College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, China.

produce their BGs because no E lysis gene has been detected in Gram-positive bacteria.

BG preparation was recently established by methods other than the E lysis gene. The protocols were able to convert viable cells to BGs, mainly on the basis of critical concentrations of chemical compounds (Amara *et al.*, 2013b; Amro *et al.*, 2014; Vinod *et al.*, 2014). BGs retain all morphological, structural, and antigenic features of the cell wall and can be used as a vaccine candidate. Besides, they can be exploited as a delivery system for proteins, which are either expressed or anchored to the envelopes before lysis or subsequently loaded (Haslberger *et al.*, 2000). To prevent *Listeria* infection, finding a new method, which can be successful, used to generate LMGs is very important.

In the present study, the Gram-positive pathogenic *L. monocytogenes* ghosts (LMGs) were produced by treatment with simple chemicals. The potential efficacy of LMGs as vaccines and their ability to induce protective immune responses against virulent *L. monocytogenes* challenge in rats were also evaluated.

Materials and Methods

Bacterial strain and culture condition

L. monocytogenes CMCC54004 was provided by Jiangsu Provincial Center for Disease Control and Prevention in the People's Republic of China. *L. monocytogenes* was propagated in brain heart infusion (BHI) medium (Land Bridge Co. Ltd., Beijing, China) under aerobic conditions at 37°C on a shaking incubator at 200 rpm. The growth of *L. monocytogenes* was spectrophotometrically measured with optical density at 600 nm (UV-vis spectrophotometer, U-3900; Hitachi, Japan).

Detection of minimum inhibition concentration

Standard experiments to determine the minimum inhibition concentration (MIC) for NaOH, SDS, and H₂O₂ were conducted (Andrews, 2001). Briefly, agar dilution plates, which contain different concentrations of NaOH, SDS, or H₂O₂, were prepared. *L. monocytogenes* was coated on these plates under aerobic conditions at 37°C, then detection of MIC of these chemicals above. The final concentration of the CaCO₃ solution used to prepare LMGs was 0.35 µg/mL as described by Amara *et al.* (2013b).

Production of LMGs

The production of LMGs was performed as previously described (Amara *et al.*, 2013a, 2013b) with some modifications. The production of LMGs is described in Figure 1. In brief, the biomass of cultivated *L. monocytogenes* was collected by centrifugation (5000 × g for 15 min) and washed

gently thrice with 0.5% sterile saline (NaCl solution). The collected *L. monocytogenes* cells were resuspended in 0.5% sterile saline to OD₆₀₀ of 0.6~0.8, which equals a cell concentration of about 10⁶ CFU/mL. A 8× stock solution was prepared for each NaOH, SDS, and H₂O₂, as determined from the MIC.

Step 1, 10 mL of 8× stock NaOH, CaCO₃, and SDS was taken and added into 50 mL of the bacterial suspension (10⁶ CFU/mL). This mixture was incubated at 37°C for 1 h on a shaking incubator at 100 rpm. After incubation, the mixture was centrifuged at 5000 × g for 20 min.

Step 2, the cell pellets were suspended in 50 mL of 0.5% sterile saline before the preprepared H₂O₂ stock solution (8×) was added, for a final concentration as determined from the MIC. This mixture was incubated at 37°C for 30 min on a shaking incubator at 100 rpm. After incubation, the mixture was centrifuged at 5000 × g for 20 min.

Step 3, the cells were suspended in 20 mL of 60% ethanol at room temperature for 30 min with gentle vortexing of 30 s each for 5 min. The mixture was centrifuged at 5000 × g for 20 min. LMGs were harvested by centrifugation (5000 × g for 20 min) and washed thrice with 0.5% sterile saline. The final pellet was resuspended in sterile phosphate-buffered saline (PBS) (137 mmol/L NaCl; 2.7 mmol/L KCl; 10 mmol/L Na₂HPO₄; 2 mmol/L KH₂PO₄, pH 7.4) and stored at 4°C until further use.

