



Prevalence and characterization of *Listeria monocytogenes* isolated from online market-purchased enoki mushrooms (*Flammulina velutipes*) in the Republic of Korea

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ABSTRACT

The aim of this study was to characterize the microbiological quality of enoki mushrooms (*Flammulina velutipes*) commercially sold via online market retailers in the Republic of Korea and to identify the antibiotic susceptibility and genetic variability of *Listeria monocytogenes* isolates. A total of 100 enoki mushrooms were purchased online from 20 different sellers. As a result, 86–100% of the samples had a mean load of total aerobic bacteria (TAB), coliform, and yeast/mold (Y/M) which reached 5.32 ± 1.52 , 3.89 ± 1.85 , and 4.23 ± 1.14 log CFU/g, respectively. Seven samples positive for *L. monocytogenes* were detected among 100 online market-purchased enoki mushrooms, followed by the enrichment step of the isolates. Among the nine *L. monocytogenes* isolates, the highest proportion (67%; 6/9) was serogrouped as $\frac{1}{2}$ a-3a. It also was found that six isolates (i.e. BMI₅₋₁, BMI₅₋₃, BMI₅₋₄, BMI₅₋₅, BMI_{B5-1}, and BMI_{B5-3}) were capable of amplifying *prfA*, *actA*, *hlyA*, *inlA*, and *inlC*. An antibiotic susceptibility test using the disc diffusion method revealed that all the *L. monocytogenes* isolates were susceptible to antibiotics (amoxycillin-clavulanic acid, gentamicin, and kanamycin) commonly used for the resolution of listeriosis infections, while being resistant to aztreonam and nalidixic acid.

1. Introduction

Listeria monocytogenes, a member of the genus *Listeria*, is a facultative, gram-positive, and nonspore-forming bacterium. The most common symptoms associated with listeriosis outbreaks range from a mild gastroenteritis (diarrhea and fever) to severe clinical syndromes, including encephalitis, meningitis, pneumonia, and septicemia (Zhu, Gooneratne, & Hussain, 2017). In the case of highly susceptible groups of the population, newborns, infants and toddlers (less than 5 years), the elderly (more than 65 years), and individuals with immunosuppressive conditions are at the greatest risk of becoming sick from listeriosis infections with a high case fatality rate of up to 20–30% (Khan et al., 2016).

In the past two decades, foods of animal sources, such as meat and milk, contaminated with *L. monocytogenes* have been implicated in the majority of human listeriosis cases (Ravindhiran, Sivarajan, Sekar, Murugesan, & Dhandapani, 2023). However, the frequency of fresh

produce-associated listeriosis outbreaks has been increasing since 2008 (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017; Tan et al., 2019). Globally, apple, cantaloupe, celery, corn, melon, lettuce, and seed sprouts have emerged as the predominant vehicles of the *L. monocytogenes* contamination as reflected by several food-borne outbreaks and recalls over the last 10 years (Garner & Kathariou, 2016; Marik, Zuchel, Schaffner, & Strawn, 2020; Zhu et al., 2017). Recently, the onset of two multistate outbreaks associated with the consumption of enoki mushrooms (*Flammulina velutipes*) contaminated with *L. monocytogenes* in the USA was attributed to extensive recall events and enormous economic losses, thereby resulting in 36 hospitalizations and four deaths (CDC, 2020; CDC, 2023).

Edible mushrooms have some health-associated benefits for consumers because these products are calorie-poor (with only approximately 30 kcal per 100 g of mushrooms), but are a good source of essential amino acids, vitamins (e.g. B₂, B₃, B₉, C, D and B₁₂), and other bioactive compounds (e.g. ergosterols, lectins, phenolics,

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polysaccharides, and terpenoids), particularly having biological effects, such as anticancer, antidiabetic, hepatoprotective, hypocholesterolemic, and immune-potentiating influences, for human diets (El-Ramady et al., 2022; Grimm, Kuenz, & Rahmann, 2021). However, since mushrooms are one of the most perishable food materials with a limited product shelf life of 1–3 d at an ambient temperature (Zhang, Pu, & Sun, 2018), a high moisture content, a water activity (0.98 or higher), and a neutral pH close to 6.1–6.8 make these products ideal for supporting microbial growth and proliferation (Martinez-Carrera et al., 1998). Indeed, previous studies (Johannessen, Loncarevic, & Kruse, 2002; Samadpour et al., 2006) have demonstrated the emergence of enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella* sp., *L. monocytogenes*, and *Staphylococcus aureus* strains recovered from edible mushrooms, posing a difficult challenge to food industry and public health. Chen et al. (2018) reported that *L. monocytogenes* predominated in *Fl. velutipes* samples (55.5%; 116/209) collected from rural Chinese markets, 2012–2016, followed by *Volvaria volvacea* (11.8%), *Hypsizygus marmoreus* (10.1%), *Pleurotus geesteranus* (8.3%), *Pleurotus ostreatus* (6.7%), *Pleurotus eryngii* (4.4%), and shiitake mushrooms (2.9%). Chen, Wu, Zhang, Wu, and Guo (2015) determined that the contamination level of *L. monocytogenes* was 31.5% (34/108) in edible mushroom-associated samples collected from 16 cities located in South China, 2011–2012, and 29 (76.3%) enoki mushroom samples were positive for this bacterium with the most probable number (MPN) exceeding 100 MPN/g. The proportion of enoki mushrooms which were found to be contaminated with *L. monocytogenes* also was high (55.6%; 45/81), and 26.7% (12/45) of the *L. monocytogenes*-positive samples had a *L. monocytogenes* density that exceeded 100 MPN/g (Wu et al., 2015). Among edible mushrooms screened, *Tuber indicum*, *Calocybe gambosa*, and *Hygrophorus limacinus* were the most frequently contaminated with *L. monocytogenes* with a high prevalence that reached 100% (6/6), 40% (8/20), and 40% (4/10), respectively (Venturini, Reyes, Rivera, Oria, & Blanco, 2011). Additionally, earlier studies (Chen et al., 2014; Murgesan, Kucerova, Knable, & Laborde, 2015) demonstrated the colonization of persistent *L. monocytogenes* clones in mushroom production and processing facilities at critical sites pertaining to food-contacting equipments, floors, floor crevices, and others. *L. monocytogenes* may enter food production and/or processing facilities through a plethora of routes that remain uncharacterized (Garner & Kathariou, 2016) so that the potential of edible mushrooms to carry food-borne pathogenic bacteria derived from a variety of different sources is high as long as these products are normally hand-harvested without being washed and commercialized in many cases at ambient temperatures (Venturini et al., 2011).

