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RESEARCH

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Quantitative release assessment for beta-lactamase-producing *Escherichia coli* of dairy origin into vegetables

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Abstract

Outbreaks of food poisoning associated with vegetables contaminated with *Escherichia coli* have been reported globally. This study was conducted to assess the probability of releasing beta-lactamase-producing *E. coli* of dairy farm origin into vegetables in Japan. A release assessment model connecting dairy farms to vegetable farms was developed. Data on the indicated diseases and antimicrobial use in dairy cattle were obtained from the agricultural insurance program in Hokkaido Prefecture between 2016 and 2019. Data on *bla*-harboring *E. coli* in cattle were obtained from the Japan Veterinary Antimicrobial Resistance Monitoring System. Microbiological field sampling was conducted at dairy and vegetable farms. Inoculation experiments in vegetables were conducted. All processes in the model simulation were iterated for 1000 times. The estimated proportion of dairy farms holding cattle with *bla*-harboring *E. coli* was 6.00% (95% CI: 4.81–7.35%). Beta-lactams were used in 71.76% (333,098/464,204 average annual cases) of indicated diseases. The estimated concentration of *bla*-harboring *E. coli* in mixed fresh manure at affected farms was 3.33 $\log_{10}$ CFU/g (95% CI: 3.33–3.34). The concentrations were reduced to 0.83 (95% CI: 0.34–1.25) and −0.54 $\log_{10}$ CFU/g (95% CI: −1.04 to −0.05) in soil after immature manure, and slurry spraying, respectively. The estimated concentrations in the soil of fields of radish, leafy vegetables, tomato, and spring-seeded and autumn-seeded onions at harvest were −1.99 (95% CI: −2.48 to −1.57), −2.87 (95% CI: −3.36 to −2.42), −3.82 (95% CI: −4.32 to −3.40), −5.36 (95% CI: −5.85 to −4.93), and −5.94 (95% CI: −6.43 to −5.52) $\log_{10}$ CFU/g, respectively. The concentrations in the bodies of leafy green spinach and lettuce were −9.88 (95% CI: −10.38 to −9.43) and −10.91 (95% CI, −11.52 to −8.05) $\log_{10}$ CFU/g, respectively. The probability of ingesting *bla*-harboring *E. coli* of dairy origin with raw vegetables in Japan was thus assessed to be very low.

One Health impact statement

This study applied a release assessment in the World Organisation for Animal Health framework for antimicrobial resistance risk assessment to connect the antimicrobial use for dairy cattle, manure production and vegetable farming. As vegetable is often consumed raw, the assessment is directly associated with human health. Although the environmental continuum was not explored, the reduction of the hazard on the soil of vegetable farms was considered. The study involved the engagement with dairy and vegetable farmers and the agriculture industry in understanding farming behavior and value chain. The approach in this study can be applied to the complex problems across the animal-human-environment interface.

Keywords: risk assessment, vegetable, *Escherichia coli*, dairy, beta-lactams, manure

Introduction

Antimicrobial resistance (AMR) among bacteria is a global threat; thus, prudent use of antimicrobials in treating livestock is necessary to ensure food safety in humans under global harmonization guidelines (WHO, 2015). The selection of antimicrobial-resistant bacteria (ARB) by the use of antimicrobials in livestock has received considerable attention. Such livestock-derived ARB may be controlled not only by more prudent use of antibiotics in animals but also by ensuring hygienic processing of livestock products, along with proper heating, which are common solutions for the prevention of food poisoning.

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Vegetables, in contrast to livestock products, are often eaten raw, and outbreaks of food poisoning associated with vegetables have been reported globally. According to a study examining the sources of foodborne illnesses in the United States between 1998 and 2008, of 9.6 million annual illnesses, 4.9 million (51.1%) could be attributed to plant commodities, with leafy vegetables serving as the most common source of illnesses, linked to >2 million annual cases (Painter *et al*., 2013). In 2011, a large outbreak of enterohemorrhagic *Escherichia coli* associated with the consumption of sprouts occurred in Germany, with 4321 cases, including 852 cases involving hemolytic-uremic syndrome, and at least 50 of the cases were fatal (Buchholz *et al*., 2011). The Shiga toxin-producing *E. coli* (STEC) strain O104:H4, which caused that outbreak, harbored the TEM-1 and CTX-M-15 beta-lactamase genes (Mellmann *et al*., 2011), suggesting there is a risk of transmission of ARB to humans through vegetables.

Bacteria and fungi cause significant plant diseases, and antimicrobials that are used in human and veterinary medicine, such as streptomycin, tetracyclines, and triazoles, have also been used to control plant diseases, especially in low- and middle-income countries (FAO, 2018). In Japan, streptomycin, kasugamycin and validamycin, which are aminoglycosides, and oxytetracycline have been registered as pesticides (MAFF, 2023b). A hydroponic experiment exposing pakchoi (*Brassica chinensis* L.) to tetracycline, cephalexin, and sulfamethoxazole revealed that pakchoi take up and accumulate these antimicrobials, and associated antimicrobial-resistance genes (ARGs) in the pakchoi endophytic system increases with the antibiotic concentration (Zhang *et al*., 2017).

In addition to antimicrobials applied in horticulture, manure from livestock may carry the risk of promoting AMR when applied to soil on which vegetables are grown. An experiment using lettuce suggested that the application of poultry or swine manure significantly increases ARG abundance in the soil and the lettuce endosphere (inside the plant) but not the lettuce phyllosphere (total above-ground surface) (Huang *et al*., 2021). Most phyllosphere microorganisms are non-pathogenic bacteria, including *Alphaproteobacteria*, *Gammaproteobacteria*, and the phyla *Bacteroides*, and *Actinobacteria* (Vorholt, 2012). However, ARGs in the phyllosphere of manure-treated lettuce are reportedly eight times more abundant than in conventionally fertilized lettuce (Zhu *et al*., 2017), suggesting that manure-derived ARGs can contaminate the plant surface as well as the endosphere. The level of contamination of the endosphere and phyllosphere of vegetables with pathogenic ARB of livestock origin appears to depend on several factors associated with antimicrobial use in animals, the quality of manure produced, the use of manure and contaminated irrigation water, and the completeness of vegetable surface washing (Doan *et al*., 2020).

There are numerous genes in *E. coli* of human and animal origin that confer resistance to beta-lactams. Some of them, such as $bla_{\text{TEM-1}}$, are widespread in *E. coli* from animals but code only for narrow-spectrum beta-lactamases that can inactivate penicillins and amino-penicillins. However, in recent years, genes that code for extended-spectrum beta-lactamases (ESBLs) or overexpressed cephalosporinases have emerged in *E. coli* from humans and animals (Poirel *et al*., 2018). Beta-lactams are widely used to treat bacterial infections in both humans and animals. The presence of beta-lactams-resistant *E. coli* poses a risk of failing to control bacterial infections in these populations.

In Japan, the dairy livestock sector has the best clinical data availability. The objective of the study was to assess the probability of releasing beta-lactamase-producing *E. coli* of dairy farm origin into vegetables in Japan.

Methods

FRAMEWORK OF THE RISK ASSESSMENT

This study employed the World Organisation for Animal Health (WOAH) risk assessment framework (WOAH, 2022). The hazard was defined as beta-lactamase-producing *E. coli* derived from dairy cattle, and the risk was defined as the probability and magnitude of the adverse health effects caused by the infection with the hazard through the consumption of raw vegetables in Japan. The risk assessment consists of release assessment, exposure assessment, consequence assessment, and risk estimation (WOAH, 2022). As the outcome of individuals infected with the hazard can be very complex and thus variable, the release assessment was selected in this study. In this study, the final outputs are the probability that the immature manure from dairy farms contains beta-lactamase-producing *E. coli* and the concentration of beta-lactamase-producing *E. coli* of dairy farm origin in and on the surface of vegetables before washing.

FAULT TREE ANALYSIS

A fault tree diagramming approach (Makita *et al*., 2018) was used to clarify the potential processes of scenarios in which the use of beta-lactam antibiotics in cattle would result in ingestion of the hazard through the consumption of raw vegetables so that critical variables are not missed in the risk simulation model. The fault tree process started with adverse human health effects associated with the hazard and traced back to the source step by step. Published literature and expert opinions obtained via interviews described in the next section were used to construct the fault tree. A list of beta-lactam antibiotics approved for use in cattle in Japan and the indicated diseases for these drugs was produced by collecting information from a published text (Japanese Society of Antimicrobials for Animals, 2013), and the list was placed in Supplementary Table 1.