Viability assay

To determine the bacterial viability at each step in producing LMGs, 50 µL of the suspension was spread onto BHI agar plates and incubated for 5 d at 37°C. At the same time, the bacterial viability kit (Molecular Probes BacLight; Thermo Fisher Scientific) was used to determine the live and dead cells during the production of LMGs according to the manufacturer's instructions and other reports (Gasol *et al.*, 1999; Stocks, 2004) by mixing the suspension with the probes and visualizing under a microscope.

DNA and protein concentration and genomic DNA extraction

To analyze the release of DNA and proteins from inside the cells in each step, the DNA concentration was determined by measuring the absorption at 260 nm (UV-vis spectrophotometer, U-3900; Hitachi, Japan). The protein concentration was determined by measuring the absorption at 280 nm (UV-vis spectrophotometer, U-3900; Hitachi, Japan).

The genomic DNA of LMGs was extracted using an EZ-10 spin column bacterial genomic DNA isolation kit (Sangon Biotech, China) according to the manufacturer's protocol. The existence of genomic DNA of LMGs was examined by

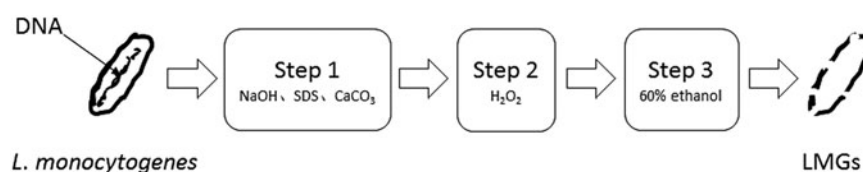


FIG. 1. schematic illustration of LMGs generation. Live *Listeria monocytogenes* cells were changed into dead LMGs after treatment of three steps. LMG, *L. monocytogenes* ghost.

1% agarose gel electrophoresis. The gel was detected by ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc.) and photographed.

Morphological features

The morphological features of LMGs were examined by scanning electron microscopy (SEM). LMG cells were deposited on cover glass fixed on carrier by conductive double-faced adhesive tape and sputter coated with gold. The SEM observation was performed with a field emission scanning electron microscope (S-4800; Hitachi, Japan) at 10 kV.

The internal structural features of LMGs were examined by transmission electron microscopy (TEM). After fixation, dehydration, and sectioning, the LMG cells were observed under a transmission electron microscope at 80 kV, according to the method of Cheng *et al.* (2014).

Experimental animals

The 6-week-old female Balb/c rats, weighing ~18 g, were maintained at 25°C in a 12-h light/12-h dark cycle with access to standard pellet diet and water. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at Beijing Kwinbon Biological Technology Co. Ltd. (Beijing, China).

Immunization and challenge experiment

To assess the immunogenicity and protective efficacy of LMGs, a group of 10 rats were equally divided into Groups A and B ($n=5$ per group). The rats in group A were non-immunized controls that received sterilized PBS. The rats in group B were immunized with 2 mL of LMGs (1×10^8 cells/mL) in sterile PBS by multipoint subcutaneous injection. The rats in groups A and B were immunized three times at 2-week intervals.

To determine the immune response, blood samples were obtained from the tail veins of individual rats at 2-week intervals during immunization and determined by an ELISA kit (Beijing Kwinbon Biological Technology Co. Ltd., Beijing, China) according to the protocol. Two weeks after the last immunization, all the rats were intravenously challenged with 2 mL of virulent *L. monocytogenes* (1×10^8 CFU/mL) in sterile PBS. The viability of the rats in groups A and B was recorded after the challenge experiment.

Statistical analyses

Statistical analyses were performed with one-way ANOVA using SPSS software (version 20.0). Data are expressed as mean \pm standard error of the mean. Differences were considered statistically significant if $p < 0.05$.

Results

MIC of chemicals

The determination of results showed that the MIC of NaOH, SDS, and H_2O_2 for *L. monocytogenes* was 1.10, 0.0675 mg/mL, and 0.035% (v/v), respectively.

