Prevalence and Virulence Characterization of *Listeria monocytogenes* in Chilled Pork in Zhejiang Province, China

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Abstract

Listeria monocytogenes is an important foodborne pathogen that can grow in refrigeration temperature and causes severe human infections. The aims of this work were to estimate the prevalence of *L. monocytogenes* in chilled pork in Zhejiang, China and to examine the virulence features of the isolates. Of 331 meat samples, 196 were positive for *Listeria* spp., with *L. innocua* accounting for 54.4%, *L. monocytogenes* for 11.5%, and *L. welshimeri* for 4.2%. The most prevalent *L. monocytogenes* serotype was 1/2c (60.5%), followed by serotypes 1/2a (28.9%), 1/2b (7.9%), and 4b (2.6%). All *L. monocytogenes* isolates contained virulence-associated genes examined. Adhesion and invasion ability of serotype 1/2c isolates was much lower than those of other serotypes. Only one isolate was defective in cell-to-cell spread. These findings are important for risk assessment of chilled pork as a source of potential transmission of *L. monocytogenes* to other food products, particularly to ready-to-eat food products.

Introduction

L IS A FOODBORNE pathogen that can cause severe listeriosis in humans with a high fatality rate (about 30%) (Alberti-Segui *et al.,* 2007). Invasive listeriosis is more frequently associated with immune-compromised individuals, the elderly, pregnant women, and neonates. Listeriosis in humans is characterized by infections of the central nervous system (meningitis or meningoencephalitis), primary bacteremia, and septicemia (Vazquez-Boland *et al.,* 2001). *L. monocytogenes* strains contain 13 serotypes and can be divided into at least four lineages (I, II, III, and IV) based on phylogenetic method (Wiedmann *et al.,* 1997; Liu, 2013). Strains belonging to lineages I (mostly serotypes 4b and 1/2b) and II (mostly serotypes 1/2a and 1/2c) account for the majority of human listeriosis cases (Chen *et al.,* 2009c).

Human listeriosis is reported around the world every year, with foodborne cases taking the lead (Scallan *et al.,* 2011). Food implicated in human listeriosis cases includes vegetables, raw milk, meat, and ready-to-eat food products (Chen *et al.,* 2009c; Lambertz *et al.,* 2012). *L. monocytogenes* can contaminate food products and grow to infectious levels prior to consumption during processing, transportation, and storage (Miettinen and Wirtanen, 2006). To prolong the shelf life of food, temperature control from farms to tables is one of the best measures, especially for fresh vegetables and meat (Shalini and Singh, 2014). Carcass meat is chilled immediately after slaughter to an internal temperature of 0–4°C, and kept below 4°C during subsequent operations (Mackey *et al.,* 1980; Zhang *et al.,* 2011). Although there were several reports on prevalence of *L. monocytogenes* in raw meats (Inoue *et al.,* 2000; Lambertz *et al.,* 2012; Ristori *et al.,* 2014), no information was available with regard to *Listeria* contamination in chilled meat, nor were there any studies that examined the major serotypes and their pathogenic potentials of *L. monocytogenes* from chilled meat. Since *L. monocytogenes* can grow in refrigeration temperatures (Liu *et al.,*

Zhejiang province with 5 samplings about 2 months apart (10–12 samples each time). Samples were placed in sterile stomacher bags (Luqiao Co., Ltd., China) and stored in a styrofoam box containing ice packs. Sample analysis was initiated immediately upon arrival in the laboratory according to our previous method (Chen *et al.,* 2009a). Briefly, samples were enriched in Fraser enrichment broths (FB1 and FB2; Luqiao) and then inoculated onto *Listeria*-selective agar plates (PALCAM agar; Luqiao) to select colonies suspected as *Listeria* spp. Ten representative colonies were picked up and subcultured in brain heart infusion broth (Luqiao), and 1 mL of each culture was taken for DNA extraction and tested by multiplex polymerase chain reaction (PCR) (Chen *et al.,* 2009a).

Serotype identification and lineage classification

Serotype identification was conducted as previously described (Doumith *et al.,* 2004a; Chen *et al.,* 2010). Briefly a multiplex PCR primer pairs (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/fpd) targeting *lmo2819*, *lmo2110*, *lmo0737*, *lmo1118,* and *lmo1134* were used. Lineage classification was determined by phylogenetic analysis of nucleotide sequences of the *actA* gene fragments between 775 and 1313, which cover the proline-rich repeats (Wiedmann *et al.,* 1997; Chen *et al.,* 2010).