The dangerous nature of *L. monocytogenes* can be magnified by its ability to survive in adverse environments (e.g. over a wide range of pH values of 4.1–9.4 and in the presence of up to 13.9% NaCl), multiply in foods stored at refrigerator temperatures as low as – 0.4, and form biofilms (which represent a source of the recurrent contamination in the food production/processing environments), making it as a causative agent responsible for major food-borne diseases and illnesses (Shabala, Lee, Cannesson, & Ross, 2008; Zhu et al., 2017). The presence of *L. monocytogenes* in edible mushrooms, however, has received less attention, and little information is available on the contamination and prevalence levels of this bacterium in enoki mushrooms purchased online. Therefore, this study aimed I) to evaluate the microbiological quality (total aerobic bacteria, *E. coli*/coliform, yeast/mold, and *L. monocytogenes*) of Korean online market-purchased enoki mushrooms both qualitatively and quantitatively, and then II) to determine the genetic and phenotypic characteristics of *L. monocytogenes* isolates according to their serogroup, virulence, and antibiotic susceptibility profiles. The results obtained from the present study would not only contribute to evaluating the microbiological risk implicated in specialty mushrooms, but also provide a basis for the development of safety management techniques to control the contamination caused by *L. monocytogenes* in highly appreciated food products, such as enoki

mushrooms.

2. Materials and methods

2.1. Sample preparation and risk assessment

Generally, enoki mushrooms are commercially produced under a controlled environment (at an optimal temperature of 18–25 °C) in polypropylene bottles filled with a substrate containing rice bran and/or (paddy and/or wheat) sawdust (Fay et al., 2023). After the commercial production, enoki mushrooms that have completed a growing cycle are pulled as a bunch from the substrates, which means that the enoki mushroom roots containing the substrates are directly included during the packaging unless otherwise noted. During 2023–2024, a total of 100 enoki mushrooms were collected from 10 different online retailers located in eight different provinces (Busan-si, Daejeon-si, Gwangju-si, Gimpo-si, Hwaseong-si, Incheon-si, Seongman-si or Seoul-si) of the Republic of Korea. During the course of this study, it took approximately 3 d for the enoki mushrooms from being produced till reaching the online retailers. All the samples were kept below 4 °C during the transportation process and used for carrying out further *L. monocytogenes* risk assessment within 6 h.

Individually, 25 g of enoki mushrooms was placed in a stomacher bag (Difco® Laboratories, Detroit, MI, USA) filled with 225 mL of buffered peptone water (BPW; Difco® Laboratories) and homogenized at 25 °C for 1 min using a stomacher (BagMixer® 400, INTERSCIENCE Laboratory Inc., Saint-Nom-la-Breche, France). Each of the bacterial suspensions was serially diluted in 0.2% peptone water (PW; Difco® Laboratories), and the aliquot (1.0 mL) was spread on three different petrifilms (3M™ Microbiology Products, St. Paul, MN, USA), such as Petrifilm™ Aerobic Count Plate, Petrifilm™ EC Count Plate, and Petrifilm™ YM Count Plate, to quantify the viable number of total aerobic bacteria (TAB), *E. coli*/coliform, and yeast/mold (Y/M), respectively. Petrifilm™ Aerobic Count Plate and Petrifilm™ EC Count Plate were incubated at 37 °C for 24–48 h. Petrifilm™ YM Count Plate was incubated at 25 °C for 72 h. One hundred µL of the diluents was spread on Oxford agars (Difco® Laboratories) supplemented with the Oxford Agar Supplement (Difco® Laboratories), following 24–48 h of incubation at 37 °C.

Each of the enoki mushrooms (weighing 25 g) was transferred to a stomacher bag (Difco® Laboratories) containing 225 mL of *Listeria* enrichment broth (LEB; Difco® Laboratories). After homogenization at 25 °C for 3 min, the samples were incubated at 30 °C for 24 h, after which 1 mL of the primary solution was added to 9 mL of Fraser broth (Oxoid™ Ltd.) amended with the Fraser Broth Supplement (Oxoid™ Ltd.), following 24 h of enrichment at 37 °C for 24 h. A 10-µL loopful of the aliquots was streaked onto Oxford agars and incubated at 37 °C for 24–48 h. Suspect colonies of *L. monocytogenes* that represented a typically blurred grey-greenish surface with black centre and halo on Oxford agars were picked up with a sterile 1-µL loopful and streaked on tryptic soy agar supplemented with 0.6% yeast extract (Difco® Laboratories) (TSAYE). After 24 h of incubation at 37 °C, 2–3 suspect colonies of *L. monocytogenes* were picked up randomly from every TSAYE and identified via 16S rRNA sequencing.

2.2. Detection of virulence determinants and serogrouping

In order to screen for the virulence determinants (*prfA*, *actA*, *hlyA*, *inlA*, and *inlC*) and differentiate the major serogroup of *L. monocytogenes* isolates recovered from enoki mushrooms, conventional or multiplex PCR-based methods were used according to a method described in a study of Doumith, Buchrieser, Glaser, Jacquet, & Martin (2004) previously. Individually, the genomic DNA of *L. monocytogenes* isolates were extracted using Bead™ Genomic DNA Prep Kit Bacterium (GD701-100; BioFact Biofactory, Yuseong-gu, Daejeon-si, Republic of Korea). Each reaction mix (20 µL) consisting of 15 µL of PowerChek™ *Listeria*

monocytogenes PCR PreMix (KogeneBioTech Co., Ltd., Seoul-si, Republic of Korea) and 5 μ L of genomic DNA was prepared according to the manufacturer's instruction so as to determine the presence of *prfA* from the *L. monocytogenes* isolates. *prfA* (450 bp) was amplified in a C1000™ thermal cycler (Bio-Rad Laboratories Inc., Hercules, California, USA) as follows: an initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Additionally, a PCR mixture (20 μ L) consisting of 1 μ L of each primer, 20 μ L of AccuPower® HotStart PCR PreMix (K-5051, Bioneer Co., Ltd., Daejeon-si, Republic of Korea), 13 μ L of deionized water, and 5 μ L of genomic DNA was prepared according to the manufacturer's instruction, and *actA* (827bp; F, GCTGATTTAAGAGATAGAGGAACA; R, TTATGTGGTTATTTGCTGTC), *hlyA* (388bp; F, GAATGTAAACTTCGGCGCAATCAG; R, GCCGTCGATGATTGAACCTTCATC), *inlA* (800 bp; F, ACGAGTAACGGGACAAATGC; R, CCCGACAGTGGTGTAGATT) or *inlC* (517bp; F, AATCCCACAGGACACAACC; R, CGGAATGCAATTTTCACTA) was amplified as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 72 °C for 5 min.