VALUE CHAIN ANALYSIS

A literature review was conducted to understand the vegetable value chains, so that the release assessment captures the real-life situations. The system for marketing vegetables was evaluated using a published text (Agricultural Marketing Society of Japan, 2019) and supplemented with information from the website of the National Federation of Agricultural Cooperative Associations (NFACA) Fruit and Vegetable Center Ltd. (National Federation of Agricultural Cooperative Associations Fruit and Vegetable Center Ltd., 2023). Information regarding the production and marketing of fertilizers was collected from the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) (MAFF, 2023a). Information regarding the proportions of vegetable farms using organic manure was collected from the press release of an investigation of enterohemorrhagic *E. coli* and *Salmonella* in vegetables in Japan conducted by the MAFF (2010). Two vegetable producers, a company selling organic fertilizer, and officers at the Betsukai Town Office were interviewed to support our understanding of the value chains of vegetables, organic fertilizers, the use of antimicrobials for horticulture, and timing of manure application and harvesting of vegetables between November 2020 and December 2021. These producers, a company employee, and officers were repeatedly involved in the discussions to better understand vegetable production and marketing systems.

FIELD EXPERIMENT

The release assessment used the data from both published literature and the field experiments conducted in this study to quantify the microbiological dynamics from cattle feces to vegetables. To quantify the reduction in the number of *E. coli* from cattle feces to immature manure, published data from a study conducted in four cattle farms in Hokkaido Prefecture, Japan, in different seasons (May, August, and November 2019 and February 2020) (Katada *et al*., 2021) were used. The reduction in the number of *Enterobacteriaceae*, including *E. coli*, was used in this study.

Six samples of mature manure were collected using sterilized bottles to quantify *E. coli* at the Rakuno Gakuen University Field Education and Research Center (FEDREC) in 2020.

To quantify the reduction in *E. coli* from cattle feces to slurry and slurry to soil, three dairy farms in Hokkaido Prefecture were visited, and one sample each of cattle feces and slurry was collected at the respective farms, and seven samples of soil (three samples from one farm and two samples from the other two farms) were collected using sterilized bottles in 2021.

To quantify the reduction in *E. coli* from manure to the soil on the day the manure was applied, a sample of liquid compost of cattle feces (after anaerobic digestion using a biogas plant) and two samples of the soil on which the liquid compost was applied were collected using sterilized bottles on the same day at the FEDREC in 2020.

To model the reduction in *E. coli* abundance in the soil after the application of manure, the soil was sampled using sterilized bottles in 2020 at six time points after manure application (0, 7, 28, 60, 100, and 160 days) from four predetermined locations of a corn field at FEDREC.

For microbiological tests, 1 g of each sample was homogenized in 9 ml of buffered peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The resulting homogenates were plated on CHROMagar™ ECC agar (CHROMagar, Paris, France), and incubated at 37°C for 20 h, and the number of *E. coli* colonies was determined according to colony color and morphology.

VEGETABLE UPTAKE EXPERIMENT

To quantify the level at which *E. coli* cells are taken up by the vegetable body, experiments were conducted using tomato, lettuce, and spinach. A suspension of *E. coli* was prepared, the bacterial concentration was determined, and the soil of pots in which spinach, lettuce, and tomato were growing was inoculated with 50, 25, or 250 ml of the suspension, respectively, based upon the size of the pots. The bacteria concentration prepared ranged between log 10 and 11 CFU/ml. In a published experiment, inoculation of 500ml of log 9 CFU/ml of *Salmonella enterica* Typhimurium strain aPTVS177 resulted in log 4 CFU/g of soil. Inoculation of 20 µl of log 4 CFU/ml of aPTVS177 on injured peduncle caused internalization in melon (Lopez-Velasco *et al*., 2012). The bacteria concentration in our experiment was selected to exceed the level used in the previously published experiment. After disinfecting the surface of the vegetables, the concentrations of *E. coli* were measured on day 1 (day of inoculation), 2, 3, 6, and 10. The concentration of *E. coli* present in the soil on day one was also measured. Seven pots each were prepared for duplicate experiments for each vegetable.

QUANTITATIVE RELEASE ASSESSMENT FOR BETA-LACTAMASE-PRODUCING *E. COLI* OF DAIRY CATTLE ORIGIN INTO VEGETABLES

A quantitative release assessment was conducted by sequentially modeling (1) the selection of beta-lactamase-producing *E. coli* in dairy cattle exposed to beta-lactam antibiotics, (2) the reduction in the number of *E. coli* resulting from composting and natural decay in the soil, and (3) uptake of *E. coli* into the vegetable body.

SELECTION OF BETA-LACTAMASE-PRODUCING *E. COLI* BY USE OF BETA-LACTAM ANTIBIOTICS ON DAIRY FARMS

Dairy cattle in Hokkaido Prefecture that were registered in the Hokkaido Agricultural Mutual Relief Association (AMRA) were used for modeling purposes. The numbers of calves, heifers, and adult cows, cases of diseases for which treatment with beta-lactam antibiotics is indicated, and the records of actual use of beta-lactam antibiotics between 2016 and 2018 were provided by the AMRA. The indicated diseases for beta-lactam antibiotics included calf and heifer pneumonia/bronchitis, calf and heifer diarrhea, clinical and sub-clinical mastitis, heifer mastitis, post-partum fever, inter-digital phlegmon, and surgery. The incidence rates of the indicated diseases were modeled using gamma distributions by the phase of growth – calf, heifer, and adult cow (Table 1). Beta-lactam antibiotics used for the treatment of the indicated diseases included ampicillin, amoxicillin, benzylpenicillin, cephazolin, cefloxim, and ceftiofur (Supplementary Table 1). The probability of treatment with beta-lactam antibiotics at the occurrence of indicated diseases during certain phases of animal growth was modeled using a beta-distribution (Table 1).

The selection of beta-lactamase-producing bacteria was modeled to occur if *E. coli* in the intestine of an animal harbors the *bla* gene. Based on the expert opinions of four AMR researchers, if a dairy farm has a cow that harbors the *bla* gene in *E. coli* in the intestine, all cattle on the farm are assumed to have *E. coli* harboring the *bla* gene. The proportion of dairy farms in Japan in which cattle have *E. coli* harboring the *bla* gene was modeled using a beta distribution with the number of *bla*-positive and -negative *E. coli*, as obtained from the Japan Veterinary Antimicrobial Resistance Monitoring (JVARM) data for fecal tests of cattle sampled at slaughterhouses between 2015 and 2019. In the JVARM, a fecal sample is collected at slaughterhouses from one cattle each from a dairy or beef cattle farm. As of 2019, there were 1.3 million cattle in 15,000 dairy farms and 2.5 million cattle in 46,300 beef cattle

Table 1. Distributions and sources of information used in the dairy farm release assessment model.

Item	Distribution	Data	Source
Incidence rates of indicated diseases (*j*) in calves, heifers, and adult cows (*i*): IR_{ji}	Gamma (cases, number of animals)	Summary shown in the results	AMRA database
Probability of treatment with the beta-lactam antibiotic *k* at the occurrence of indicated diseases (*j*) in a growth phase *i*: $Puse_{jki}$	Beta (treatment+1, cases–treatment+1)	Summary shown in the results	AMRA database
Proportion of dairy farms with cattle harboring the *bla* gene, under the assumption that all cattle in the positive farm harbor-resistant *E. coli*	Beta (positive+1, negative+1)	75/1251 *E. coli* isolates were *bla* positive	JVARM 2015–2019
Change in the concentration of beta-lactamase-producing *E. coli* in the year following treatment with beta-lactam antibiotics	Discrete (same value for 5 days each from the treatment until day 35, then fixed value of day 0)	3.28 (days 1–5), 4.94, 4.50, 4.17, 4.00, 3.78, 3.61 (days 31–35)	Ahmed *et al*. (2016)
Volume of cattle feces in a growth phase *i*: $Feces_i$	Point estimates: Calf = 8 kg; Heifer = 16 kg; Adult cow = 40 kg	Milking cows >700 kg = 50 kg; milking cows 600–700 kg = 36 kg; dry period = 21 kg; heifer = 16 kg	Livestock Industry's Environmental Improvement Organization, 1998

farms in Japan (MAFF, 2020). There is a limitation in the JVARM that the data of *E. coli* only in dairy cattle are not available. The JVARM uses the published methodology for the detection of *bla* gene (Dallenne *et al*., 2010).

The estimated change in the number of resistant *E. coli* in the intestine of a live animal after treatment with a beta-lactam antibiotic has been published elsewhere (Ahmed *et al*., 2016). According to that study, the initial count of resistant *E. coli*, at 3.28 $\log_{10}$ CFU/g, immediately increases to approximately 4.94 $\log_{10}$ CFU/g and then decreases to the original level by approximately day 40 after intra-muscular injection of the antibiotic. The dynamics of the $\log_{10}$ CFU/g bacterial counts were modeled every 5 days from the injection of antibiotics up to day 35, and between days 36 and 365, the baseline bacterial count of 3.28 $\log_{10}$ CFU/g was maintained.