PCR typing of virulence-associated genes

Virulence-associated genes were screened according to our previous reports (Chen *et al.,* 2009b, 2010) by PCR typing (Supplementary Table S2) on six stress response genes (*danK*, *groEL*, *bsh*, *gadD1*, *gadD2*, and *gadD3*), three adhesion and invasion genes (*inlA*, *inlB*, and *inlJ*), six intracellular growth–associated genes (*plcA*, *hly*, *mpl*, *actA*, *plcB*, and *hpt*), and two regulatory genes (*prfA* and *sigB*).

Virulence characterization

Adhesion and invasion assays on Caco-2 cells were conducted to evaluate the infection potential of *L. monocytogenes* isolates according to a previous method (Burkholder and Bhunia, 2010). Adhesion index was expressed as the ratio of recovered colonies to the actual inoculum, while invasion index was calculated as the ratio of recovered colonies after gentamicin treatment to the actual inoculum. Plaque formation was performed on mouse fibroblast L929 cell monolayers in six-well plates (Corning, USA) according to the method previously described (Jiang *et al.,* 2006). Actin tail formation was conducted as previously described (Talman *et al.,* 2014). The bacterial cells were stained with polyclonal antibodies to *L. monocytogenes* and then probed with Alexa Fluor 488 conjugated donkey anti-rabbit antibody (Abcam, UK). F-actin was stained with phalloidin-Alexa Fluor 568 (Abcam). DAPI (Invitrogen, USA) was used to stain the cell nuclei. Actin tails were visualized by confocal microscope (Olympus FLV 1000, Japan). L929 and Caco-2 cells used in the above experiments were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2. Three independent experiments were performed on each strain (in triplicate wells per strain) for the adhesion and invasion assay.

Statistical analysis

The confidence intervals (CIs) for prevalence of *Listeria* spp. in chilled pork were calculated as previously reported (Ross, 2003). The adhesion and invasion indexes were presented as relative to the reference strain EGDe set as 100%, and data were analyzed using the two-tailed Student *t*-test. Differences were considered significant at a significance level $p < 0.05$. The statistical software SPSS (version 18.0) (International Business Machines Corporation, USA) was used.

Results

Of the 331 chilled pork samples examined, 196 were identified as *Listeria* spp. positive with a recovery rate of 59.2% (i.e., 196/331, 95% CI: 53.7%–64.6%) (Fig. 1A). Among the six *Listeria* species targeted for detection, only *L. monocytogenes*, *L. innocua*, and *L. welshimeri* were identified. *L. innocua* was the most prevalent species (180 samples positive) with 95% CI between 48.8% and 59.8%, followed by 38 positive samples for *L. monocytogenes* (95% CI: 8.3–15.4%) and 14 positive samples for *L. welshimeri* (95% CI: 2.3–7.0%). There were samples that contained more than one species of *Listeria*: 22 samples carried *L. monocytogenes* and

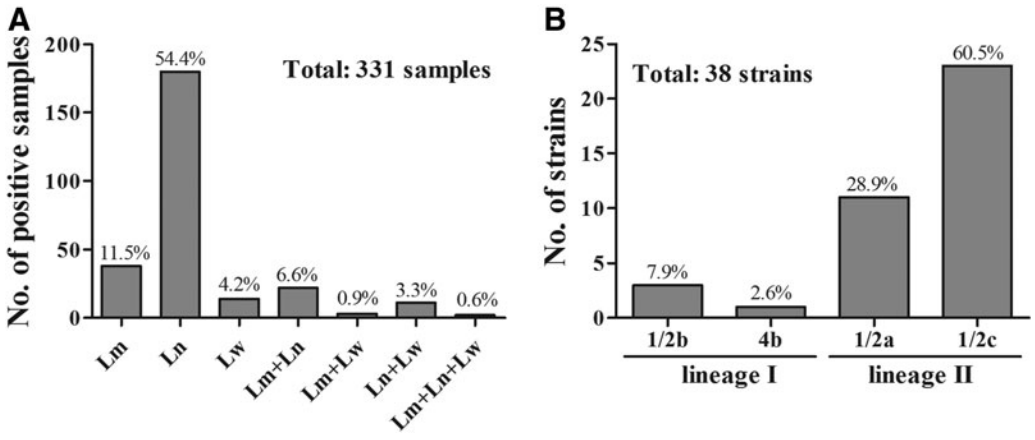

FIG. 1. Prevalence of *Listeria* species in chilled pork **(A)** and serotype distribution of *Listeria monocytogenes* isolates **(B)**. Lm, *Listeria monocytogenes*; Ln, *L. innocua*; Lw, *L. welshimeri*.