Viability

In generation of LMGs, the live *L. monocytogenes* cells will be changed into dead LMGs. The viability of *L. mono-*

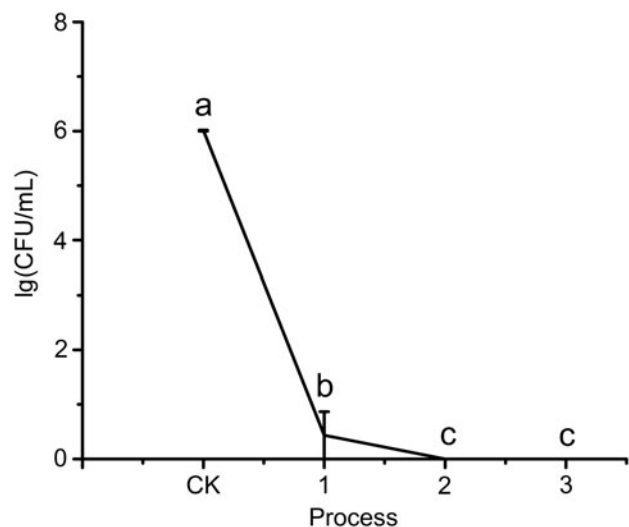


FIG. 2. Viability of LMG cells at different steps. The cells were spread onto BHI agar plates and incubated for 5 d at 37°C. The X-axis indicates the steps of LMG production. Data are presented as the mean \pm SE of three independent experiments. Different letters in the same column indicate a statistical difference between the mean values ($p < 0.05$). BHI, brain heart infusion; LMG, *L. monocytogenes* ghost; SE, standard error.

cytogenes cells/LMGs changed from 1×10^6 CFU/mL in the first processing step to 0 CFU/mL in the last processing step ($p < 0.05$) in Figure 2 (numerical variation of the logarithm). Nonliving LMGs were detected in the third-step process. In the present study, the aforementioned method showed 100% lysis efficiency.

Figure 2 shows the determination of living or dead *L. monocytogenes* cells during LMG production. After step-by-step treatment, the number of live *L. monocytogenes* cells was gradually reduced; ultimately, no live *L. monocytogenes* cells remained. These results were also confirmed by using the bacterial viability kit, as there were all red cells left after

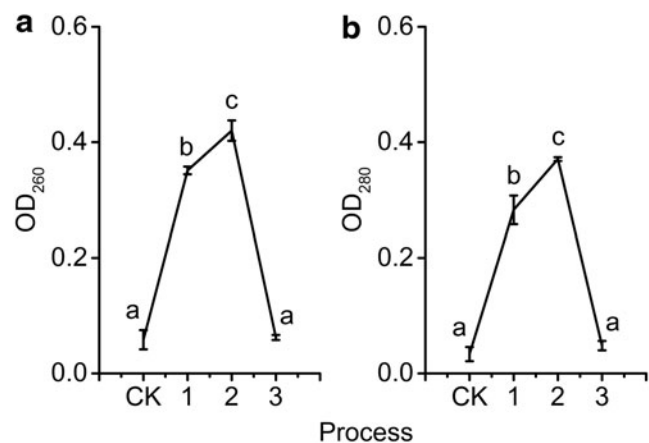


FIG. 3. Release of LMG DNA (a) and protein (b) from cells during LMG generation. The X-axis indicates the production steps. Data are presented as mean \pm SE of three independent experiments. Different letters in the same column indicate a statistical difference between the mean values ($p < 0.05$). LMG, *L. monocytogenes* ghost; SE, standard error.