The $\log_{10}$ CFU/g of beta-lactamase-producing *E. coli* in fresh feces at dairy farms with cattle harboring *E. coli* with the *bla* gene was estimated as follows (Eqns 1 and 2):

$$\log_{10} CFU/g_i = \sum_{j=1}^{m}\sum_{k=1}^{n} IR_{ji} Puse_{jki} \times \log_{10} CFU/g_{BLused} + \left(1 - \sum_{j=1}^{m}\sum_{k=1}^{n} IR_{ji} Puse_{jki}\right) \times \log_{10} CFU/g_{base}, \quad (1)$$

where IR_{ji} denotes the incidence rate of an indicate disease *j*, $Puse_{jki}$ denotes the probability of using beta-lactam antibiotic *k* when indicated disease *j* occurs during growth phase *i* (calf, heifer, or adult cow), and $\log_{10} CFU / g_{BLused}$ averaged bacterial concentration of a cow treated with a beta-lactam antibiotics. The concentration of beta-lactamase-producing *E. coli* differs between calves, heifers, and adult cows due to differences in the incidence rates of diseases and differences in the probability of treatment with beta-lactam antibiotics. In reality, feces from calves, heifers, and adult cows are mixed and sent to either a slurry tank or compost shed, and the concentration of beta-lactamase-producing *E. coli* in combined fresh feces ($\log_{10} CFU / g_{bla}$) can be calculated as shown in Eqn 2:

$$\log_{10} CFU/g_{bla} = \log_{10} \frac{\sum_{i=1}^{3} (10^{\log_{10} CFU/g_i} \times N_i Feces_i)}{\sum_{i=1}^{3} N_i Feces_i} \quad (2)$$

where N_i and $Feces_i$ denote the number of animals and volume of feces from an animal in growth phase *i* (calves, heifers, and adult cows), respectively.

REDUCTION IN *E. COLI* BY COMPOSTING AND NATURAL DECAY IN SOIL

The $\log_{10}$ CFU/g of *Enterobacteriaceae* after composting was subtracted from the bacterial concentration before composting for all 15 pairs in the published data (Katada *et al*., 2021) to calculate the extent of bacterial reduction due to composting. The *Enterobacteriaceae* data were used because data regarding *E. coli* were not available. Here, we faced the limitation of the use of data for the reduction in *Enterobacteriaceae* to simulate the reduction in *E. coli*. The mean was calculated 1000 times for 30 randomly sampled values from the bacterial reduction levels for 15 pairs in the log scale. A set of the 1000 mean reduction levels in $\log_{10}$ CFU/g due to composting was deducted from the set of 1000 simulation results for $\log_{10}$ CFU/g of *E. coli* in fresh feces at dairy farms to obtain the estimated $\log_{10}$ CFU/g of *E. coli* in immature manure after composting.

Similarly, the mean was calculated 1000 times for 30 randomly sampled values for the level of bacterial reduction from fresh feces to slurry at three dairy farms. A set of the 1000 mean reduction levels in $\log_{10}$ CFU/g resulting from slurry decomposition was deducted from a set of 1000 simulation results of $\log_{10}$ CFU/g of *E. coli* in fresh feces at the dairy farms to obtain the estimated $\log_{10}$ CFU/g of *E. coli* in slurry.

To model the reduction in *E. coli* from manure to the soil upon which immature manure was applied on that day, the level of reduction in the number of *E. coli* from the biogas plant to two field sites was analyzed to calculate the mean for 30 resampled $\log_{10}$ CFU/g values 1000 times. Similarly, to model the reduction in *E. coli* from the slurry to the soil upon which the slurry was applied on that day, the level of reduction at seven field sites on three dairy farms upon which the slurry was applied on that day was analyzed to calculate the mean for 30 resampled $\log_{10}$ CFU/g values 1000 times. Using the abovementioned steps, the concentrations of *E. coli* in soils upon which both immature manure and slurry or fresh feces were applied on that day were simulated.

To predict the concentration of *E. coli* in the soil on a given day after application of manure, slurry, or fresh feces, a mixed-effects generalized linear regression with Poisson errors was performed, selecting the number of *E. coli* as the outcome variable, days after the application of manure from the biogas plant as the explanatory variable, and the field sites as a random effect, using the field experimental data. Using slope in the log scale, the concentrations of *E. coli* after application of manure, slurry, or fresh feces with simulated bacterial concentration were predicted for the day of harvesting for leafy vegetables, tomato, radish, spring-seeded onion, and autumn-seeded onion. These simulations estimated the bacterial concentration on the surface of vegetables, which can be reduced by washing or removing the outer portion in a reality. All statistical analyses and simulations were performed using the statistical software R, version 4.2.2. (R Core Team, 2021).

UPTAKE OF *E. COLI* INTO THE BODY OF VEGETABLES

In the experimental application of *E. coli* on soil in which vegetables were grown, the level of uptake in the edible part was highest on day 2 (see Results). The level of uptake was calculated by deducting the $\log_{10}$ CFU/g for day 2 in the vegetable body from the value in soil on day 1 after the application of *E. coli*. The concentration of *E. coli* in the vegetable body was predicted using the predicted number of *E. coli* in the soil on the day of harvesting and the level of uptake.

As an additional sensitive analysis, the concentration of *E. coli*, including non-beta-lactamase-producing *E. coli*, was estimated, as in reality, any *E. coli* can cause infection in humans. In addition, the concentration of beta-lactamase-producing *E. coli* was estimated in a scenario in which untreated slurry was applied to the soil of a vegetable-growing field. For this simulation, the bacterial concentration was modeled by deducting the level of reduction from the slurry to the soil upon which the slurry was applied on that day from the concentration of beta-lactamase-producing *E. coli* in fresh feces.

Results

FAULT TREE

Figure 1 shows the fault tree for adverse health effects due to the consumption of vegetables contaminated with beta-lactamase-producing bacteria of dairy farm origin. Adverse health effects, including prolongation of symptoms or death, can occur when beta-lactam antibiotics prescribed by a physician are not effective in treating cases of either food poisoning or extra-intestinal infections caused by beta-lactamase-producing bacteria (central and right branches of Fig. 1a). The first line treatment of bacterial diarrhea is rehydration, and the use of antibiotics should be avoided for STEC, as antimicrobial treatment may cause hemolytic uremic syndrome. However, antibiotics are used for infants and immunocompromised people with severe illness and bloody diarrhea (Shane *et al*., 2017). In cases involving food poisoning due to toxins produced by beta-lactamase–producing bacteria, this branch was not traced further (left branch of Fig. 1a), as antibiotics are not used for the treatment of illness related to toxin exposure. Bacterial food poisoning occurs due to intestinal infection with beta-lactamase–producing bacteria colonizing either the interior of the vegetable body or the surface of the vegetable (central branch of Fig. 1a). Extra-intestinal

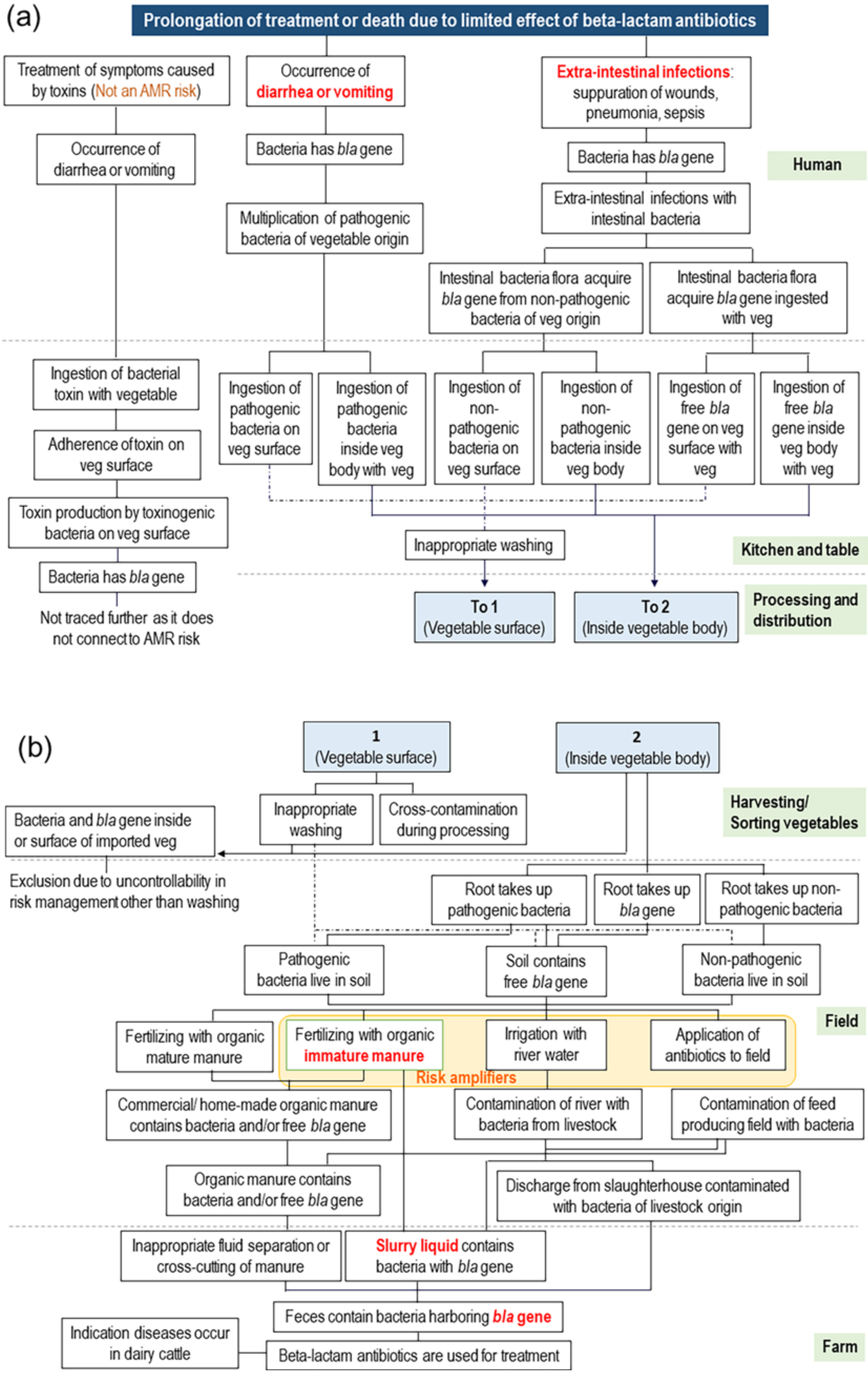

Fig. 1. Fault tree for adverse health effects due to consumption of vegetables contaminated with beta-lactamase-producing bacteria of dairy farm origin: (a) from adverse health effects in humans, kitchen and table, to processing and distribution of vegetables, and (b) from harvesting/sorting of vegetables, vegetable fields, to dairy farms.