All 9 *L. monocytogenes* isolates recovered from enoki mushrooms were analyzed by 16S rRNA sequencing (SolGent Co., Ltd., Yuseong-gu, Daejeon-si, Republic of Korea) with 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') universal primers. Each genomic DNA template was amplified with EF-Taq at an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. Each finished product was then analyzed with ABI Prism 3730XL DNA analyzer (Applied Biosystems, Carlsbad, California, USA).

2.3. Susceptibility to antibiotics

Individually, *L. monocytogenes* isolates grown in the brain heart infusion broth (BHI; Oxoid™ Ltd.) overnight at 37 °C were diluted in a saline solution containing 0.85% NaCl equivalent to a 0.5 McFarland turbidity standard using a nephelometer (PhoenixSpec™, Becton Dickinson Diagnostics, Sparks, MD, USA) and 0.5 Remel™ McFarland Equivalence Turbidity Standards (Remel™, Lenexa, KS), following spreading on Mueller Hinton agars (Oxoid™ Ltd.). Then, a total of 15 different antibiotic disks containing amoxycillin-clavulanic acid (AMC; 30 μ g; CT0223B; Oxoid™ Ltd.), ampicillin (AMP; 10 μ g; CT0003; Oxoid™ Ltd.), aztreonam (ATM; 30 μ g; CT0264; Oxoid™ Ltd.), cefepime (FEP; 30 μ g; CT0771; Oxoid™ Ltd.), chloramphenicol (C; 30 μ g; CT0013B; Oxoid™ Ltd.), ciprofloxacin (CIP; 5 μ g; CT0425B; Oxoid™ Ltd.), gentamicin (CN; 10 μ g; CT0024; Oxoid™ Ltd.), kanamycin (K; 30 μ g; CT0026B; Oxoid™ Ltd.), nalidixic acid (NA; 30 μ g; CT0031B; Oxoid™ Ltd.), ofloxacin (OFX; 5 μ g; CT0446B; Oxoid™ Ltd.), penicillin (P; 10 μ g; CT0043; Oxoid™ Ltd.), rifampicin (RD; 5 μ g; CT0207B; Oxoid™ Ltd.), streptomycin (S; 10 μ g; CT0047B; Oxoid™ Ltd.), sulphamethox-trimethoprim (SXT; 25 μ g; CT0052; Oxoid™ Ltd.) or tetracycline (TE; 30 μ g; CT0054; Oxoid™ Ltd.) were loaded on the surface of the Mueller Hinton agar plates. After 24 h of incubation at 37 °C, the diameter (mm) of the inhibition zones for each antibiotic disk was measured, and the tested *L. monocytogenes* isolates were designated to sensitive (S), intermediate (I) or resistant (R) according to a protocol described by the Clinical and Laboratory Standards Institute® (CLSI) guidelines. This experiment was repeated three times with duplicate samples.

2.4. Analysis of the obtained data

All experiments were repeated three times, and the results obtained were expressed as the mean $\pm$ standard deviation. Analysis of variances (ANOVA) with the Duncan's multiple range test was carried out via SAS version 10 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Risk assessment

Fig. 1 and the Supplementary Table S1 show the abundance of TAB, coliform, *E. coli*, Y/M, and *L. monocytogenes* recovered from online market-purchased enoki mushrooms. The mean counts for TAB analyzed were around 5.32 ± 1.52 log CFU/g with a range of 2.51–7.80 log CFU/g. Enoki mushrooms obtained from the seller E had the highest numbers of TAB, ranging from 7.63 to 8.16 log CFU/g. Enoki mushrooms obtained from the seller I tended to have significantly ($p < 0.05$) lower TAB counts (mean: 2.51 ± 0.65 log CFU/g; range: 1.78–3.56 log CFU/g) than the others. About 89% (89/100) of the samples had mean coliform counts, ranging from 1.14 to 6.52 log CFU/g. In general, enoki mushrooms obtained from the seller N were highly contaminated with coliforms with a mean count of 6.52 ± 0.16 log CFU/g, followed by those obtained from the seller E (6.38 ± 0.99 log CFU/g), the seller G (6.27 ± 0.09 log CFU/g), the seller D (5.57 ± 0.85 log CFU/g), the seller B (5.01 ± 0.95 log CFU/g), and the seller P (4.67 ± 0.78 log CFU/g). The mean counts of Y/M were 4.23 ± 1.14 log CFU/g in enoki mushrooms. The samples obtained from the sellers Q, N, and J were the items with the highest mean counts of Y/M (5.99 ± 0.37 , 5.63 ± 0.39 , and 5.06 ± 0.55 , respectively). All of the enoki mushrooms screened contained no detectable *E. coli* at a lower detection limit of 1.0 log CFU/g. Additionally, Table 1 describes the frequency of TAB, coliform, *E. coli*, Y/M, and *L. monocytogenes* recovered from online market-purchased enoki mushrooms. As a result, TAB, coliform, and Y/M had a prevalence rate of 100% (100/100), 86% (86/100), and 100% (100/100), respectively.

3.2. Characterization of the *L. monocytogenes* isolates

In this study, enoki mushroom samples obtained from the seller E and F were found to become contaminated with *L. monocytogenes* with a range of ≤ 3.79 and ≤ 1.48 log CFU/g, respectively, as determined using the quantitative analysis (Fig. 1E and the Supplementary Table S1). As shown in Table 1, seven samples were found to be positive for *L. monocytogenes* out of 100 enoki mushrooms distributed through an online market in the Republic of Korea as determined by the qualitative analysis followed by the enrichment procedure of each of the samples. Especially, we found that most of the samples (4/5) obtained from the seller E were highly contaminated with *L. monocytogenes*.