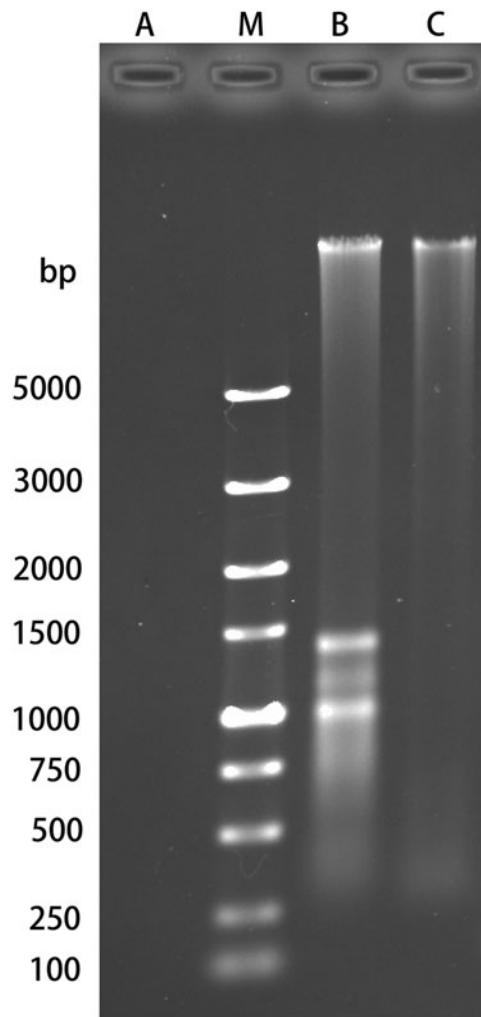


FIG. 4. Presence of genomic DNA in LMGs by agarose gel electrophoresis. Lane A, negative control (without DNA); Lane M, DNA standard; Lane B, genomic DNA of LMGs; Lane C, genomic DNA of *L. monocytogenes*. LMG, *L. monocytogenes* ghost

the third-step treatment (data not shown). These data suggested that the MIC of NaOH, SDS, and H₂O₂ would be sufficient to produce inactivated bacteria from *L. monocytogenes*. Nonliving LMGs suggested its safe use in the immune experiment.

DNA and protein concentration and genomic DNA extraction

As shown in Figure 3a, the total DNA content was well comparable at the beginning with the untreated control and chemical-treated *L. monocytogenes* cells. During the LMG production, the DNA content increased and then declined ($p < 0.05$); the protein content followed a similar trend during the processing (Fig. 3b). The change in the DNA and protein concentration indicated the occurrence of cell lysis, which was monitored during the preparation of BGs.

The presence of genomic DNA from LMGs was examined by agarose gel electrophoresis, detected by the ChemiDoc XRS+ system, and photographed. Figure 4 shows the existence of genomic DNA of *L. monocytogenes* and LMGs. The genomic DNA of *L. monocytogenes* was much more than LMGs. The genomic DNA of *L. monocytogenes* was probably degraded during LMG production.

Morphological features

As shown in Figure 5, the LMGs had a morphological structure similar to that of *L. monocytogenes*. Therefore, LMGs may have all the immunostimulatory surface properties of the original host strain, *L. monocytogenes*. The internal structural features of LMs were examined by TEM. As shown in Figure 6, the LMGs are empty cell envelopes produced by releasing the bacterial cytoplasm through a channel in the cell envelope. The morphological features of LMGs indicate the successful production of LMGs.

Immunization and challenge experiment

In this study, the rats in group B produced a significant immune response ($p < 0.05$), with an antibody titer of serum up to 10^4 U (Fig. 7). The viability of rats from groups A and B