Table 1. Adhesion and Invasion Ability of *L* Isolates

Serovar	*Strain*	*Adhesion (% of EGDe)*	*Invasion (% of EGDe)*
1/2a	EGDe	100.0 ± 6.1	100.0 ± 7.1
1/2a	Lm345	147.5 ± 29.1	851.4 ± 590.3
	Lm354	163.9 ± 30.2*	481.8 ± 136.3*
	Lm364	328.0 ± 55.5*	987.1 ± 157.9*
	Lm374	482.1 ± 10.2*	402.8 ± 35.6*
	Lm376	138.6 ± 32.9	115.9 ± 30.3
	Lm396	242.2 ± 101.2	631.3 ± 378.4
	Lm402	8.3 ± 0.9*	5.7 ± 2.0*
	Lm405	10.8 ± 1.9*	3.5 ± 0.5*
	Lm413	299.9 ± 82.0*	377.8 ± 113.7*
	Lm427	158.9 ± 62.7	193.6 ± 26.4*
	Lm428	172.2 ± 38.7*	136.4 ± 15.1*
1/2c	Lm342	9.3 ± 1.7*	3.8 ± 3.8*
	Lm343	8.3 ± 5.6*	2.1 ± 1.9*
	Lm344	5.8 ± 2.9*	2.2 ± 1.8*
	Lm349	5.3 ± 2.3*	3.8 ± 2.3*
	Lm352	6.2 ± 3.7*	2.5 ± 0.9*
	Lm355	8.0 ± 4.9*	2.7 ± 0.8*
	Lm358	9.4 ± 0.4*	22.7 ± 3.3*
	Lm362	10.3 ± 0.4*	17.2 ± 1.8*
	Lm363	6.5 ± 0.5*	10.0 ± 1.4*
	Lm368	9.8 ± 0.1*	10.9 ± 3.1*
	Lm372	11.4 ± 0.2*	23.0 ± 2.4*
	Lm373	15.1 ± 0.9*	6.9 ± 1.9*
	Lm378	10.7 ± 1.3*	3.0 ± 0.9*
	Lm388	9.8 ± 2.7*	3.3 ± 1.0*
	Lm393	10.8 ± 3.6*	3.8 ± 2.3*
	Lm394	12.8 ± 2.7*	2.1 ± 0.9*
	Lm398	10.8 ± 2.4*	2.7 ± 1.2*
	Lm400	8.4 ± 5.1*	2.1 ± 1.7*
	Lm406	11.2 ± 1.5*	2.5 ± 0.6*
	Lm407	7.0 ± 1.8*	2.5 ± 0.9*
	Lm414	8.5 ± 0.6*	5.0 ± 1.1*
	Lm421	8.2 ± 2.9*	3.4 ± 0.6*
	Lm424	10.1 ± 2.9*	3.1 ± 1.9*
1/2b	Lm346	257.6 ± 66.8*	1307.2 ± 172.8*
	Lm415	250.9 ± 42.8*	574.7 ± 121.5*
	Lm418	167.2 ± 11.4*	145.0 ± 50.2
4b	Lm410	4574.7 ± 944.4*	3555.6 ± 427.3*

Comparing with strain EGDe, $p < 0.05$ is marked as *.

L. innocua, 3 samples harbored *L. monocytogenes* and *L. welshimeri*, and 11 samples possessed *L. innocua* and *L. welshimeri*. Two samples were contaminated with all three species (Fig. 1A).

By PCR serotyping of the 38 *L. monocytogenes* strains, we found that serotypes 1/2c and 1/2a accounted for 23 and 11, respectively (Fig. 1B). There were three strains of serotypes 1/2b and one strain of 4b. Phylogenetic analysis showed that the majority of the strains (89.4%, 34/38) belonged to lineage II with the rest (10.6%, 4/38) grouping into lineage I.

All virulence-associated genes, except *gadD3*, were present in all 38 *L. monocytogenes* strains. The gene *gadD3* encoding one of the glutamate decarboxylases was not present in three serotype 1/2c strains (Lm373, Lm394, and Lm406) (data not shown). The *actA* gene in Lm418 had a 105-bp deletion that encodes 35 amino acids in the proline-rich repeat region (data not shown).