The isolates suspected to be positive for *L. monocytogenes* were further identified by carrying out a 16S rRNA sequencing and serogrouped via a multiplex-based PCR method in Table 2. As a result, a 16S rRNA sequence analysis confirmed that all nine *L. monocytogenes* isolates were assigned to *L. monocytogenes* (*L. monocytogenes* BfR-LI-00752, *L. monocytogenes* CFSAN023459, *L. monocytogenes* LM5, *L. monocytogenes* LM11, and *L. monocytogenes* S3), regardless of their isolation sources. Additionally, it was found that the most common serogroup was $\frac{1}{2}$ a-3a, accounting for 67% out of the total of 9 *L. monocytogenes* isolates.

All the *L. monocytogenes* isolates are screened for the determination of virulence attributes in Table 3. Six isolates (i.e. BMI₅₋₁, BMI₅₋₃, BMI₅₋₄, BMI₅₋₅, BMI_{B5-1}, and BMI_{B5-3}) were capable of amplifying *prfA*, *actA*, *hlyA*, *inlA*, and *inlC*. BMI₁₈₋₄ and BMI₁₉₋₂ were found to possess only *prfA*. Additionally, four virulence genes, such as *prfA*, *hlyA*, *inlA*, and *inlC*, were detected in EMI₆₋₁.

Herein, as shown in Table 4, we observed that a vast majority of *L. monocytogenes* isolates had a clear hierarchy of resistance to aztreonam, cefepime, nalidixic acid, and penicillin albeit 100% of the isolates were found to be susceptible to amoxycillin-clavulanic acid, gentamicin, and kanamycin. Approximately 56–89% of the *L. monocytogenes* remained more prone to be intermediately resistant to ciprofloxacin and ofloxacin.

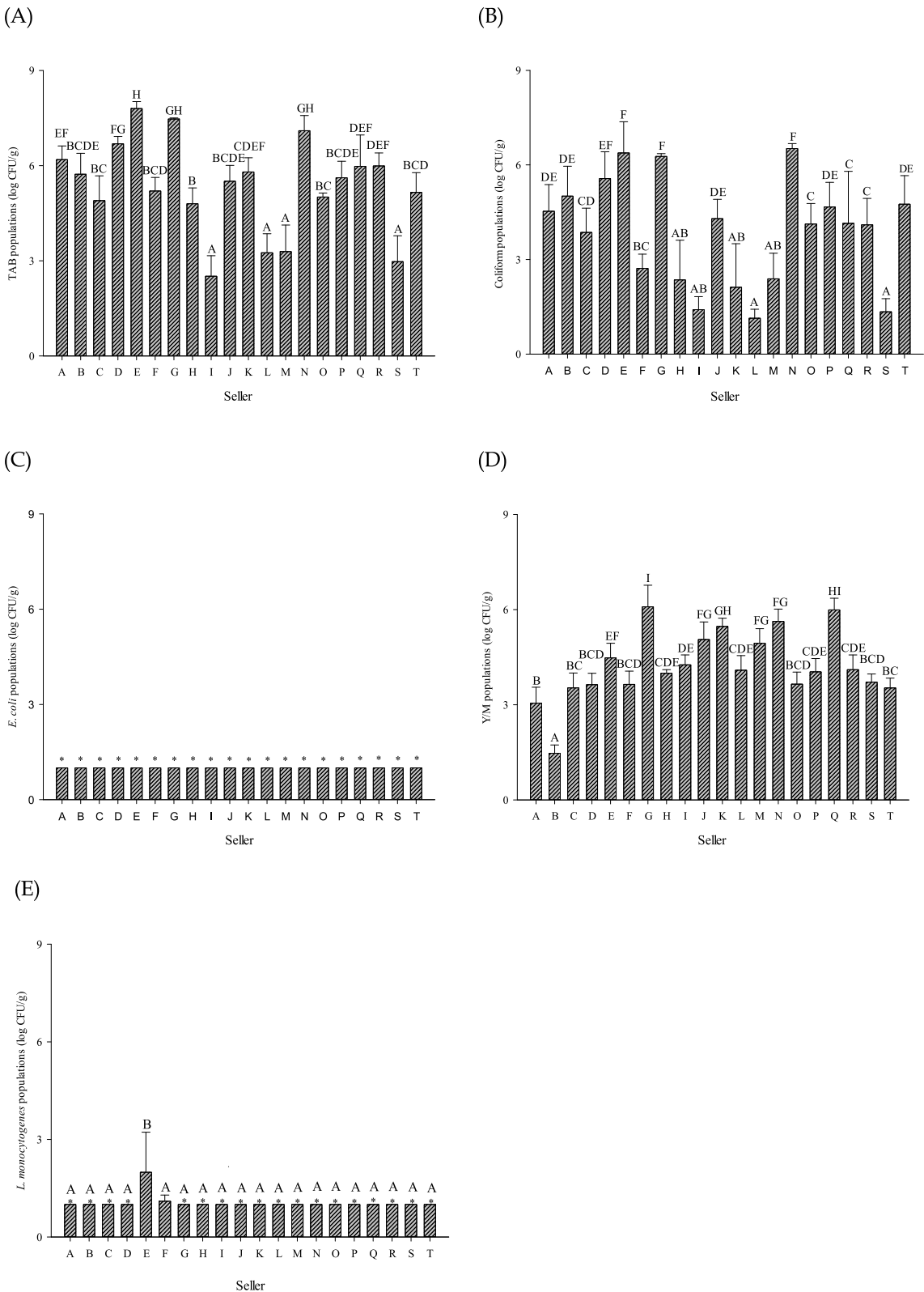


Fig. 1. Quantification (log CFU/g) of (A) TAB, (B) coliform, (C) *E. coli*, (D) Y/M, and (E) *L. monocytogenes* recovered from online market-purchased enoki mushrooms, followed by direct-plating on culture media. *, Below the detection limits (≤ 0.95 log CFU/g). ^A Mean values with different uppercase letters within each of the groups denote significant ($p < 0.05$) differences.

Table 1

Frequency of TAB, coliform, *E. coli*, Y/M, and *L. monocytogenes* recovered from online market-purchased enoki mushrooms, followed by the enrichment procedure of the samples.

