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ESTIMATION OF THE LIMIT OF DETECTION OF *SALMONELLA* SPP. IN ARTIFICIALLY CONTAMINATED CHICKEN PASTE

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Abstract. *Salmonellosis is the illness caused by the Salmonella bacteria. The usual symptoms of Salmonella infections are diarrhoea, fever, abdominal cramps, chills, headache, nausea or vomiting. These symptoms typically appear after consuming contaminated food. Effective isolation of Salmonella from food enables preventive action or the identification of the causative agent of the disease. This study aimed to determine the limit of detection of Salmonella spp. in artificially contaminated samples of chicken paste. To contaminate the sample, a bacterial suspension of Salmonella enterica subsp. enterica serovar Paratyphi B (ATCC 8759) was used. The detection of Salmonella spp. performed following SRPS EN 6579-1:2017+A1:2020, including three selective enrichment broths (Muller Kauffmann Tetrathionate-Novobiocin broth, Rappaport-Vassiliadis broth, Selenite Cystine broth) and three solid media for isolation (Salmonella Shigella agar, Xylose Lysine Deoxycholate agar, Bismut Sulfite agar). Four concentrations of bacterial suspension were prepared, and samples of chicken paste were artificially contaminated at four different levels. Five of the samples were tested with no previous contamination, as the zero contamination level. The number of positive findings of Salmonellae spp. and the total number of samples per inoculation level were used for calculation by the PODLOD_ver12.xls EXCEL program by Wirlich and Wilrich. This program estimates the probability of detection (POD) function and the limit of detection (LOD) of qualitative microbiological methods. The results of the detection of Salmonella spp. showed a* $LOD_{50\%}$ *0.028 [0.017; 0.047] CFU in 1 g, and* $LOD_{95\%}$ *0.122 [0.074; 0.202] CFU in 1 g. The results of the detection of Salmonella spp. expressed per tested portion were* $LOD_{50\%}$ *0.706 [0.426; 1.171] CFU in 25 g, and* $LOD_{95\%}$ *was 3.051 [1.839; 5.062] in 25 g. The combinations of applied enrichment broths and solid media for isolation did not show a difference in the detection results.*

Key words*: chicken paste, limit of detection, microbiological analysis, Salmonella spp.*

Introduction

Salmonella spp. are Gram-negative, facultative anaerobic, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. They are widely distributed in the environment and are recognised as major zoonotic pathogens, primarily transmitted through contaminated food and water [1]. *Salmonella* strains exhibit the ability to form biofilms and to survive under stressful environmental conditions, including low pH and desiccation, which contributes to their public health significance and frequent involvement in foodborne outbreaks [2]. Globally, *Salmonella* is estimated to cause around 155000 deaths each year, most of which are linked to the consumption of contaminated food [3]. *Salmonella* is among the leading foodborne pathogens transmitted through products of animal origin, particularly meat, poultry, eggs, and dairy. According to the European Food Safety Authority and ECDC [4], poultry and poultry-derived products remain the main reservoirs of *Salmonella*, frequently linked to human outbreaks. Antunes et al. [5] highlighted that contamination may occur during slaughtering, processing, or food handling, with cross-contamination and insufficient cooking as critical risk factors. Moreover, as reported by Fardsanei et al. [6], the persistence of *Salmonella enterica* in food environments is further enhanced by its antimicrobial resistance and virulence determinants, which increase the public health burden associated with animal-derived foods.

Salmonella is a significant foodborne pathogen commonly associated with poultry products, including chicken meat and liver pâté. Contamination can occur at various stages, including slaughter, processing, and handling, and undercooking of these products is a major risk factor for human infection [7, 8]. Even apparently healthy chickens can carry *Salmonella spp.*, which may spread to surfaces, utensils, and other foods in the environment, increasing the risk of cross-contamination [9]. Therefore, proper cooking to an internal temperature of 165°F (74°C) and strict hygiene practices are essential measures to prevent *Salmonella*-related illness. Shaltout et al. [10] reported that *Salmonella Typhimurium* was detected in 13.3%, 20%, and 6.7% of breast, thigh, and pane samples, respectively. *Salmonella Anatum* was found in 7% of nugget samples, while *Salmonella Enteritidis* was identified in 13.3% of breast samples and in 6.7% of both thigh and pane samples. In addition, *Salmonella Heidelberg*, *Salmonella Muenster*, and *Salmonella Kentucky* were each isolated from 6.7% of the examined samples.

