Summary

It was relatively recent that *Campylobacter* species (C spp.) and *Listeria* species (L spp.) were found to cause food poisoning, leaving many unclear points and problems to be solved. In Chapter 1, combined contamination by C spp. and L spp. was investigated in different chicken categories. In Chapter 2, the presence of benzalkonium chloride (BC)-resistant Listeria monocytogenes (Lm) strains was examined in 219 randomly selected strains from the strains preserved at the Public Health Laboratory of Nippon Veterinary and Life Science University. Furthermore, in Chapter 3, the relationships between distribution channels, rearing systems, and bacterial contamination were investigated, and subsequently, an attempt was made to analyze the relationships among factors, such as the processing plants, retail stores, continuous contamination, etc., based on the concept of hazard analysis and critical control points (HACCP).

Chapter 1

Retail chicken were categorized into general broiler chicken ('General'), Meigara-brand chicken (Meigara), and Japanese local chicken (Jidori), and the contamination rates of C spp. and L spp. were compared.

Materials and Methods: From September 2017 to August 2018, 90 chicken meat samples were purchased from 16 stores in Tokyo and tested for the presence of C spp. L spp. was also tried to isolate together with C spp. from 65 samples from 13 stores purchased after

December 2017. The isolation of C spp. and examinations of biochemical characteristics were performed according to routine procedures, and the bacterial species were determined by polymerase chain reaction. L spp. was also isolated, and the bacterial species were determined by biochemical tests and hemolysis examinations. For *Listeria monocytogenes* (Lm), serotyping was performed. Statistical analyses were performed by two-way analysis of variance.

Results: Although chicken samples were purchased from a total of 16 stores, the chicken were classified into 20 different distribution channels as some stores sold chicken from different processing plants. The total number of production areas and types was 17 and 22, respectively, for Jidori and Meigara. Regarding C spp., 165 strains of *C. jejuni* (Cj) and 66 strains of *C. coli* (Cc) were isolated. More than 50% of the samples of Jidori and Meigara were contaminated with Cj. The Cc contamination rate in 'General' tended to be low. A total of 175 strains of L spp. were isolated, including *L. innocua* (Li; 89 strains), *L. gray* (Lg; 58 strains), Lm (22 strains), and *L. welshimeri* (Lw; 6 strains). The isolation rate of L spp. was highest in Jidori (86%), and lowest in 'General'. The ratio of positive samples was Jidori > Meigara > 'General'. By bacterial type, the contamination rates of Li and Lg were high, and contamination by either or both species was confirmed in 83% of the positive samples. Additionally, contamination by all three of Li, Lm, and Lw was observed in one sample of 'General'. A total of 22 strains of Lm (1/2a: 11 strains; 1/2b: 7 strains; 1/2c: 4 strains) were isolated from seven samples (10.8%). Combined contamination by both C spp. and L spp. was found in 28 of the 65 samples (43%). In all categories of chicken, the rate of combined contamination was

approximately 40% with no statically significant difference between categories ($P > 0.05$). By type, contamination by both Cj and Li was the most common combined contamination. Cj was isolated from 4 of the 5 samples from which Lm was isolated, and Cc was isolated from the remaining sample, but there was no sample contaminated by Lm, Cj, and Cc. The rate of contamination by only L spp. (21 samples) was three times higher than that by only C spp. (7 samples).

Discussion: In total, 232 strains of C spp. were isolated from 57 chicken samples that were purchased from 13 of the 16 stores, and Cj contamination was the most common, being not inconsistent with previous reports. Among the categories of chicken, 'General' had the lowest contamination rate. The low isolation rate of Lm was thought be due to stop the plate culturing time at 48 h. The result showing that many Lm spp. of serotype 1/2 were isolated from chicken may help the elucidation of the infection routes of listeriosis arising in Japan.

Chapter 2

In this chapter, screening tests were conducted for BC resistance in the Lm strains preserved at the Veterinary Public Health Laboratory of Nippon Veterinary and Life Science University, and drug susceptibility tests were conducted on the resistant strains.

Materials and Methods: Among the Lm 547 strains stored at -80°C, a total of 219 strains were randomly selected and tested, including 17 from beef, 5 from minced meat of beef and pork, 61 from pork, and 136 from chicken samples. For strains that showed BC resistance in the screening test, and for the Lm EGD-e strain that was used as the negative control the minimal inhibitory concentration (MIC) was

further determined by the disk diffusion method and the trace liquid dilution method. Excel 2010 (Microsoft Japan) was used for statistical calculations. The diameters obtained by the disk diffusion method are expressed as the average value ± standard error in millimeters, and the significance of differences were tested by the Student's t-test.

Results: The preserved Lm strains used in this chapter were randomly selected from the strains that had been isolated from a total of 820 meat samples purchased from 105 stores in Tokyo and six prefectures between June 1996 and August 2018; in total 219 preserved Lm strains originating from 85 stores were tested. In the screening test using the drop test, the EGD-e strain did not grow on 2% sheep blood-added Mueller-Hinton medium containing 10 µl/ml BC, although three strains (1.4%), i.e., 633C3, 779C2, and 868C4, did grow on the medium. With the streak culture method, the EGD-e strain did not grow, but clear growth was observed for the three BC-resistant strains, and the three strains also showed β-hemolysis on stab culture. With the disc diffusion method, a clear inhibition circle was formed by the EGD-e strain at a BC concentration of more than 10^2 µg/ml, whereas concentration-dependent inhibition circles were formed by 633C3, 772C2, and 868C4 at concentrations of more than 10^3 µg/ml. With the broth microdilution method, the MIC of the EGD-e strain was 8 µg/ml, whereas the MIC of 633C3, 772C2, and 868C4 was 32 µg/ml. The MIC of the 824C3 strain was also 8 µg/ml. Comparing the appearance frequency of resistant bacteria for 11 years from June 1996 to June 2007 and for 9 months from December 2017 to August 2018, the former was two of 197 strains (1.0%) while the latter was two of 22 (9.1%). The resistant strains were all chicken-derived isolates. Two strains,