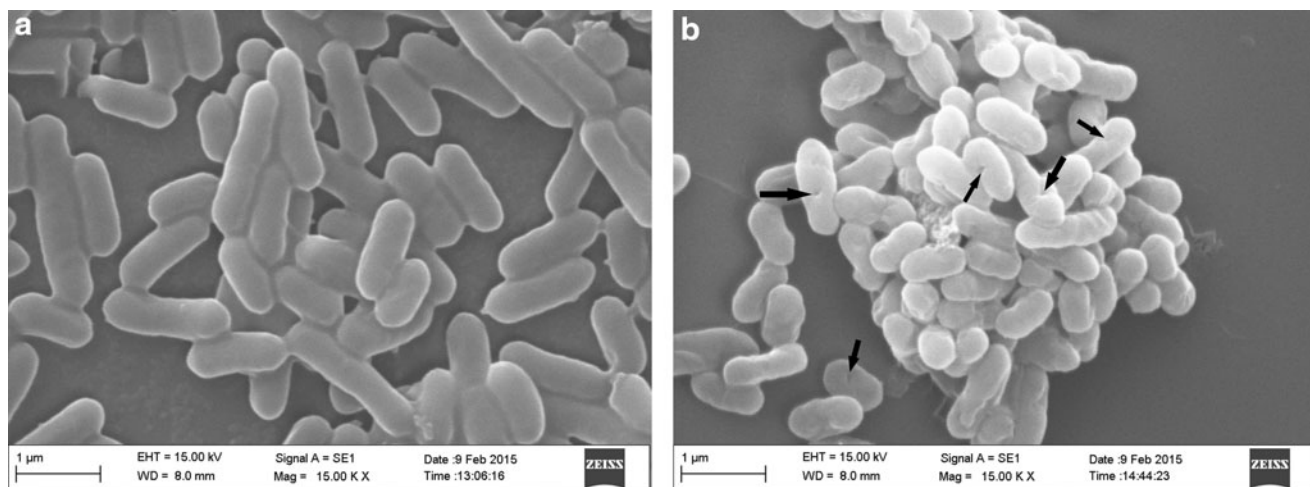


FIG. 5. Morphological features of (a) *L. monocytogenes* and (b) LMG cells by SEM. Arrows indicate transmembrane lysis tunnels. LMG, *L. monocytogenes* ghost; SEM, scanning electron microscopy.

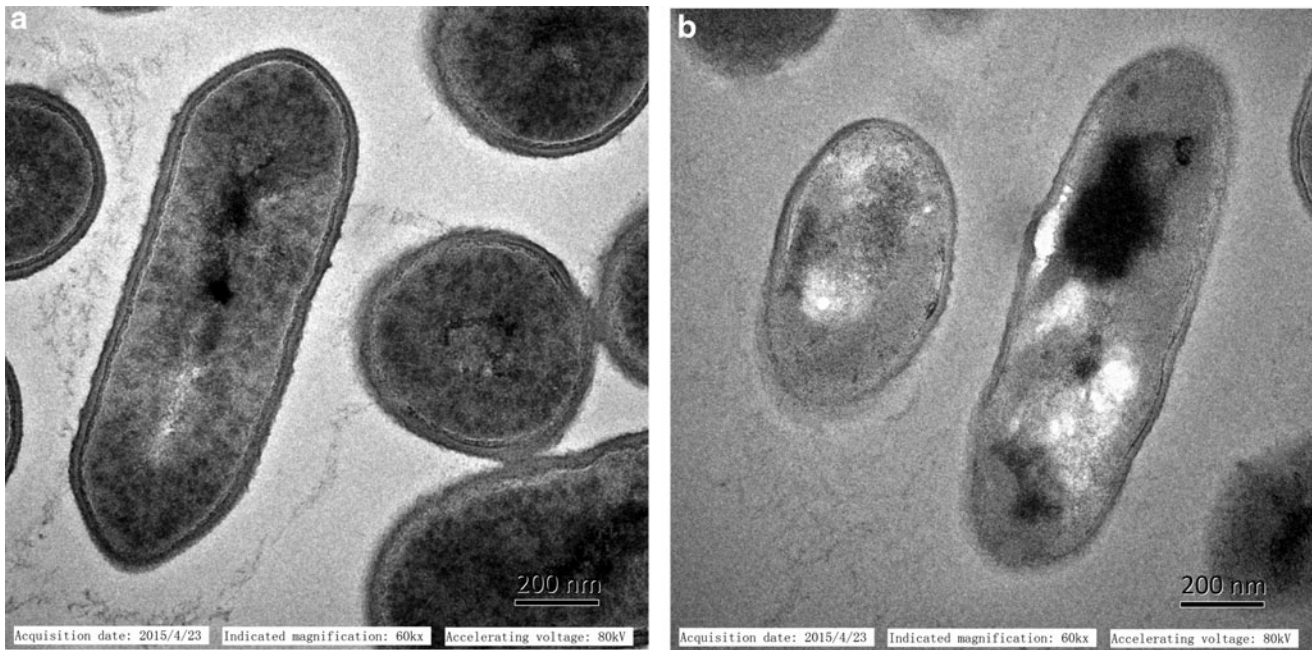


FIG. 6. Morphological features of (a) *L. monocytogenes* and (b) LMG cells by TEM. The LMG cells contain a minimal amount of cytoplasm. LMG, *L. monocytogenes* ghost; TEM, transmission electron microscopy.

is shown in Figure 8. All the rats in group A failed to pass the challenge experiment (0% survival), whereas 100% survival was achieved in group B. LMGs could be used to induce protective immune responses against virulent *L. monocytogenes* challenge in rats.