Considering the mentioned facts regarding the presence of *Salmonella* spp. in foods of animal origin and the necessity for its timely detection, this study aimed to evaluate the detection limits and the matrix effect on the determination of *Salmonella* spp. in artificially contaminated chicken paste samples.

Materials and methods

Preparation of bacterial suspension

The bacterial suspension was prepared from cultures of *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (ATCC 8759) grown on tryptone soya agar plates, incubated at 37 °C for 24 hours. The prepared bacterial suspension with a density of 0.5 McF was tenfold diluted, and 1 mL of dilutions 10^{-6} and 10^{-7} were transferred to

a Petri plate in duplicate and poured with Plate Count Agar (PCA) (HiMedia, India) following ISO 4833-1 [11]. The Petri plates were incubated at 37 °C for 72 h. After incubation, the number of *Salmonella* bacteria cells in suspension was counted to estimate the cell count in the contaminated samples.

Artificial contamination of samples

A total of five non-contaminated samples and 40 samples at four different contamination levels were prepared. Contamination was performed by adding a bacterial suspension of *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (ATCC 8759) (Microbiologics, USA) to a homogenate consisting of 25 g of sample and 225 g of Buffered Peptone Water (BPW)(HiMedia, India). Specifically, five samples received no bacterial suspension (Level 0, non-contaminated), five samples received 1 mL from a 10^{-8} dilution of the bacterial suspension (Level I), five samples received 800 µL from a 10^{-8} dilution (Level II), twenty samples received 500 µL from a 10^{-8} dilution (Level III), and ten samples received 300 µL from a 10^{-8} dilution (Level IV).

Detection of *Salmonella* spp.

Detection of *Salmonella spp.* was performed according to SRPS EN ISO 6579-1:2017 [12] and amendment SRPS EN ISO 6579-1:2017/A1:2020 [13]. Therefore, the procedure for detecting *Salmonella* spp. included in the parallel application of the method described in Annex D of the mentioned standard. The samples were first subjected to pre-enrichment in BPW between 34 °C and 38 °C for 18 h to allow recovery of potentially stressed cells. For selective enrichment, aliquots of the pre-enrichment culture were inoculated into Rappaport-Vassiliadis (RV) broth (HiMedia, India) and Müller-Kaufmann tetrathionate-novobiocin (MKTTn) broth (HiMedia, India), incubated at 41.5 °C and between 34 °C and 38 °C for 24 h, respectively. In parallel, selective enrichment was also performed in selenite broth incubated between 34 °C and 38 °C for 24 h. From the enrichment cultures, streaking was performed onto selective solid media: Salmonella–Shigella (SS) agar (HiMedia, India) and Xylose Lysine Deoxycholate (XLD) agar (HiMedia, India), as well as bismuth sulfite (BS) agar (HiMedia, India). All plates were incubated between 34 °C and 38 °C for 24 h and 48 h. Presumptive colonies of *Salmonella* spp. were identified by their typical morphology (black colonies on SS agar, red to transparent colonies with black centres on XLD agar, and black/brown colonies with a metallic sheen on BS agar). Confirmation of suspected isolates was carried out by subculturing onto non-selective agar, followed by biochemical characterisation and serological identification in accordance with SRPS EN ISO 6579-1:2017 and its Annex D [12, 13].

POD and LOD calculation

The number of positive findings of *Salmonella* spp. on the applied level of contamination was used for calculation by the PODLOD_ver12.xls EXCEL program by Wilrich and Wilrich [14]. This program estimates the probability of detection (POD) function and the limit of detection (LOD) of qualitative microbiological methods [15].

Results and Discussion

After the experiments were conducted, the results of *Salmonella* spp. detection in both uncontaminated and artificially contaminated samples are presented in the Tables 1-9.