Seller	Location	Sampling periods	Prevalence of microorganisms				
			TAB	Coliform	<i>E. coli</i>	Y/M	<i>L. monocytogenes</i>
Seller A	Seoul-si	2023.10.30	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller B	Seoul-si	2023.12.02	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller C	Seongnam-si	2023.12.02	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller D	Seoul-si	2023.11.20	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller E	Seoul-si	2023.11.27	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	4/5 (80%)
Seller F	Seoul-si	2023.11.27	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	1/5 (20%)
Seller G	Incheon-si	2023.12.08	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller H	Daejeon-si	2023.01.10	5/5 (100%)	3/5 (60%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller I	Seongnam-si	2024.02.13	5/5 (100%)	3/5 (60%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller J	Incheon-si	2024.02.13	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller K	Seongnam-si	2024.02.14	5/5 (100%)	2/5 (40%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller L	Busan-si	2024.02.14	5/5 (100%)	1/5 (20%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller M	Gimpo-si	2024.02.15	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller N	Hwaseong-si	2024.02.15	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller O	Busan-si	2024.02.19	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller P	Seongnam-si	2024.02.19	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller Q	Gwangju-si	2024.02.22	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Seller R	Seoul-si	2024.02.22	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	1/5 (20%)
Seller S	Seoul-si	2024.02.23	5/5 (100%)	2/5 (40%)	0/5 (0%)	5/5 (100%)	1/5 (20%)
Seller T	Yeoju-si	2024.02.27	5/5 (100%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
Total			100/100 (100%)	86/100 (86%)	0/100 (0%)	100/100 (100%)	7/100 (7%)

Table 2

Identification and serogrouping of *L. monocytogenes* isolates recovered from online market-purchased enoki mushrooms.

Strain	Seller	Identification ^a	Serogroup					Lineage
			ORF2819	lmo0737	lmo1118	ORF2110	prs	
ATCC 19117	ND ^b	<i>L. monocytogenes</i> LM11	+	–	–	+	+	(4bde), ($\frac{1}{2}$ b-3b-7)
ATCC 19114	ND	<i>L. monocytogenes</i> LM11	+	–	–	–	+	($\frac{1}{2}$ b-3b-7)
EMI 5-1	Seller E	<i>L. monocytogenes</i> BfR-LI-00752	–	+	–	–	+	($\frac{1}{2}$ a-3a)
EMI 5-3	Seller E	<i>L. monocytogenes</i> LM11	–	+	–	–	+	($\frac{1}{2}$ a-3a)
EMI 5-4	Seller E	<i>L. monocytogenes</i> LM11	–	+	–	–	+	($\frac{1}{2}$ a-3a)
EMI 5-5	Seller E	<i>L. monocytogenes</i> CFSAN023459	–	+	+	–	+	($\frac{1}{2}$ a-3a), ($\frac{1}{2}$ c-3c)
EMI 6-1	Seller F	<i>L. monocytogenes</i> LM5	–	+	–	–	+	($\frac{1}{2}$ a-3a)
EMI 18-4	Seller R	<i>L. monocytogenes</i> LM5	–	–	–	–	+	ND
EMI 19-2	Seller S	<i>L. monocytogenes</i> LM5	–	–	–	–	+	ND
EMI B5-1	Seller E	<i>L. monocytogenes</i> S3	–	+	–	–	+	($\frac{1}{2}$ a-3a)
EMI B5-3	Seller E	<i>L. monocytogenes</i> BfR-LI-00752	–	+	–	–	+	($\frac{1}{2}$ a-3a)

^a All isolates were identified as *L. monocytogenes* via a 16S rRNA sequencing.

^b ND, not determined.

Table 3

Detection of virulence determinants (*prfA*, *actA*, *hlyA*, *inlA*, and *inlC*) of *L. monocytogenes* isolates recovered from online market-purchased enoki mushrooms.

Strain	Seller	Identification ¹⁾	Virulence-associated determinants				
			<i>prfA</i>	<i>actA</i>	<i>hlyA</i>	<i>inlA</i>	<i>inlC</i>
ATCC 19117	ND ³⁾	<i>L. monocytogenes</i> LM11	+	+	+	+	+
ATCC 19114	ND	<i>L. monocytogenes</i> LM11	+	+	+	+	+
EMI 5-1	Seller E	<i>L. monocytogenes</i> BfR-LI-00752	+	+	+	+	+
EMI 5-3	Seller E	<i>L. monocytogenes</i> LM11	+	+	+	+	+
EMI 5-4	Seller E	<i>L. monocytogenes</i> LM11	+	+	+	+	+
EMI 5-5	Seller E	<i>L. monocytogenes</i> CFSAN023459	+	+	+	+	+
EMI 6-1	Seller F	<i>L. monocytogenes</i> LM5	+	–	+	+	+
EMI 18-4	Seller R	<i>L. monocytogenes</i> LM5	+	–	–	–	–
EMI 19-2	Seller S	<i>L. monocytogenes</i> LM5	+	–	–	–	–
EMI B5-1	Seller E	<i>L. monocytogenes</i> S3	+	+	+	+	+
EMI B5-3	Seller E	<i>L. monocytogenes</i> BfR-LI-00752	+	+	+	+	+

Table 4
An antimicrobial susceptibility profile of 9 *L. monocytogenes* isolates recovered from online market-purchased enoki mushrooms against 16 antibiotics.