infections, such as suppuration of wounds, pneumonia, and sepsis, can occur when the skin or internal organs or systematic body are infected with intestinal beta-lactamase–producing bacteria that do not cause food poisoning. Theoretically, acquisition of the *bla* gene by humans can occur either when an individual ingests non-pathogenic (for intestines) beta-lactamase-producing bacteria colonizing the interior of the vegetable body or the vegetable surface or when intestinal bacteria flora acquires free *bla* genes present inside the vegetable body or on the surface (right branch of Fig. 1a). The ingestion of beta-lactamase-producing bacteria and the free *bla* genes on the surface of vegetables can be largely prevented by careful washing of vegetables before consumption.

The scenario in which a human ingests beta-lactamase-producing bacteria present either inside or on the surface of vegetables imported into Japan was not traced further, as the risk cannot be managed domestically other than by washing (top left of Fig. 1b). Pathogenic and non-pathogenic beta-lactamase-producing bacteria that live in soil and free *bla* genes in the soil that contaminate the interior or surface of vegetables can be derived from either manure or irrigation water, and the application of antibiotics onto soil may select such bacteria, thus acting as a risk amplifier (Fig. 1b). Immature manure can come from either composted manure or slurry from dairy farms. Rivers may be contaminated with beta-lactamase-producing bacteria through either inappropriate discharge from dairy farms or slaughterhouses and overflow from slurry tanks. Finally, the selection of beta-lactamase-producing bacteria on dairy farms was traced to the use of beta-lactam antibiotics for the treatment of indicated diseases (bottom of Fig. 1b).

FERTILIZER AND VEGETABLE VALUE CHAINS

Figure 2 shows the fertilizer and vegetable value chains in Japan. Organic manure is distributed to vegetable producers through Japan Agricultural (JA) Cooperatives, public fertilizer centers, fertilizer retailers, or directly by livestock farms. According to a study by the MAFF (MAFF, 2010), 74, 46, 35, 67, and 69% of lettuce, cabbage, spring onion, tomato, and cucumber farms, respectively, used cattle manure.

The majority of vegetable farmers sell vegetables to JA Cooperatives. The JA Cooperatives then sell the vegetables through wholesalers in wholesale markets and the NFACA Fruit and Vegetable Center Ltd., which belongs to the JA Cooperative group. Intermediate wholesalers and merchandisers sell the vegetables to retailers, processors, and food service industries, who deliver whole or cut raw vegetables and ready-to-eat foods to consumers. Some vegetable farmers directly market vegetables to consumers through farm stands or home delivery using transportation services (Fig. 2).

QUANTITATIVE RELEASE ASSESSMENT FOR BETA-LACTAMASE-PRODUCING *E. COLI* OF DAIRY CATTLE ORIGIN INTO VEGETABLES

According to the Hokkaido AMRA, 791,000, 779,400, and 785,700 heads of dairy cattle were registered and insured in Hokkaido Prefecture in 2016, 2017, and 2018, respectively. Of these animals, there were 164,800 (20.8%), 153,300 (19.7%), and 158,000 (20.1%) calves, respectively, and 164,700 (20.8%), 166,700 (21.4%), and 156,800 (20.0%) heifers, respectively. On average, there were 464,204 annual cases of indicated diseases, and beta-lactams were used in 333,098 of these cases (71.76%).

Table 2 shows the annual incidence rates of diseases for which the use of beta-lactam antibiotics is indicated among relevant sub-populations. Notable diseases with a high frequency included calf pneumonia (incidence rate [IR] 37.0, and 95% credible interval [CI]: 36.9–37.2%), calf diarrhea (IR = 31.7, and 95% CI: 31.6–31.9%), and clinical and sub-clinical mastitis (IR = 57.6, and 95% CI: 57.5–57.8%). Surgery conducted on adult cows was also common (IR = 12.9, and 95% CI: 12.8–13.0%).

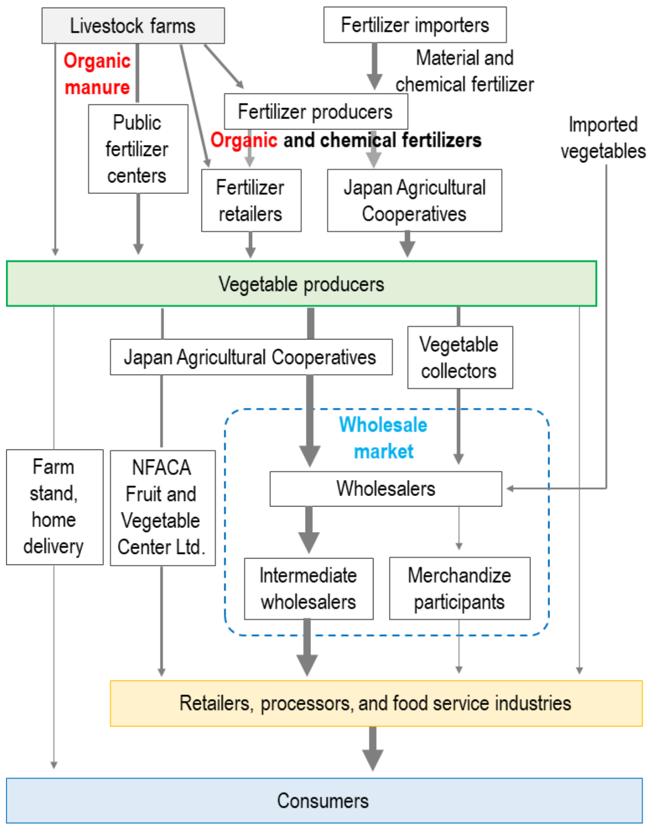

Fig. 2. Fertilizer and vegetable value chains in Japan.

Figure 3 shows a comparison of the probability of the use of beta-lactam antibiotics upon the occurrence of frequently indicated diseases. Intra-mammary injection of cefazolin for adult cow mastitis exhibited the highest probability of use (73.23, and 95% CI: 73.14–73.34%). Intra-mammary injection of benzylpenicillin for adult cow mastitis had the second highest probability of use (32.70, and 95% CI: 32.59–32.79%). Intra-mammary injection of cefuroxime was used in 12.87% (95% CI: 12.80–12.97%) of mastitis cases, suggesting that intra-mammary injection of beta-lactam antibiotics such as cefazolin, benzylpenicillin, and cefuroxime is performed at almost all occurrences of adult cow mastitis. The third highest probability of beta-lactam antibiotic use was the administration of benzylpenicillin at surgery (29.27, and 95% CI: 29.04–29.46%).

Between 2015 and 2019, 75 of 1261 *E. coli* isolated from cattle by the JVARM harbored the *bla* gene. In JVARM, one cattle each from a farm is sampled in slaughterhouses. Of these 75 isolates, 72 harbored bla_{TEM} (96.0%), and bla_{SHV}, $bla_{CTX\text{-}M1}$, $bla_{CTX\text{-}M9}$, and bla_{CIT} were harbored by one isolate each. Based on the assumption that all cattle on a dairy farm will harbor *E. coli* with *bla* if the gene is detected in the herd, the proportion of dairy farms that have cattle with *E. coli* harboring the *bla* gene (i.e., beta-lactamase-producing *E. coli*) was estimated at 6.00% (95% CI: 4.81–7.35%).

Figure 4 shows the concentrations of beta-lactamase-producing *E. coli* in fresh feces, immature manure, and slurry tanks on dairy farms and in soil from fields on the day immature manure and slurry were applied. The concentration declined from 3.33 $\log_{10}$CFU/g (95% CI: 3.33–3.34) in fresh feces to 1.52 $\log_{10}$CFU/g (95% CI: 1.01–1.94) in immature manure and 1.20 $\log_{10}$CFU/g (95% CI: 0.89–1.51) in slurry. No *E. coli* were detected in mature manure in this study, so *E. coli* would not be transferred to the soil upon application of mature manure. According to an interview with a representative of a company selling manure, the maturity of manure is regularly assessed as a quality assurance measure, and all the manure

Table 2. Annual incidence rates of diseases for which the use of beta-lactam antibiotics is indicated among relevant sub-populations (data are shown to 2 decimal places due to narrow CIs).