Discussion

In the past, BG was previously created through the controlled production of the bacteriophage phi X174 lysis protein

E (Witte *et al.*, 1992; Mader *et al.*, 1997; Eko *et al.*, 1999), which exists in Gram-negative bacteria. In the present study, the Gram-positive pathogenic LMGs were produced by a novel method without phi X174 lysis protein E. It may bring new opportunities to produce Gram-positive BGs.

The application of BGs as vaccines was limited because of their low lysis efficiency and production within ghost preparations (Paukner *et al.*, 2005; Zhang and Zhaoshan, 2006). The new method in this study has much higher lysis efficiency and production. The results in this study strongly suggest that this chemical method could be used to generate Gram-positive BGs. Recently, *Staphylococcus aureus* Ghost, a Gram-positive BG, was successfully generated by NaOH (Vinod *et al.*, 2015). It was similar with the method used in our study. The method in this study could be a common method for generation of Gram-negative and Gram-positive

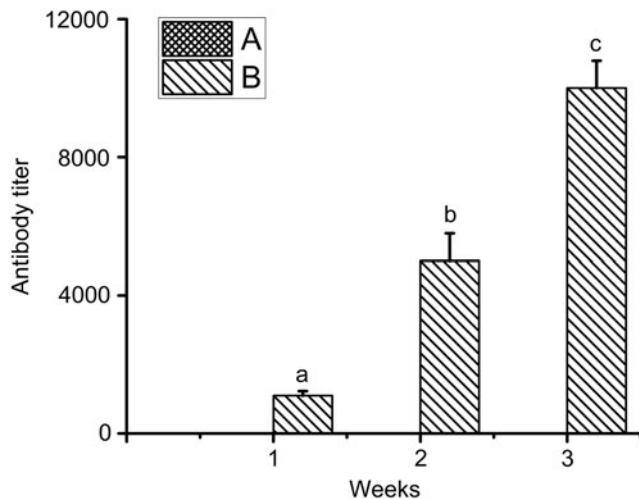


FIG. 7. Antibody titer of rats immunized by LMGs. Antibody titer was not detected in group A (black) rats immunized by PBS. Data are presented as mean \pm SE of three independent experiments. Different letters in the same column indicate statistical difference between the mean values ($p < 0.05$). LMG, *L. monocytogenes* ghost; PBS, phosphate-buffered saline.

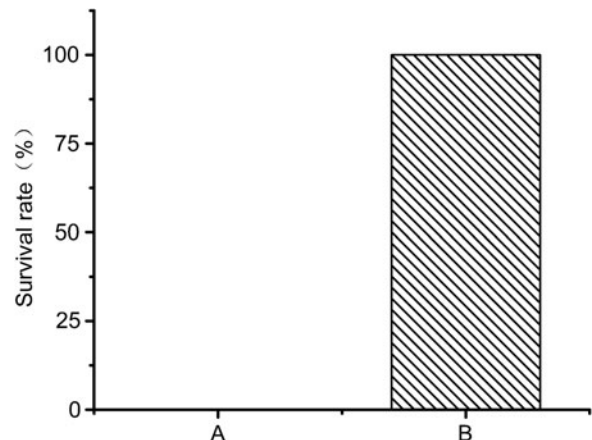


FIG. 8. Survival of rats in groups A and B of the challenge experiment. The survival rate of rats in group A is 0%.

BGs. A major problem for generation of BGs seems to be the choice of appropriate concentrations of these chemicals above. The easy manufacturing process and low production costs provide additional advantages for mass implementation of this method.

BGs possess all the immunostimulatory surface properties of the original host strain, but none of the infectious threats associated with live vaccines (Eko *et al.*, 1999; Lubitz *et al.*, 1999). In this study, LMGs were produced with the correct morphological features and intact antigens on the cell membrane, which are necessary of protective immune responses against virulent strains. The potential efficacy of LMGs as vaccines and their ability to induce protective immune responses against virulent *L. monocytogenes* challenge in rats were evaluated. LMGs could be used to induce a protective immune response against virulent *L. monocytogenes* challenge in rats.