To assess the pathogenicity of *L. monocytogenes* isolates from chilled pork, we analyzed their ability to adhere to and invade into epithelial Caco-2 cells and to spread from cell to cell. All serotype 1/2c strains showed much lower adhesion and invasion indexes than those of the reference strain EGDe ($p < 0.05$) (Table 1 and Supplementary Table S3). The serotype 1/2a strains seemed to be more infective than EGDe, as judged from median adhesion of about 1.6-fold and median invasion of about 3.8-fold higher than EGDe, although there were significant variations among strains. The only serotype 4b strain, Lm410, displayed the highest ability of adhesion and invasion, significantly higher than that of EGDe. Plaque assay showed that visible plaques were apparent with all tested isolates except one (Lm418) (Fig. 2A). Confocal imaging confirmed the defect of Lm418 in forming the actin tails in Caco-2 cells (Fig. 2B).

Discussion and Conclusions

L. monocytogenes is one of the most important foodborne pathogens because of the severity of its infections in humans. Its prevalence data in food could become part of the risk assessment and control strategy for public health policymakers and the food-processing industry. Food transported and stored at refrigeration temperature can largely reduce the risk of many foodborne diseases (Shalini and Singh, 2014).

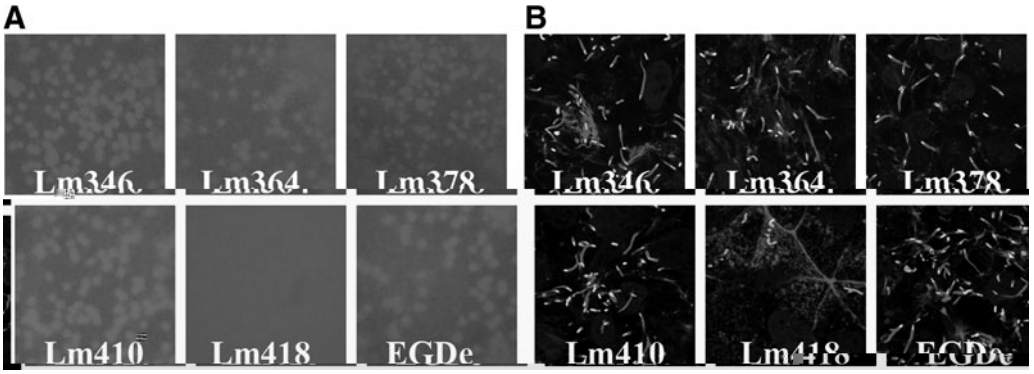

FIG. 2. Cell-to-cell spread of representative *Listeria monocytogenes* isolates showing the defect phenotype of the isolate Lm418. **A:** Plaque formation in fibroblast L929; **B:** Actin-tail formation in Caco-2 cells; *Listeria* were stained as green (shown in white), F-actin were stained as red (shown in light gray), and cell nuclei were stained as blue (shown in dark gray). The serotypes of strains Lm346, Lm364, Lm378, Lm410, Lm418, and EGDe were 1/2b, 1/2a, 1/2c, 4b, 1/2b, and 1/2a, respectively.

However, the risk of listeriosis remains because *L. monocytogenes* can survive and grow in refrigeration temperatures (Liu *et al.,* 2002).

Our results showed that the overall prevalence of *L. monocytogenes* in chilled pork was 11.5%, much higher than that found in raw meat from Yangzhou, China (4.83%) (Zhou and Jiao, 2006) and Sweden (1.2%) (Lambertz *et al.,* 2012), but lower than that found in meat products in Japan (25%) (Inoue *et al.,* 2000) and Brazil (48.7%) (Ristori *et al.,* 2014). The most frequently isolated species was *L. innocua* (54.4%), a finding similar to previous reports (Chen *et al.,* 2009c; Derra *et al.,* 2013). The majority of the *L. monocytogenes*–positive samples were also concomitantly contaminated with *L. innocua*. Therefore, *L. innocua* contamination could be a marker of poor hygiene during food processing and handling (Takahashi *et al.,* 2014).