Disease	Sub-population	50th percentile of the mean (%)	2.5–97.5th percentile (%)
Calf pneumonia	Calves	37.04	36.87–37.20
Heifer pneumonia	Heifers	4.29	4.23–4.35
Calf diarrhea	Calves	31.74	31.58–31.91
Heifer diarrhea	Heifers	1.01	0.99–1.04
Clinical and sub-clinical mastitis	Adult cows	57.63	57.50–57.75
Heifer mastitis	Heifers	0.18	0.17–0.19
Postpartum fever	Adult cows	2.58	2.56–2.61
Inter-digital phlegmon	Adult cows	1.51	1.49–1.53
Surgery (for any disease)	Adult cows	12.89	12.83–12.95

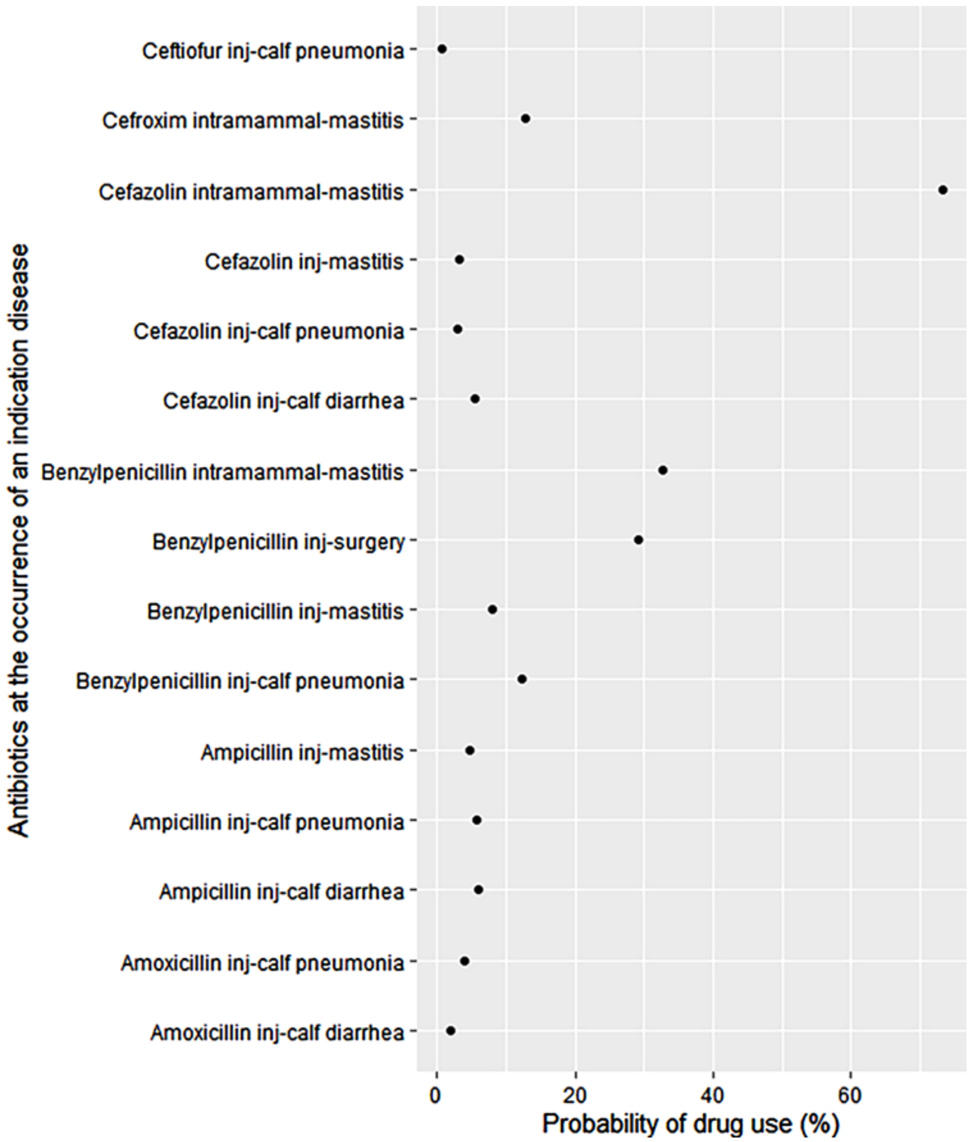

Fig. 3. Probability of the use of beta-lactam antibiotics at the occurrences of frequent indicated diseases. The credible interval is not shown, as the data were extensive and the intervals were <1%.

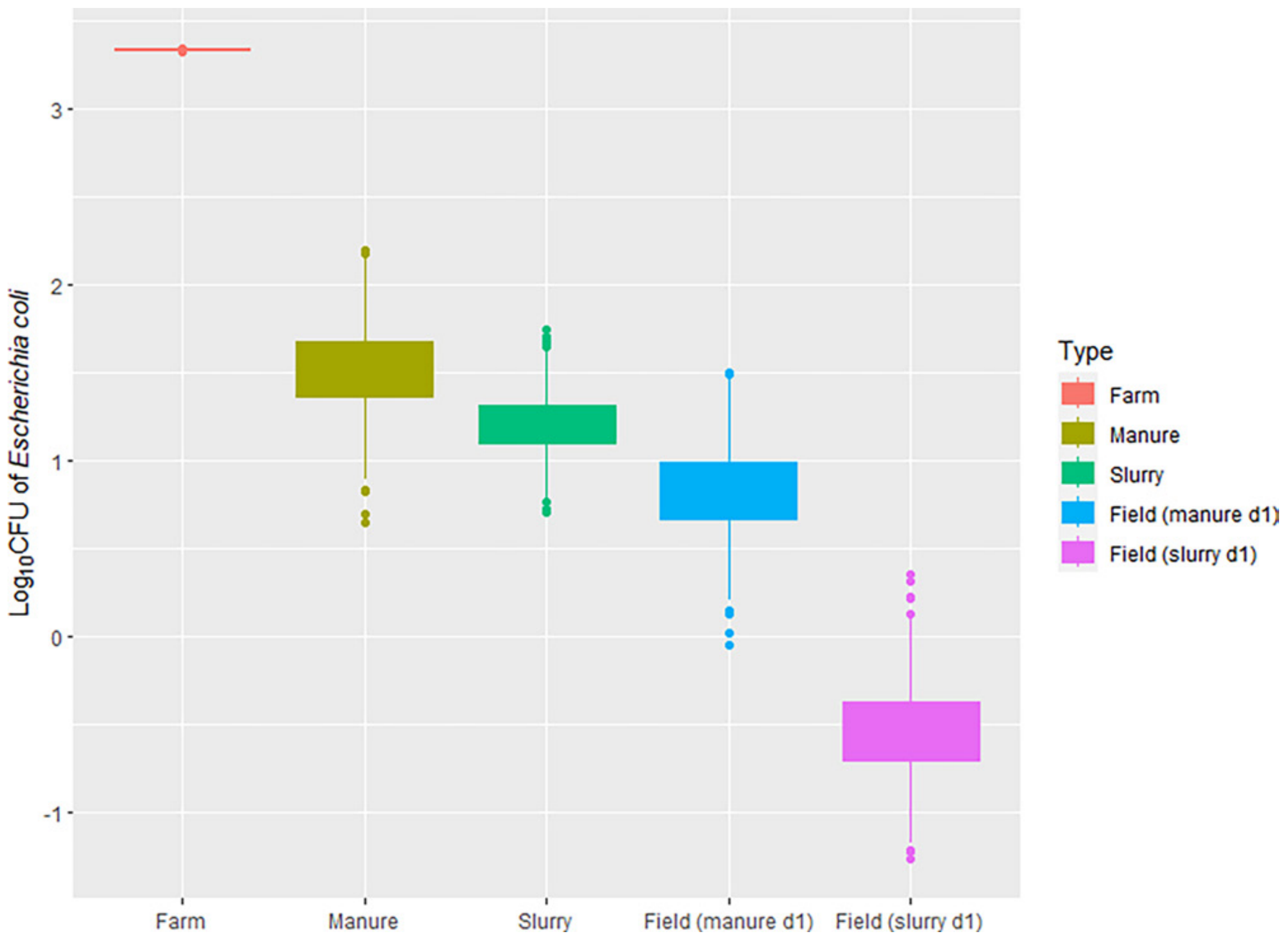

Fig. 4. Bacterial concentrations in log_{10}CFU/g of beta-lactamase-producing *E. coli* in fresh feces, immature manure, and slurry tanks on dairy farms and in field soil on the day of immature manure and slurry application.

products are mature when sold. However, in another interview with an organic farmer, it was revealed that immature manure obtained from livestock farms is sometimes applied on vegetable farms. This study thus assessed the bacterial concentrations under a scenario in which immature manure is applied onto the soil of a vegetable-growing field. In soil upon which immature manure and slurry were applied, the bacteria counts on the day of application were 0.83 log_{10}CFU/g (95% CI: 0.34–1.25) and −0.54 log_{10}CFU/g (95% CI: −1.04 to −0.05), respectively (Fig. 4).