It was reported that the existence of BG genomic DNA is a potential risk in the immune system (Ebensen *et al.*, 2004; Paukner *et al.*, 2005). In this study, we find there was a little genomic DNA in LMGs, and this LMGs generated can be used in rats immunization against virulent *L. monocytogenes*. In the present study, the Gram-positive pathogenic LMGs were produced by treatment with simple chemicals containing NaOH, CaCO₃, SDS, and H₂O₂. To precisely control the lysis of BGs, the time of each step process and no genomic DNA in BGs need further study in the follow-up research.

The BGs of *Escherichia coli* (Witte *et al.*, 1992), *Vibrio cholera* (Eko *et al.*, 2000), *Pasteurella multocida* (Marchart *et al.*, 2003a), *Pasteurella haemolytica* (Marchart *et al.*, 2003b), and *Helicobacter pylori* (Mayr *et al.*, 2005) have been successfully produced and can be used to induce protective immune responses against corresponding virulent bacteria. In the present study, the Gram-positive foodborne pathogen *L. monocytogenes* was added to the BG family.

Conclusion

The LMGs were produced with the correct morphological features and retained intact antigens on the cell membrane with simple chemical treatment. The LMGs produced by this method can be used to induce a protective immune response against virulent *L. monocytogenes* challenge in rats.

Acknowledgment

This project was financially supported by the National Natural Science Foundation of China (no. 31271930) and the Innovation of Graduate Culturing of Jiangsu Province (KYLX_1167).

Disclosure Statement

No competing financial interests exist.

References

Alvarez OA, Leong D, Hickey B, Beaufort A, Jordan K. The challenge of challenge testing to monitor *Listeria monocytogenes* growth on ready-to-eat foods in Europe by following the European Commission (2014) Technical Guidance document. *Food Res Int* 2015;75:233–243.

Amara AA, Salem-Bekhit MM, Alanazi FK. Preparation of bacterial ghosts for *E. coli* JM109 using sponge like reduced protocol. *Asian J Biol Sci* 2013a;6:363–369.

Amara AA, Salem-Bekhit MM, Alanazi FK. Sponge-like: A new protocol for preparing bacterial ghosts. *Scient World J* 2013b;2013:545741.

Amro AA, Salem-Bekhit MM, Alanazi FK. Plackett–Burman randomization method for bacterial ghosts preparation form *E. coli* JM109. *Saudi Pharm J* 2014;22:273–279.

Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001;48:5–16.

Chen J, Li N, She F. *Helicobacter pylori* outer inflammatory protein DNA vaccine-loaded bacterial ghost enhances immune protective efficacy in C57BL/6 mice. *Vaccine* 2014;32:6054–6060.

Cheng HY, Soo VWC, Islam S, McAnulty MJ, Benedik MJ, Wood T.K. Toxin GhoT of the GhoT/GhoS toxin/antitoxin system damages the cell membrane to reduce adenosine triphosphate and to reduce growth under stress. *Environ Microbiol* 2014;16:1741–1754.

Ebensen T, Paukner S, Link C, *et al.* Bacterial ghosts are an efficient delivery system for DNA vaccines. *J Immunol* 2004;172:6858–6865.

Eko FO, Mayr UB, Attridge SR, Lubitz W. Characterization and immunogenicity of *Vibrio cholerae* ghosts expressing toxin-coregulated pili. *J Biotechnol* 2000;83:115–123.

Eko FO, Witte A, Huter V, *et al.* New strategies for combination vaccines based on the extended recombinant bacterial ghost system. *Vaccine* 1999;17:1643–1649.

Farber JM, Peterkin PI. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* 1991;55:476–511.

Gandhi M, Chikindas ML. *Listeria*: A foodborne pathogen that knows how to survive. *Int J Food Microbiol* 2007;113:1–15.

Gasol JM, Zweifel UL, Peters F, Fuhrman JA, Hagström A. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. *Appl Environ Microbiol* 1999;65:4475–4483.

Gray ML, Killinger AH. *Listeria monocytogenes* and listeric infections. *Bacteriol Rev* 1966;30:309–382.