Previous studies showed that different lineages of *L. monocytogenes* exhibited different pathogenic potential (Doumith *et al.,* 2004b; Zhou *et al.,* 2005). We observed that lineage II isolates exhibited much lower adhesion and invasion ability than those of lineage I strains, which is in agreement with previous reports (Jacquet *et al.,* 2004; Zhou *et al.,* 2005). Lineage II strains of low pathogenic potential were predominant in our study and elsewhere (Zhou and Jiao, 2006; De Cesare *et al.,* 2007; Chen *et al.,* 2009c; Lomonaco *et al.,* 2009; Hadjilouka *et al.,* 2014). It is tempting to speculate that humans might be more often exposed to less virulent strains, because the majority of human listeriosis cases are often associated with lineage I strains (mostly serotypes 4b and 1/2b) (Goulet *et al.,* 2006; Leite *et al.,* 2006; Orsi *et al.,* 2011). This might partially explain the discrepancy between the prevalence of *L. monocytogenes* in food products being high while the incidence of listeriosis is relatively low, especially in China. However, caution should be exercised with this argument since there were reports that lineage I strains accounted for over half of their isolates from cheese or blue crab and related environments (Leite *et al.,* 2006; Pagadala *et al.,* 2012).

The reason why the strain Lm418 failed to form visible plaque and actin tails requires further examination since this 35-amino-acids deletion could be found in many other *L. monocytogenes* strains and did not seem to affect cell-to-cell spread (Skoble *et al.,* 2001).

In summary, this study presented some information on prevalence and major serotypes of *L. monocytogenes* isolates from chilled pork in the coastal Chinese province of Zhejiang as well as on the virulence potentials of these strains relevant to their serotypes. Such data are useful for future risk reassessment of chilled pork products as part of the food chain.

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Disclosure Statement

No competing financial interests exist.

References

Alberti-Segui C, Goeden KR, Higgins DE. Differential function of *Listeria monocytogenes* listeriolysin O and phospholipases C in vacuolar dissolution following cell-to-cell spread. Cell Microbiol 2007;9:179–195.

Burkholder KM, Bhunia AK. *Listeria monocytogenes* uses *Listeria* adhesion protein (LAP) to promote bacterial transepithelial translocation and induces expression of LAP receptor Hsp60. Infect Immun 2010;78:5062–5073.

Chen J, Chen Q, Jiang J, Hu H, Ye J, Fang W. Serovar 4b complex predominates among *Listeria monocytogenes* isolates from imported aquatic products in China. Foodborne Pathog Dis 2010;7:31–41.

Chen J, Jiang L, Chen Q, *et al*. lmo0038 is involved in acid and heat stress responses and specific for *Listeria monocytogenes* lineages I and II, and *Listeria ivanovii*. Foodborne Pathog Dis 2009a;6:365–376.

Chen J, Luo X, Jiang L, *et al*. Molecular characteristics and virulence potential of *Listeria monocytogenes* isolates from Chinese food systems. Food Microbiol 2009b;26:103–111.

Chen J, Zhang X, Mei L, Jiang L, Fang W. Prevalence of *Listeria* in Chinese food products from 13 provinces between 2000 and 2007 and virulence characterization of *Listeria monocytogenes* isolates. Foodborne Pathog Dis 2009c;6:7–14.

De Cesare A, Mioni R, Manfreda G. Prevalence of *Listeria monocytogenes* in fresh and fermented Italian sausages and ribotyping of contaminating strains. Int J Food Microbiol 2007;120:124–130.

Derra FA, Karlsmose S, Monga DP, *et al*. Occurrence of *Listeria* spp. in retail meat and dairy products in the area of Addis Ababa, Ethiopia. Foodborne Pathog Dis 2013;10:577–579.

Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J Clin Microbiol 2004a;42:3819–3822.

Doumith M, Cazalet C, Simoes N, *et al*. New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. Infect Immun 2004b;72:1072–1083.

Goulet V, Jacquet C, Martin P, Vaillant V, Laurent E, de Valk H. Surveillance of human listeriosis in France, 2001–2003. Euro Surveill 2006;11:79–81.

Hadjilouka A, Andritsos ND, Paramithiotis S, Mataragas M, Drosinos EH. *Listeria monocytogenes* serotype prevalence and biodiversity in diverse food products. J Food Prot 2014;77:2115–2120.

Inoue S, Nakama A, Arai Y, *et al*. Prevalence and contamination levels of *Listeria monocytogenes* in retail foods in Japan. Int J Food Microbiol 2000;59:73–77.

Jacquet C, Doumith M, Gordon JI, Martin PM, Cossart P, Lecuit M. A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. J Infect Dis 2004;189:2094–2100.