According to the field experiment data, the *E. coli* concentration in soil decreased significantly over time (unit of time: day, slope in log = −0.05, standard error = 0.003, $p<0.001$). Based upon the slope, the reduction in the concentration of beta-lactamase-producing *E. coli* after spraying immature manure and slurry on the soil of vegetable-growing fields was simulated as shown in Fig. 5.

Manure is applied in the beginning of December for leafy vegetables, tomato, and onion that are seeded in spring, the beginning of June for radish, and the beginning of July for onion that is seeded in autumn. The timing of harvest is the beginning and middle of May for autumn-seeded onion and leafy vegetables, respectively, late June for tomato, the beginning of September for spring-seeded onion, and the beginning of October for radish. The bacterial concentration at harvest was the highest in soil growing radish (−1.99 log_{10}CFU/g [95% CI: −2.48 to −1.57]), followed by soil growing leafy vegetables (−2.87 [95% CI: −3.36 to −2.42]), tomato (−3.82 [95% CI: −4.31 to −3.40]), and spring-seeded (−5.36 [95% CI: −5.85 to −4.93]) and autumn-seeded onions (−5.94 [95% CI: −6.43 to −5.52], Fig. 6).

In the experiments in which soil growing spinach and lettuce were inoculated with *E. coli*, uptake of *E. coli* in the vegetable body occurred in 1 spinach sample and 6 lettuce samples. For the spinach sample, the bacterial concentrations in the soil and day 2 spinach body were 2.08 × 10^8 and 20 CFU/g, respectively. For the lettuce samples, the mean bacterial concentrations in the soil and the lettuce body were 8.38 log_{10}CFU/g and 25.17 CFU/g, respectively. The reduction in bacterial concentration in log_{10}CFU/g was calculated only using these samples that contained *E. coli* in the vegetable body. The concentrations of beta-lactamase-producing *E. coli* in the bodies of leafy vegetables at harvest were estimated to be very low in spinach (−9.88 log_{10}CFU/g [−10.38 to −9.43]) and lettuce (−10.91 log_{10}CFU/g [95%CI: −11.52 to −8.05], Fig. 7).

The results presented above show the concentrations of beta-lactamase-producing *E. coli*, but in reality, all *E. coli* can cause infections in humans. Table 3 shows the estimated concentrations of *E. coli*, including *E. coli* strains that do not produce beta-lactamases, under different conditions. At the time of harvest, soils growing leafy vegetables, tomato, and radish were estimated to harbor more than one *E. coli* cell per gram. The bacterial concentration in the vegetable body was still very low, however (−5.21 log_{10}CFU/g for spinach and −6.23 log_{10}CFU/g for lettuce).

In the scenario in which untreated slurry was applied onto the soil of vegetable-growing fields, at harvesting, the soil was estimated to contain less than one beta-lactamase-producing *E. coli* cell for all the vegetables examined, and the concentrations in vegetable bodies were very low (Table 4).

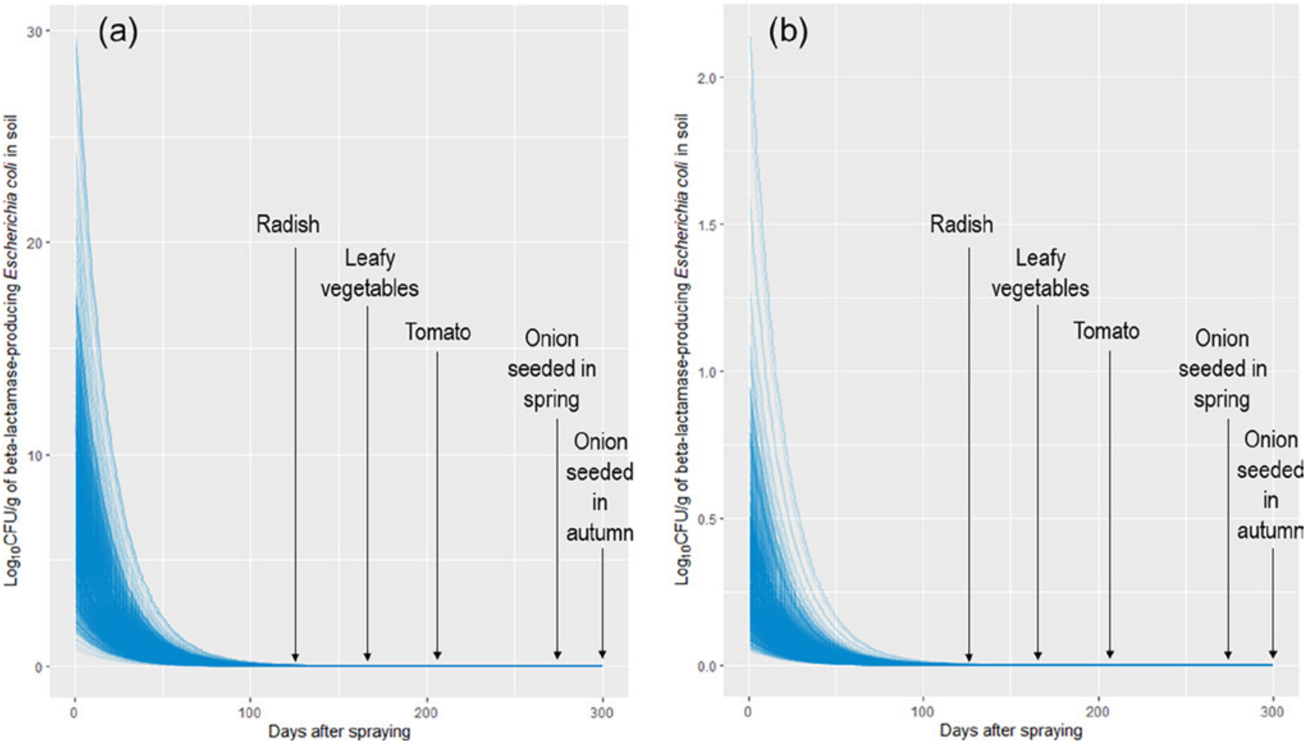

Fig. 5. Reduction in the concentration of beta-lactamase-producing *Escherichia coli* in $\log_{10}$CFU/g over time in soil after spraying (a) immature manure, and (b) slurry on vegetable-growing fields, and the concentration at harvest of radish, leafy vegetables, tomato, and onion seeded in spring and autumn.

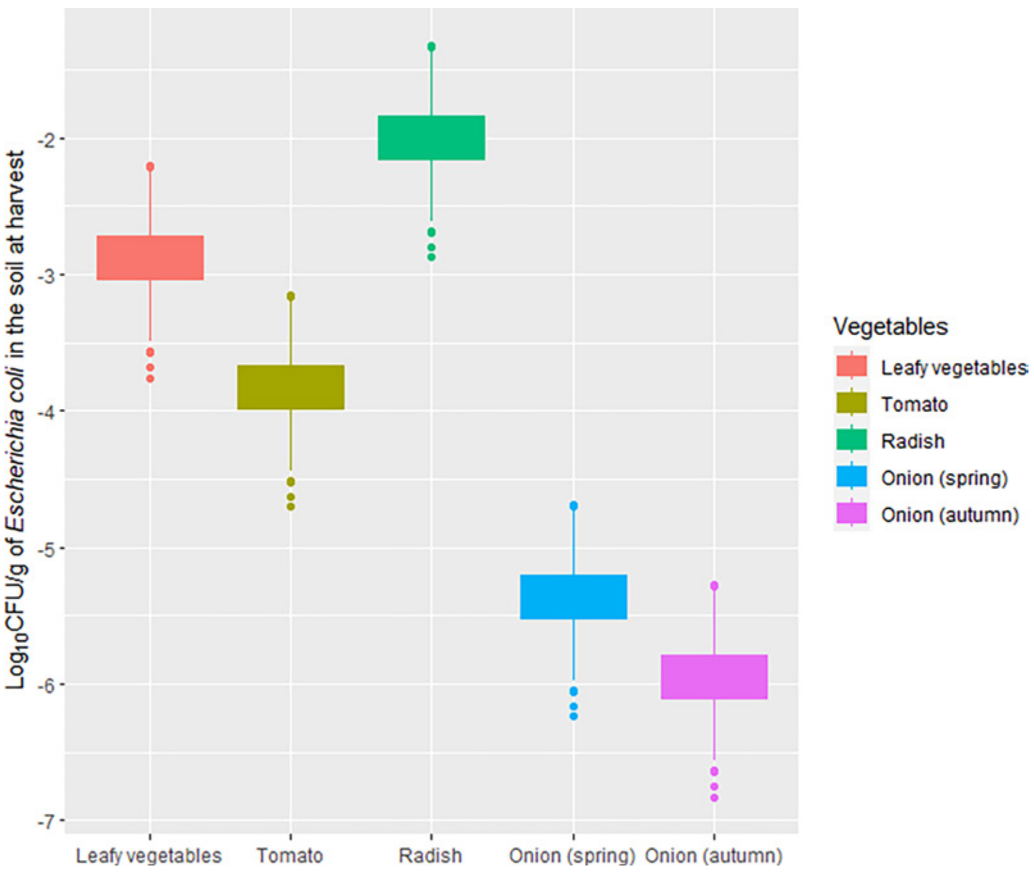

Fig. 6. Concentrations of beta-lactamase-producing *E. coli* in $\log_{10}$CFU/g at harvest in soil growing leafy vegetables, tomato, radish, and spring- and autumn-seeded onions predicted by the simulation model.