Agent	Profile		
	Susceptible	Intermediate	Resistant
Ampicillin (10 µg)	6 (67%)	3 (33%)	—
Amoxycillin-clavulanic acid (30 µg)	9 (100%)	—	—
Aztreonam (30 µg)	—	—	9 (100%)
Cefepime (30 µg)	—	—	9 (100%)
Chloramphenicol (30 µg)	4 (44%)	3 (33%)	2 (22%)
Ciprofloxacin (5 µg)	4 (44%)	5 (56%)	—
Gentamicin (10 µg)	9(100%)	—	—
Kanamycin (30 µg)	9(100%)	—	—
Nalidixic acid (30 µg)	—	—	9 (100%)
Ofloxacin (5 µg)	1 (11%)	8 (89%)	—
Penicillin G (10 units)	—	—	9 (100%)
Rifampicin (5 µg)	8 (89%)	1 (11%)	—
Streptomycin (10 µg)	9 (100%)	—	—
Sulfamethoxazole-trimethoprim (25 µg)	8 (89%)	1 (11%)	—
Tetracycline (30 µg)	9 (100%)	—	—

4. Discussion

To date, the information on the microbiological quality of edible mushrooms has rarely been reported, particularly for online market-purchased/distributed samples, such as enoki mushrooms (*F. velutipes*). The mean count of mesophilic microorganisms was 7.1 log CFU/g in enoki mushrooms purchased from retail markets located in Zaragoza, Spain (Venturini et al., 2011). Öncül and Çiftçi (2023) also evidenced that all edible mushroom samples collected from local markets in the Fethiye region of Muğla/Türkiye exhibited a high quantity of total mesophilic aerobic bacteria (TMAB) counts, ranging from 4.13 to 8.83 log CFU/g. In a study conducted by Ergönül, Kalyoncu, and Akata (2018) who quantified the density of TMAB present in 8 different edible mushrooms, the mean counts were between 6.9 and 9.2 log CFU/g, which have supported our findings. Among the different species of edible mushrooms commercialized in the Republic of Korea, *F. velutipes* had significantly higher TMAB counts (4.12 log CFU/g) than did *Agaricus bisporus* (3.88 log CFU/g), *Pleurotus ostreatus* (3.20 log CFU/g), and *Pleurotus eryngii* (2.96 log CFU/g) (Meng, Jang, Song, & Lee, 2024). Jiang et al. (2018) revealed that the loads of TAMB detected in all sliced mushroom (*A. bisporus*) samples acquired by eight different point-of-sales located in Perugia, Italy were always over 7.87 log CFU/g. As described previously in a study conducted by Mansur and Oh (2015), TAMB counts can be a useful indicator for determining the shelf life of minimally processed vegetables, such as kale, stored at refrigerator temperatures. The authors (Mansur & Oh, 2015) pointed out that kale reached the unacceptable limit when its TAMB counts exceeded ≥ 7.0 log CFU/g, which corresponded well to the severe deterioration on the overall visual quality of fresh-cut kale products. Similarly, the microbiological quality of fruits and vegetables is regarded as unsuitable as long as the TMAB populations exceeding 7 log CFU/g are rated as poor (Tango et al., 2018). From the bacteriological points of view, some enoki mushrooms sampled from the sellers E, G, and N were poor with a mean TAB range of 7.10 ± 0.48 – 7.80 ± 0.22 log CFU/g, whereas the others remained good within a microbiologically acceptable limit.

Despite that the presence of microorganisms in foods is not necessarily an indicator of hazards to the consumers, the presence of (fecal) coliforms detected can indicate a poor hygienic manufacturing practice of food items (Kamal, Begum, & Khair, 2010). Previously, Öncül and Çiftçi (2023) reported that approximately 80% (16/20) of edible mushrooms were found to be contaminated by coliforms with 6 samples being in the range of 3.07–5.07 log CFU/g, and the coliform counts detected in enoki mushrooms exceeded 2.55 log CFU/g (Meng et al., 2024). Kamal et al. (2010) highlighted that 40% (6/16) oyster mushrooms collected through a randomized sampling from local markets

located in Savar thana, Dhaka recognized as unsafe and unconsumable for consumers with maximum total bacterial and coliform counts being $\geq 10^8$ CFU/g since the contamination source for fresh oyster mushrooms would have result from the unhygienic processing condition and the improper handling of employees. In this study, coliform counts greatly varied among the 100 of the online market-purchased enoki mushrooms within a range of 1.14 ± 0.28 – 6.52 ± 0.16 log CFU/g.

Eight different species of edible mushrooms sold in Turkey were shown to have Y/M counts with a range of 1.9–3.3 log CFU/g (Ergönül et al., 2018). Y/M was detected from 70% to 88% of the shiitake mushrooms (*Lentinus edodes*) purchased from local markets located in Petersburg, USA with a mean count of 6.90 log CFU/g (Kim et al., 2016). It should be also noted that the presence of Y/M counts exceeding 5.7 log CFU/g in fruits and vegetables may pose a potential health hazard, leading to the spoilage of food items, which was accompanied by the production of mycotoxins (Tango et al., 2018). Santana, Vanetti, and Kasuya (2008) have concluded that shiitake mushrooms suspected to become spoiled may give a higher Y/M count of $\leq 10^6$ CFU/g. As such, it was clear that enoki mushrooms used in this study contained relatively lower numbers of Y/M with 95% of the samples having mean counts of less than 6.00 log CFU/g, with the exception of those from the seller G (mean counts: 6.09 ± 0.68). Comprehensively, the microbiological quality of enoki mushrooms varies largely, and this may have resulted from store-to-store variations in storage conditions and differences in age/location of the products at the sampling time, indicating that the vast majority of enoki mushrooms procured from online markets would have a lower potential for the contamination of TAB, *E. coli*, and/or Y/M.

L. monocytogenes was widely diffused in natural environments and foods, especially in edible mushrooms, with the detection rate as high as 41.1% (Li et al., 2021). In the present study, we collected a total of 100 enoki mushrooms and found 7 samples to be contaminated with *L. monocytogenes*. Chen et al. (2014) found a high occurrence (18.6%; 55/295) of *L. monocytogenes* in three enoki mushroom production plants and associated environments (especially, the mycelium-scraping machinery was the likely primary source for the *L. monocytogenes* contamination and was found to result in the cross contamination of upstream food-contacting equipments and packaged final products), which is consistent with the findings of Li et al. (2021). Xu et al. (2023) represented the presence of pathogenic *L. monocytogenes* strains in 13.3% (8/60) of the king oyster mushroom samples with the persistence of some *L. monocytogenes* isolates in mushroom production plants. Johannessen et al. (2002) detected *L. monocytogenes* in 0.6% (1/156) of the edible mushroom samples collected from Norwegian markets. Despite the comparatively low contamination rates of *L. monocytogenes* in online market-purchased enoki mushrooms, a specialty mushroom, such as enoki mushrooms, would be a possible contributor to the *L. monocytogenes* contamination, which gives a clear indication on the emerging relevance of the consumption of enoki mushrooms with the food safety issue on an emphasis on implementing a regular food contamination surveillance of *L. monocytogenes* to minimize the cross contamination by this bacterium in the cultivation and processing of enoki mushrooms.