Jiang LL, Xu JJ, Chen N, Shuai JB, Fang WH. Virulence phenotyping and molecular characterization of a low-pathogenicity isolate of *Listeria monocytogenes* from cow's milk. Acta Biochim Biophys Sin (Shanghai) 2006;38:262–270.

Lambertz ST, Nilsson C, Bradenmark A, *et al*. Prevalence and level of *Listeria monocytogenes* in ready-to-eat foods in Sweden 2010. Int J Food Microbiol 2012;160:24–31.

Leite P, Rodrigues R, Ferreira M, *et al*. Comparative characterization of *Listeria monocytogenes* isolated from Portuguese farmhouse ewe's cheese and from humans. Int J Food Microbiol 2006;106:111–121.

Liu D. Molecular approaches to the identification of pathogenic and nonpathogenic Listeriae. Microbiol Insights 2013;6:59–69.

Liu S, Graham JE, Bigelow L, Morse PD 2nd, Wilkinson BJ. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. Appl Environ Microbiol 2002;68:1697–1705.

Lomonaco S, Decastelli L, Nucera D, Gallina S, Manila Bianchi D, Civera T. *Listeria monocytogenes* in Gorgonzola: Subtypes, diversity and persistence over time. Int J Food Microbiol 2009;128:516–520.

Mackey BM, Roberts TA, Mansfield J, Farkas G. Growth of *Salmonella* on chilled meat. J Hygiene 1980;85:115–124.

Miettinen H, Wirtanen G. Ecology of *Listeria* spp. in a fish farm and molecular typing of *Listeria monocytogenes* from fish farming and processing companies. Int J Food Microbiol 2006;112:138–146.

Orsi RH, den Bakker HC, Wiedmann M. *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int J Med Microbiol 2011;301:79–96.

Pagadala S, Parveen S, Rippen T, *et al*. Prevalence, characterization and sources of *Listeria monocytogenes* in blue crab (*Callinectus sapidus*) meat and blue crab processing plants. Food Microbiol 2012;31:263–270.

Ristori CA, Rowlands RE, Martins CG, Barbosa ML, Yoshida JT, de Melo Franco BD. Prevalence and populations of *Listeria monocytogenes* in meat products retailed in Sao Paulo, Brazil. Foodborne Pathog Dis 2014;11:969–973.

Ross TD. Accurate confidence intervals for binomial proportion and Poisson rate estimation. Computers Biol Med 2003;33: 509–531.

Scallan E, Hoekstra RM, Angulo FJ, *et al*. Foodborne illness acquired in the United States–Major pathogens. Emerg Infect Dis 2011;17:7–15.

Shalini R, Singh S. Effect of hurdle technology in food preservation: A review. Crit Rev Food Sci Nutr 2014 Sep. 15. [Epub ahead of print]

Skoble J, Auerbuch V, Goley ED, Welch MD, Portnoy DA. Pivotal role of VASP in Arp2/3 complex-mediated actin nucleation, actin branch-formation, and *Listeria monocytogenes* motility. J Cell Biol 2001;155:89–100.

Takahashi H, Ohshima C, Nakagawa M, *et al*. Development of new multilocus variable number of tandem repeat analysis (MLVA) for *Listeria innocua* and its application in a food processing plant. PLoS One 2014;9:e105803.

Talman AM, Chong R, Chia J, Svitkina T, Agaisse H. Actin network disassembly powers dissemination of *Listeria monocytogenes*. J Cell Sci 2014;127:240–249.

Vazquez-Boland JA, Dominguez-Bernal G, Gonzalez-Zorn B, Kreft J, Goebel W. Pathogenicity islands and virulence evolution in *Listeria*. Microbes Infect 2001;3:571–584.

Wiedmann M, Bruce JL, Keating C, Johnson AE, McDonough PL, Batt CA. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect Immun 1997;65:2707–2716.

Zhang P, Liu Y, Fang C, Yu Y, Chen J, Fang W. Comparison of growth models of Listeria monocytogenes in chilled pork. Wei Sheng Wu Xue Bao 2011;51:1625–1631. [in Chinese]

Zhou X, Jiao X. Prevalence and lineages of *Listeria monocytogenes* in Chinese food products. Lett Appl Microbiol 2006;43:554–559.

Zhou X, Jiao X, Wiedmann M. *Listeria monocytogenes* in the Chinese food system: Strain characterization through partial actA sequencing and tissue-culture pathogenicity assays. J Med Microbiol 2005;54:217–224.

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