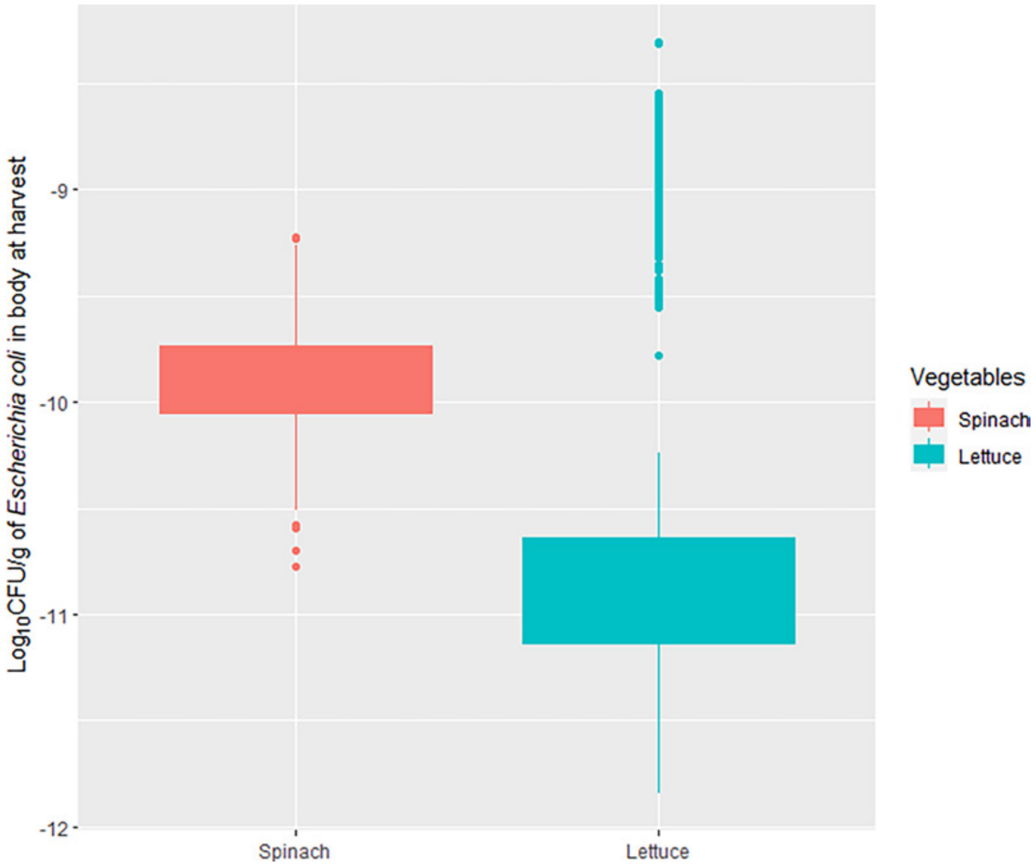

Fig. 7. Concentrations of beta-lactamase–producing *Escherichia coli* in $\log_{10}$CFU/g in the body of spinach and lettuce at harvest.

Table 3. Estimated concentrations of both beta-lactamase–producing and non-producing *Escherichia coli* under different conditions.

Condition	50th percentile in $\log_{10}$CFU/g (%)	2.5–97.5th percentiles (%)
Immature manure	6.20	5.70–6.65
Slurry	5.87	5.58–6.20
Soil on day of immature manure application	5.51	5.00–5.96
Soil on the day of slurry application	4.11	3.64–4.56
Soil at harvesting of leafy vegetables	1.81	1.30–2.26
Soil at harvesting of tomato	0.86	0.35–1.31
Soil at harvesting of radish	2.69	2.18–3.14
Soil at harvesting of spring-seeded onion	−0.67	−1.18 to −0.22
Soil at harvesting of autumn-seeded onion	−1.26	−1.77 to −0.81
Vegetable body of spinach at harvesting	−5.21	−5.72 to −4.75
Vegetable body of lettuce at harvesting	−6.23	−6.88 to −4.06

Discussion

Quantitative risk assessment modeling has been used to assess the risks of AMR arising from animal-source foods (Makita *et al*., 2020). This study applied a modeling technique to assess the extent of release of beta-lactamase-producing *E. coli* of dairy farm origin into vegetables.

In our study, the estimated farm-level prevalence of cattle with beta-lactamase-producing *E. coli* was 6.0%. This suggests that 6.0% of organic manure of dairy farm origin could contain beta-lactamase-producing *E. coli* when manure is not matured at the farm. Much higher prevalence rates of ESBL-producing *E. coli* in cattle have been reported: 86.7% at the farm level (39/45 farms, 30 dairy and beef mixed farms and 15 beef farms) in Germany (Schmid *et al*., 2013), and 70.7% (58/82) at the animal level in China (Liu *et al*., 2022). According to the JVARM data, most beta-lactamase-producing *E. coli* harbored the *bla* gene, and our study thus used *bla* as the indicator of beta-lactamase-producing *E. coli* in the release assessment. Given the estimated farm-level prevalence 6.0%, the assumption that all cattle in a farm contaminated with

Table 4. Estimated concentrations of beta-lactamase–producing *Escherichia coli* after untreated slurry was applied onto the soil of vegetable-growing fields under different conditions.

Condition	50th percentile in $\log_{10}$CFU/g (%)	2.5–97.5th percentiles (%)
Soil on the day of untreated slurry application	2.63	2.59–2.66
Soil at harvesting of leafy vegetables	−1.11	−1.07 to −1.04
Soil at harvesting of tomato	−2.02	−2.06 to −1.99
Soil at harvesting of radish	−0.19	−0.23 to −0.16
Soil at harvesting of spring-seeded onion	−3.56	−3.59 to −3.53
Soil at harvesting of autumn-seeded onion	−4.15	−4.18 to −4.11
Vegetable body of spinach at harvesting	−8.09	−8.13 to −8.06
Vegetable body of lettuce at harvesting	−9.10	−9.38 to −7.16

E. coli harboring bla gene may have the *E. coli*, and that JVARM collect fecal sample from one cattle each from a farm, animal-level prevalence may be similar to the farm-level prevalence. Therefore, the proportion of cattle with beta-lactamase-producing *E. coli* may be lower than that reported in these other countries.

As the composting experiment results showed, completely mature manure does not contain live *E. coli*. Commercially produced manure is quality controlled, but an unknown proportion of manure produced either in prefectural compost centers or on dairy farms may contain beta-lactamase-producing *E. coli*. Therefore, the proportion of organic manure of dairy farm origin with beta-lactamase-producing *E. coli* would be much lower than 6.0%, which is the proportion estimated in this study based on the sampling of cattle feces at slaughterhouses. However, the soils upon which immature manure was applied were estimated to harbor more than 100 *E. coli* cells per gram at the harvesting of leafy vegetables and radish, although the concentration of beta-lactamase-producing *E. coli* was less than one cell per gram. The probability of infection is considered to be dose-dependent (Buchanan *et al*., 2000), and the infectious dose of *E. coli* is reportedly 10–100 organisms (Gianella *et al*., 1972, Peterson *et al*., 1989). This suggests that infection with beta-lactamase-producing *E. coli* can occur via the consumption of inadequately washed raw vegetables. Moreover, in Japan, the farm- and animal-level prevalence of Shiga toxin-producing *E. coli* O157 among beef cattle was reportedly 27.1% (110/406 farms) and 8.9% (218/2436 cattle) (Sasaki *et al*., 2011). Consuming unwashed vegetables grown in soil upon which immature manure has been applied can thus result in infection. In the case the *E. coli* infecting a human is a beta-lactamase-producing strain, it can cause adverse health effects associated with AMR. However, considering the difference in bacterial concentrations estimated in the soil of radish-growing fields at time of harvest between all *E. coli* (2.69 $\log_{10}$CFU/g) and beta-lactamase-producing *E. coli* (−1.99 $\log_{10}$CFU/g), the population of beta-lactamase-producing *E. coli* would be $10^{4.68}$ times smaller than that of all *E. coli*, and the risk of AMR-related effects would actually be very low. The present study was conducted for the release assessment, and much more information is needed for quantitative exposure and consequence assessments for the entire risk assessment. Of course, from a public health perspective, food-borne diseases resulting from the consumption of raw vegetables contaminated with *E. coli* should be prevented, and therefore, public messaging urging consumers to wash vegetables before consumption should be regularly conducted.