L. monocytogenes can be generally divided into 13 serotypes of which are divided into 4 evolutionary lineages I ($\frac{1}{2}$ b, 3b, 4b, 4d, 4e, and 7), II ($\frac{1}{2}$ a, $\frac{1}{2}$ c, 3a, and 3c), III ($\frac{1}{2}$ a, 4a, 4b, and 4c), and IV (4a, 4b, and 4c) (Ravindhiran et al., 2023). Lineages I (encoding the so-called listeriolysin virulence factor) and II (possessing lots of plasmids which are resistant to heavy metals) are mostly clinically relevant strains involved in the major listeriosis epidemics (Ravindhiran et al., 2023). In the present study, the most prevalent serotype was $\frac{1}{2}$ a-3a with a frequency rate of 69% albeit two *L. monocytogenes* isolates failed to amplify the serotype-associated gene clusters. Ok et al. (2024) reported that the prevalence rates of *L. monocytogenes* among the enoki mushrooms sampled from rural and open-air markets located in Busan-si, Republic of Korea were 12.5% (10/80) in which the serotype of 10-positive

samples was $\frac{1}{2}$ ab. Chen et al. (2015) found that while *L. monocytogenes* isolates recovered from edible mushrooms, such as *Coriandrum sativum*, *Fl. velutipes*, *Hypsizygus marmoreus*, *Lentinus edodes*, *Pleurotus eryngii*, and *Pleurotus geesteranus*, only belonged to serogroups I.1 ($\frac{1}{2}$ a-3a) and II.2 ($\frac{1}{2}$ b-3b-7), the serogroup III pertaining to 4a-4c dominant in the fishery products, suggesting that specific serogroups of *L. monocytogenes* isolates may have distinct ecological niches. All 279 isolates obtained from mushroom production facilities were confirmed as *L. monocytogenes*; 40.8, 30.1, and 29.1% were distributed into $\frac{1}{2}$ a-3a, $\frac{1}{2}$ b-3b-7, and 4b-4d-4e, respectively (Pennone, Leahy, Coffey, McAuliffe, & Jordon, 2018). Particularly, it was reported that a vast majority of the *L. monocytogenes* isolates closely involved in food-borne listeriosis outbreaks (which was caused by the consumption of contaminated beef, crab, pork, salmon, and squid), Republic of Korea, 2005–2001 were serogrouped as $\frac{1}{2}$ a, $\frac{1}{2}$ b, $\frac{1}{2}$ c or 4b (Lee, Lee, Park, & Koo, 2023). As well documented by Farber and Peterkin (1991), serotypes $\frac{1}{2}$ a, $\frac{1}{2}$ b, and 4b are mainly responsible for more than 90% of human listeriosis cases, which becomes a matter of concerns for mushroom growers and consumers.

Previous studies have primarily focused on the prevalence and antimicrobial susceptibility of *L. monocytogenes* in raw or processed food products of animal origins (Arslan & Baytur, 2018; Fei et al., 2016; Sosnowski et al., 2019; Su et al., 2016; Wu et al., 2015). However, there are only a few publications on the occurrence and antibiotic resistance of pathogenic bacteria, such as *L. monocytogenes*, isolated from online market-purchased edible mushrooms, such as enoki mushrooms, in the Republic of Korea. The incidence of listeriosis infections has been reported to be higher particularly in the immunocompromised patients over 60 years in the Republic of Korea, ranging from 0.6 listeriosis cases in 2010 to 5.6 cases in 2015 per 100,000 admissions (Choi et al., 2018). Ampicillin or penicillin alone or incorporated with aminoglycoside and trimethoprim-sulfamethoxazole has been suggested as the regular way to control the listeriosis infections (Choi et al., 2018; Sosnowski, Lachata, Wiczorek, & Osek, 2019). Other antibiotics, such as chloramphenicol, rifampicin, tetracycline, and fluoroquinolones, can be proposed as alternatives for the listeriosis therapy (Olaimat et al., 2018). However, the emergence of *L. monocytogenes* isolates which are highly resistant to one or more antibiotics ordinarily used to treat patients infected by this bacterium has stressed the selective pressure for the resolution of listeriosis infections as documented by Abatcha (2017) and Olaimat et al. (2018). Previously, *L. monocytogenes* isolates recovered from edible mushrooms exhibited a high frequency of resistance to more than two antibiotics, particularly ampicillin, ciprofloxacin, and penicillin (Chen et al., 2015). Additionally, approximately 18 (34.6%), and 12 (23.0%) *L. monocytogenes* strains isolated from raw milk and cheese samples were highly resistant to tetracycline and penicillin, respectively (Kevenk & Gulel, 2016). A total of 137 *L. monocytogenes* strains isolated from Egyptian dairy cattle farms had a high resistant rate of 100.0%, 100.0%, 100.0%, 100.0%, 94.2%, and 91.2% against amoxicillin, ceftioxin, neomycin, penicillin, nalidixic acid, and cloxacillin, respectively, while 92.7% (127/137) of the *L. monocytogenes* isolates were found to contain *bla*CTX-M, a resistant-conferring gene to antibiotics, which may indicate a challenge in the treatment of listeriosis infections (Elsayed, Elkenany, Zakaria, & Badawy, 2022). All *Listeria* spp. isolated from vegetable samples showed the highest resistance (100.0%) against clindamycin, penicillin, and tetracycline (Bilung, Chai, Tahar, Ted, & Apun, 2018). As such, a wide range of food items, such as edible mushrooms, may serve as the potential reservoirs of multidrug-resistant *L. monocytogenes* strains. Treatment of *L. monocytogenes* infections is reliant upon the commonly used antibiotics in the medication of clinical and veterinary infections, including ampicillin, gentamicin, penicillin, tetracycline, and others, which indicates that some of the *L. monocytogenes* isolates that were cultured from a facility environment may have been previously exposed to these agents and consequently elaborated a higher resistance. The emergence of multidrug-resistant *L. monocytogenes* isolates has stressed the selective pressure for the

resolution of listeriosis infections (Olaimat et al., 2018). So far, considering that listeriosis cases in humans are frequently transmitted via food products, an active surveillance program should be maintained for the regular monitoring of the emergence of multidrug-resistant *L. monocytogenes* strains to prevent the onset of human listeriosis infections.