633C3 and 779C2, were isolated in 2004 and 2007, respectively, and both were serotype 1/2a. Meanwhile, the serotype of the 868C4 strain isolated in 2018 was 1/2b. It was confirmed that the chicken from which the 633C3 strain and the 779C2 strain were isolated was purchased from different stores of the same affiliated one, and the processing plants were also different. The 868C4 strain was also a chicken-derived isolate, and the meat was purchased from a completely different store than the stores that sold the chickens with 779C2 and 633C3 contamination.

Discussion: Three strains, i.e., 633C3, 772C2, and 868C4, were BC-resistant, indicating that BC-resistant Lm strains have been in Japan since at least 2004. The appearance frequency of resistant strains was 1.0% in the 11 years from June 1996 to June 2007, and 4.5% in the 9 months from December 2017 to August 2018; however, if the 824C3 strain isolated from chicken purchased by the author in January 2018 is included, the latter would increase to 2 out of 22 strains (9.1%), which is comparable to the 10% reported overseas. In addition, it was inferred from the analysis of distribution channels and the appearance frequencies that the BC-resistant Lm strains are distributed in a considerable range related to food.

Chapter 3

The sources and histories of the meat examined in Chapter 1 were further subdivided, and the contamination statuses of C spp. and L spp. in the Jidori, Meigara, and 'General' categories were analyzed for their relationship with HACCP certification.

Materials and Methods: The retail chicken and data from Chapter 1

were used for the analysis in this chapter. Necessary information on the HACCP-related certification system, information on Jidori and Meigara, and relevant laws and regulations were obtained through direct telephone interviews, the websites of ministries and agencies, various databases, and the Internet.

Results: Although chicken samples were purchased from a total of 16 stores, some stores brought in poultry meat from two or three different processing plants; thus, which were classified in 16 distribution routes (pathways) of chicken considered for L spp. and 20 routes of chicken considered for C spp. In L spp., all chickens from 13 stores that purchased more than two samples were contaminated. L spp. were isolated from all purchased specimens in six pathways and from one pathway with a high percentage of L spp (85.7%). However, some of the contamination rates varied greatly depending on the processing site. C spp. contamination was found in 13 of 15 distribution channels from which more than two samples were purchased. Three routes showed a high isolation rate of 100%, other three routes also showed a high rate of more than 70%, and the isolation rate of also L spp. was high in these routes. Of the sample purchased more than two, there were 12 types of Jidori and Meigara that were examined for L spp., and six had a contamination rate of 100%. Furthermore, two types in the Meigara category were contaminated with L spp. at a high rate of 80%, and only two types were negative. Similarly, among the 17 types of Jidori and Meigara that were purchased more than two samples and examined for C spp., nine (52.9%) were contaminated at a high rate of 100%, which was slightly higher than the contamination rate of L spp. In three types of the Meigara category, L spp. and C spp. were isolated from all

samples, and in two types of Jidori, both L spp. and C spp. showed a contamination rate of more than 70%. In each of the distribution channels, route-specific contamination conditions were observed even for the same types of Jidori and Meigara. As for the relationship between HACCP certification and the isolation rate of C spp. or L spp., the isolation rate of C spp. tended to be lower in stores that were certified than in stores that were not. Furthermore, regardless of whether certification was acquired, there was a significantly higher rate of C spp. contamination in stores that dealt with Jidori and Meigara, and in the group with the rate of lower contamination many of the rearing patterns were windowless.

Discussion: These results indicated that the probability of combined contamination with L spp. and C spp. is very high, which is in line with the fact that L spp. are common bacteria found in the environment and C spp. are widely distributed in the intestinal tract of chickens. The results also revealed typical distribution channel-dependent contamination, and the isolation rates of C spp. in distribution channels that were not HACCP-certified tended to be higher than those in HACCP-certified distribution channels. Furthermore, regardless of whether or not they were certified, the contamination rates were higher in the distribution channels that handled Jidori and Meigara. Based on the results of a survey by the Japanese Ministry of Agriculture, Forestry and Fisheries, which showed that hygienic measures were less advanced on Jidori and Meigara poultry farms than on broiler poultry farms, and the results of this study, which showed that the contamination rate tended to be low with windowless rearing systems, these factors were thought to be reflected in the high

contamination rates of both Meigara and Jidori by bacteria of both genera.

Conclusion: In Chapter 1, the actual situation of combined contamination by C spp. and L spp. was clarified from the examination of C spp. and L spp. contamination in retail chicken categorized into 'General', Meigara, and Jidori categories, and in Chapter 2, it was shown for the first time that Lm resistant to BC, a disinfectant, has been present in Japan since at least 2004, and that its appearance frequency has been increasing in recent years. Furthermore, in Chapter 3, based on the results obtained in Chapter 1, distribution channel-dependent contamination was identified, and in particular, delays in the implementation of sanitary measures in the distribution channels and rearing systems of Meigara and Jidori were highlighted. Japanese local chicken (Jidori) is a brand that has become globally marketed and accepted as '***Jidori***'.

Given that HACCP is scheduled to be fully mandatory from June 2021, the author believes that HACCP sanitation management should be strengthened in the future by focusing on the small-scale distribution and sales channels handling Jidori and Meigara.