Haslberger AG, Kohl G, Felnerova D, Mayr UB, Furst-Ladani S, Lubitz W. Activation, stimulation and uptake of bacterial ghosts in antigen presenting cells. *J Biotechnol* 2000;83:57–66.

Jechlinger W, Szostak MP, Witte A, Lubitz W. Altered temperature induction sensitivity of the lambda p(R)/cI857 system for controlled gene E expression in *Escherichia coli*. *FEMS Microbiol Lett* 1999;173:347–352.

Lebreton A, Stavru F, Cossart P. Organelle targeting during bacterial infection: Insights from *Listeria*. *Trends Cell Biol* 2015;25:330–338.

Lubitz W, Witte A, Eko FO, *et al.* Extended recombinant bacterial ghost system. *J Biotechnol* 1999;73:261–273.

Mader HJ, Szostak MP, Hensel A, Lubitz W, Haslberger AG. Endotoxicity does not limit the use of bacterial ghosts as candidate vaccines. *Vaccine* 1997;15:195–202.

Marchart J, Dropmann G, Lechleitner S, *et al.* *Pasteurella multocida*- and *Pasteurella haemolytica*-ghosts: New vaccine candidates. *Vaccine* 2003a;21:3988–3997.

Marchart J, Rehagen M, Dropmann G, *et al.* Protective immunity against pasteurellosis in cattle, induced by *Pasteurella haemolytica* ghosts. *Vaccine* 2003b;21:1415–1422.

Mayr UB, Haller C, Haidinger W, *et al.* Bacterial ghosts as an oral vaccine: a single dose of *Escherichia coli* O157: H7 bacterial ghosts protects mice against lethal challenge. *Infect Immun* 2005;73:4810–4817.

- Pamer EG. Immune responses to *Listeria monocytogenes*. *Nat Rev Immunol* 2004;4:812–823.
- Panthel K, Jechlinger W, Matis A, *et al.* Generation of *Helicobacter pylori* ghosts by PhiX protein E-mediated inactivation and their evaluation as vaccine candidates. *Infect Immun* 2003;71:109–116.
- Paukner S, Kudela P, Kohl G, Schlapp T, Friedrichs S, Lubitz W. DNA-loaded bacterial ghosts efficiently mediate reporter gene transfer and expression in macrophages. *Mol Ther* 2005;11:215–223.
- Peng W, Si W, Yin L, *et al.* *Salmonella enteritidis* ghost vaccine induces effective protection against lethal challenge in specific-pathogen-free chicks. *Immunobiology* 2011;216:558–565.
- Portnoy DA, Chakraborty T, Goebel W, Cossart P. Molecular determinants of *listeria-monocytogenes* pathogenesis. *Infect Immun* 1992;60:1263–1267.
- Stocks SM. Mechanism and use of the commercially available viability stain, BacLight. *Cytometry A* 2004;61:189–195.
- Vinod N, Oh S, Kim S, Choi CW, Kim SC, Jung C-H. Chemically induced *Salmonella enteritidis* ghosts as a novel vaccine candidate against virulent challenge in a rat model. *Vaccine* 2014;32:3249–3255.
- Vinod N, Oh S, Park HJ, Koo JM, Choi CW, Kim SC. Generation of a novel *Staphylococcus aureus* ghost vaccine and examination of its immunogenicity against virulent challenge in rats. *Infect Immun* 2015;83:2957–2965.
- Wang X, Lu C. Mice orally vaccinated with *Edwardsiella tarda* ghosts are significantly protected against infection. *Vaccine* 2009;27:1571–1578.
- Witte A, Wanner G, Sulzner M, Lubitz W. Dynamics of PhiX174 protein E-mediated lysis of *Escherichia coli*. *Arch Microbiol* 1992;157:381–388.
- Zhang R, Zhaoshan Z. Advances on the novel drug deliver system: Bacterial ghost. *Progr Biochem Biophys* 2006;33:622–626.

Address correspondence to:

Zhengxing Chen, PhD

School of Food Science and Technology

Jiangnan University

Wuxi 214122

China

E-mail: zxchen_2008@126.com

Xingrong Ju, PhD

College of Food Science and Engineering

Nanjing University of Finance and Economics

Nanjing 210023

China

E-mail: xingrongju@163.com