Until now, there are no guidelines for the qualitative and quantitative detection of *L. monocytogenes* in edible mushrooms and mushroom production/processing facilities in the Republic of Korea. As stated earlier, *L. monocytogenes* can persist in food production/processing environments where the food-contacting surface of certain equipments can act as a reservoir of this bacterium (Sun et al., 2021). Indeed, the mycelium-scraping machinery may be suspected to serve as the primary *L. monocytogenes* contamination source in mushroom production facilities, and then edible mushrooms may be a potential transmission vehicle of the *L. monocytogenes* infection for consumers (Xu et al., 2023), giving rise to an increased awareness among the mushroom producers/processors of this potential hazard. It is, therefore, important to provide a basis for strategies to control the *L. monocytogenes* contamination in food production and processing environments. Pennone et al. (2020) implemented some hygiene practices to minimize the cross contamination of *L. monocytogenes* in several mushroom production-associated environments, including the use of disinfectants/sanitizers and the building of physical barriers between each sector. Particularly, the authors introduced a live “steam cookout” step into growing rooms after the harvesting process, and its aim was to reduce or eliminate all pathogens and pests by achieving a temperature of 60–70 °C in the concrete floors. Lappi et al. (2004) also implemented *Listeria*-targeted intervention programs to establish a clean and hygienic environment of smoked fish processing plants with a common presence of one or more persistent *L. monocytogenes* ribotypes; the use of a 1200-ppm Quat disinfectant/sanitizer in trench drains, the washing of waste totes with ClO₂, and the training of staffs provoking the awareness of floor mats as a potential contamination source in the handling areas by plant managers. Importantly, such actions were effective in significantly reducing the abundance and prevalence of *L. monocytogenes*, indicating that strict control measures must be put in place to prevent the preharvest and postharvest contamination of final products with *L. monocytogenes* along with the whole mushroom production and processing chain. In order to help maintain the safety of edible mushrooms, it should be also noted that washing agricultural products in antibacterial agents, such as chlorine dioxide (ClO₂) and sodium hypochlorite (NaClO), is an important step that can detach or remove field-acquired contaminants and guarantee product quality and safety without additional thermal treatments. The lethality of a variety of aqueous disinfectants/sanitizers for inactivating *L. monocytogenes* inoculated in edible mushrooms has been validated in previous studies (Chung et al., 2023; Murray, Wu, Aktar, Namvar, & Warriner, 2015; Yoon et al., 2021a, 2021b; Yuk, Yoo, Yoon, Marshall, & Oh, 2007). Yoon, Jeong, Lee, Chio, et al. (2021) determined that dipping in 3% malic acid (MA) at an ambient temperature for 15 min resulted in a 3.01 log-reduction in the viable numbers of *L. monocytogenes* inoculated in oyster mushrooms (*Pleurotus eryngii*). Particularly, Yoon, Jeong, Lee, and Kim (2021) represented a strong synergistic interaction between 3% lactic acid (LA), 0.1% nisin, and 40 kHz ultrasound against *L. monocytogenes* inoculated in enoki mushrooms, showing by more than a 5.0 log-reduction of this bacterium. In this regard, washing in organic acids would be an effective alternative to chlorine-based disinfectant/sanitizers for controlling *L. monocytogenes* inoculated in edible mushrooms. Nevertheless, more in-depth studies are required to formulate a set of standard operation protocols for ensuring the microbiological safety of mushroom products and their surrounding environments with the aim of addressing the issue involved in the cross contamination of spoilage and/or pathogenic bacteria at each mushroom production/processing phases.

In conclusion, enoki mushrooms were found to be contaminated with TAB (mean counts: 5.32 ± 1.52 log CFU/g), *E. coli* (mean counts: ≥ 1.00

log CFU/g), and Y/M (mean counts: 4.23 ± 1.14 log CFU/g). A majority of enoki mushrooms were found to harbor coliforms, with 89% of samples having more than 3.89 ± 1.85 log CFU/g. The qualitative experiment showed that the overall prevalence of *L. monocytogenes* present on enoki mushrooms was 2% with a mean count of 1.55 ± 0.63 log CFU/g. Particularly, we determined that seven *L. monocytogenes* strains (EMI₅₋₁, EMI₅₋₃, EMI₅₋₄, EMI₅₋₅, EMI₆₋₁, EMI₁₈₋₄, and EMI₁₉₋₂) were recovered from enoki mushroom samples, followed by the enrichment step of the isolates. There was a high variability on the presence of TAB, coliform, Y/M, and *L. monocytogenes* among the samples of each seller, and consuming raw enoki mushrooms would pose a potential risk of causing listeriosis infections in humans, indicating the need for establishing strict preventive management systems to improve the microbiological quality of edible mushrooms, such as enoki mushrooms. Additionally, it is assumed that as each of the enoki mushroom cultivation procedures (i.e. preparation of substrates → inoculation of spawns → mycelium infiltration → scraping → collaring → harvest) may represent a critical point of the *L. monocytogenes* contamination from equipment and spawn or caused by the incomplete sterilization of substrates (Grocholl, Ferguson, Hughes, Trujillo, & Burall, 2024), the origin of the *L. monocytogenes* contamination in enoki mushrooms sold via online market retailers would be closely involved in insufficient heating problems or inadequate hygienic manipulations. To our knowledge, few data are available on the microbiological quality and the presence of food-borne pathogenic bacteria, such as *L. monocytogenes*, in enoki mushrooms sold online. The results obtained from the present study will promote the awareness of the *L. monocytogenes* contamination in edible mushrooms for the improvement of hygienic practices since enoki mushrooms are considered as a ready-to-eat (RTE) food where the products are consumed without undergoing the appropriate processing that would normally reduce or eliminate *L. monocytogenes*.

CRedit authorship contribution statement

Sang-Woo Lee: Writing – original draft, Investigation, Formal analysis. **Sa-Rang Park:** Investigation, Formal analysis. **Se-Gyeong Yoon:** Investigation, Formal analysis. **Hyun-Dong Cho:** Writing – review & editing, Data curation. **Mi-Kyung Lee:** Writing – review & editing, Data curation. **Sun-Young Lee:** Writing – review & editing, Data curation. **Jae-Hyun Yoon:** Validation, Supervision, Project administration, Conceptualization.

Declaration of competing interest

We have nothing to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2024.117235>.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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