In the scenario in which untreated slurry was applied onto the soil of radish-growing fields, the concentration of beta-lactamase-producing *E. coli* was $10^{1.80}$ times higher than that of soil onto which immature manure was applied: −0.19 $\log_{10}$CFU/g versus −1.99 $\log_{10}$CFU/g. As it is unlikely a consumer will ingest a large amount of soil with vegetables, the probability of infection with beta-lactamase-producing *E. coli* in this case may not be high. However, application of untreated slurry may pose a risk of accumulation and emission of antimicrobial drug resistance genes in the environment (Fatoba *et al*., 2022), and farmers lose the nutritional benefits of applying manure if it is not properly composted (Cáceres *et al*., 2018). According to our interviews with farmers, organic farmers are aware of the risk of damage to the vegetables and vegetable seeds by the application of immature manure. These farmers take adequate time between the application of manure and seeding or planting to avoid the risk of damage, and this also reduces the probability of the soil containing a high concentration of *E. coli*. Although the probability of ingesting beta-lactamase-producing *E. coli* with soil remaining on the vegetable surface is low, it is important to maintain the monitoring system and guidance for bacteriology and AMR, so that livestock farmers produce mature manure and slurry.

Our simulations demonstrated that the concentration of beta-lactamase-producing *E. coli* as well as total *E. coli* in the vegetable body is very low. The uptake of human enteric pathogens through the roots into the intercellular spaces between plant cells and into the plant vasculature tissues, xylem and phloem, is known as internalization (Hirneisen *et al*., 2012). There are many reports of studies involving inoculation of soil or hydroponic solutions with human pathogenic bacteria with examination of internalization (Hirneisen *et al*., 2012). In many cases, internalization occurs in the roots but not the aerial parts, for example, with *E. coli* O157:H7 in leafy vegetables (Erickson *et al*., 2010) and spinach (Hora *et al*., 2005) in a soil system, with *E. coli* O157:H7 in lettuce (Franz *et al*., 2007), and with *Salmonella* in lettuce in hydroponic solution (Franz *et al*., 2007). However, internalization up to the aerial parts of vegetables has also been reported. Internationalization in sterilized leaf samples of lettuce was observed in 29%, 23%, and 15% of samples for *E. coli* 157:H7, *S*. Typhimurium 110, and *S*. Typhimurium 119, with mean concentrations of 3.95, 2.57, and 2.37 $\log_{10}$CFU/g, respectively, after inoculation of soil with 3 ml of 9 $\log_{10}$CFU/ml solutions of each bacterium (Franz *et al*., 2007). Internalization of *E. coli* O157:H7 and *Listeria monocytogenes* at significantly higher levels than *Salmonella* occurred in hydroponic solution-cultivated seedlings of cress, radish, and spinach, but these bacteria were not recovered within the tissues of mature plants (Jablasone *et al*., 2005). This suggested that the introduction of bacteria that are pathogenic to humans at an early stage of plant development may enhance internalization in seedlings, but the bacteria may not persist up to harvesting. For tomatoes, an experimental study reported that *Salmonella* was detected only in a few root samples but not in other parts (Miles *et al*., 2009), but another study reported detecting *Salmonella* in the hypocotyl and cotyledons (Guo *et al*., 2002). In our experiments, the internalization of *E. coli* could not be confirmed in tomatoes.

Wounding of the root tips may affect bacterial uptake through roots. Internalization of *S*. Newport in lettuce leaves was more common in plants with damaged roots than plants with intact roots (Bernstein *et al*., 2007). Internalization in vegetables seems to be highly variable (Golberg *et al*., 2011), and it is important to maintain low levels of bacteria pathogenic to humans in the soil of vegetable-growing fields, particularly when vegetables are at an early stage of development. As livestock harbor bacteria that are pathogenic to humans (Black *et al*., 2021) and their manure is directly applied to vegetable fields, careful composting plays an important role in reducing the probability of contamination. However, in contrast to other countries in which *Enterobacteriaceae*, which are found in animal feces, predominate among isolates from vegetables (Reuland *et al*., 2014; Al-Kharousi *et al*., 2016; Padovani *et al*., 2023) and *Pseudomonas* spp., which are typically environmental bacteria, dominate the isolates from vegetables sold in supermarkets in Japan (Usui *et al*., 2019). These *Pseudomonas* spp. isolates were shown to harbor the $bla_{SHV\text{-}12}$ and bla_{TEM116} genes (Usui *et al*., 2019), which are not strongly related to Japanese livestock. Our release assessment targeted beta-lactamase-producing *E. coli* of dairy farm origin, but the role and level of contamination with beta-lactamase-producing bacteria of environmental origin remain unknown.

This study is limited to a release assessment, and there will be some challenges to extending it into the full risk assessment. The bacterial flora in the soil is not rich in human pathogens, and some of the bacteria are non-pathogenic to humans, such as *Rahnella* spp. (Hamilton-Miller and Shah, 2001; Usui *et al*., 2019), are resistant to beta-lactams. *Pantoea agglomerans* is a common plant and soil bacterium, but it is a rare opportunistic pathogen implicated in nosocomial outbreaks affecting neonates and immunocompromised patients (Gajdács 2019). *P. agglomerans* is mostly sensitive to beta-lactams (Hamilton-Miller and Shah, 2001; Usui *et al*., 2019), but the emergence of beta-lactamase-producing *P. agglomerans* has been reported from vegetables in Nigeria (Aibinu *et al*., 2012) and human patients in Hungary (Gajdács 2019). Cattle-derived bacteria that are pathogenic to humans and the bacterial flora in soil can interact, for example by the uptake of free ARGs derived from bacteria in cattle. Similarly, the *bla* gene can be transmitted from *E. coli* of cattle origin to the intestinal flora in humans via plasmids or conjugation through the consumption of contaminated vegetables. However, complex mechanisms govern such events, and addressing this issue in the risk assessment is a challenge.

There are also some other limitations in the release assessment. Although Hokkaido Prefecture has 61.7% (846,100/1,371,000 heads) of Japanese dairy cattle population (Japanese Government, 2023), the pattern of antimicrobial use may not necessarily be representative of Japan. In a study from Gifu Prefecture, the proportion of beta-lactam antibiotics used for the treatment of mastitis by weight (gram) of antimicrobials was 49.2% (913.7 of 1857.8 g used for mastitis in 50 dairy farms), and macrolides and fluoroquinolones were used for 18.7 and 24.3%, respectively (Terashi *et al*., 2023). The *Enterobacteriaceae* data were used to estimate the level of bacterial reduction from fresh cattle feces to immature manure because data regarding *E. coli* were not available. Soil bacteria such as *Pseudomonas* spp., *Xanthomonas campestris*, *P. agglomerans*, and *Erwinia carotovora* cause plant bacterial diseases in vegetables (Haynes *et al*., 2020), and streptomycin and oxytetracycline, which are used in both animals and humans, are registered in Japan as pesticides (MAFF, 2023b). As these antibiotics are not beta-lactams, our study did not include the selection of ARB in the simulation model. However, there can be co-selection of *bla* genes in soil/plant bacteria resulting from the use of these antibiotics, and such antimicrobial use may facilitate the selection of beta-lactamase-producing bacteria in soil. A link between irrigation water and contamination of soil and vegetables in Ecuador (Montero *et al*., 2021) and another link between sewage treatment plant wastewater and contamination of rivers in Japan (Azuma *et al*., 2022) with ESBL-producing *E. coli* have been suggested. In our release assessment, the effect of irrigation of vegetable-growing fields on contamination with beta-lactamase-producing *E. coli* was not considered. Another limitation is that bacterial inoculation experiments were not performed for radish, which was estimated to have the highest soil bacterial concentration at harvesting. As mentioned above, a previous experiment suggested that internalized *E. coli* O157:H7 and *L. monocytogenes* were cleared from mature radish plants (Jablasone *et al*., 2005), but additional experiments may be needed to more precisely define this phenomenon.

CONCLUSION

In conclusion, this study examined the extent of releasing pathogenic bacteria of livestock origin into vegetables using a quantitative release assessment approach. The extent of releasing beta-lactamase-producing *E. coli* of dairy cattle origin into vegetables was assessed as being low. However, prudent use of antibiotics in dairy cattle and mature composting practices should be maintained.

CONFLICT OF INTEREST

The content is solely the responsibility of the authors and does not necessarily represent the official views of the Food Safety Commission of Japan.

ETHICS STATEMENT

This study was approved by the Food Safety Commission, Cabinet Office, Government of Japan. All the respondents of the interviews were commissioned as the research collaborators for the project by the Chairperson of the Board of Trustees of the Educational Foundation Rakuno Gakuen with written consents.

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AUTHOR CONTRIBUTIONS

All author contributed equally to the development of this article.

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DATA AVAILABILITY

The data are presented in this manuscript, and the detailed data may be available upon the request to the authors.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the online version of this article.

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