Table 1. The results for the combinations of selective broths and solid media for level zero – negative control

Level zero – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-

Table 2. The results for the combinations of selective broths and solid media for the level I

Level I – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
1	+	+	+	+	+	+	+	+	+	+
2	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+

Table 3. The results for the combinations of selective broths and solid media for the level II

Level II – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
5	-	-	-	-	-	-	-	-	-	-

Table 4. The results for the combinations of selective broths and solid media for the level III (samples 1-5)

Level III – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
1	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
5	-	-	-	-	-	-	-	-	-	-

Table 5. The results for the combinations of selective broths and solid media for the level III (samples 6-10)

Level III – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
6	+	+	+	+	+	+	+	+	+	+
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-

Table 6. The results for the combinations of selective broths and solid media for the level III (samples 11-15)

Level III – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
11	+	+	+	+	+	+	+	+	+	+
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+
15	-	-	-	-	-	-	-	-	-	-

Table 7. The results for the combinations of selective broths and solid media for the level III (samples 16-20)

Level III – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+
20	-	-	-	-	-	-	-	-	-	-

Table 8. The results for the combinations of selective broths and solid media for the level IV (samples 1-10)

Level IV – sample number	RVS			MKTTn			SC (BS after 24 hours)		SC (BS after 48 hours)	
	XLD	SS	BS	XLD	SS	BS	XLD	BS	XLD	BS
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+
6	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-

Rappaport-Vassiliadis (RV) broth is a selective enrichment medium designed for the isolation of *Salmonella* spp., based on high osmotic pressure, low pH, and inhibitory agents that suppress competing flora. It is particularly effective for samples with low numbers of *Salmonella* spp. and is recommended by ISO 6579-1:2017 [12] as a secondary enrichment medium, often used in combination with Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth [16, 17, 18]. The study conducted by Hyeon et al. [19] demonstrated that the choice of selective enrichment broth significantly influenced the sensitivity and specificity of the plating media, confirming that RVS represents the most suitable enrichment broth for investigating chicken carcasses.

Müller–Kauffmann tetrathionate broth (MKTTn) was originally developed by Müller [20] and later modified by Kauffmann [21] through the incorporation of ox bile and brilliant green to enhance its selectivity. A subsequent modification involved the addition of novobiocin at a concentration of 40 mg/L, which was shown to suppress the growth of commensal organisms, particularly *Proteus* spp., thereby improving the isolation of *Salmonella* spp. [22].

Selenite Cystine (SC) broth is a selective enrichment medium used for the isolation of *Salmonella* spp. from food and clinical samples. The presence of selenite inhibits the growth of many competing Gram-negative and Gram-positive bacteria, while cystine enhances the recovery of injured *Salmonella* cells. SC broth is recommended in international standards, such as ISO 6579-1:2017 [12], as a selective enrichment medium, particularly for faecal and food samples with high background flora [23, 24, 25].

Salmonella-Shigella (SS) agar is a selective and differential medium designed for the isolation of *Salmonella* spp. and *Shigella* spp. from clinical and food samples. Bile salts and brilliant green inhibit Gram-positive and many Gram-negative bacteria, while lactose fermentation and hydrogen sulfide production allow differentiation:

Salmonella spp. typically forms colourless colonies with black centres, and *Shigella* forms colourless colonies without blackening [26, 27, 28].

Xylose Lysine Deoxycholate (XLD) agar is a selective and differential medium widely used for the isolation of *Salmonella* spp. and *Shigella* spp. from food and clinical samples. It contains xylose, lactose, and sucrose as fermentable carbohydrates, lysine for decarboxylation, and sodium thiosulfate with ferric ammonium citrate for the detection of hydrogen sulfide production. *Salmonella* spp. typically appears as red colonies with a black centre, while *Shigella* forms red colonies without blackening, which enables effective differentiation from other *Enterobacteriaceae* [27, 28, 29].

Bismuth Sulfite (BS) agar is a highly selective medium primarily used for the isolation of *Salmonella* Typhi from clinical and food samples. The medium contains bismuth sulfite and brilliant green, which inhibit most Gram-positive and many Gram-negative bacteria, while ferrous sulfate allows the detection of hydrogen sulfide production. *Salmonella* Typhi typically forms brown to black colonies with a metallic sheen, facilitating differentiation from other enteric bacteria [27, 30].

Typical morphology of presumptive colonies of *Salmonella* spp. was as follows: black colonies on SS agar, red to transparent colonies with black centers on XLD agar, and black/brown colonies with a metallic sheen on BS agar (Figure 1).

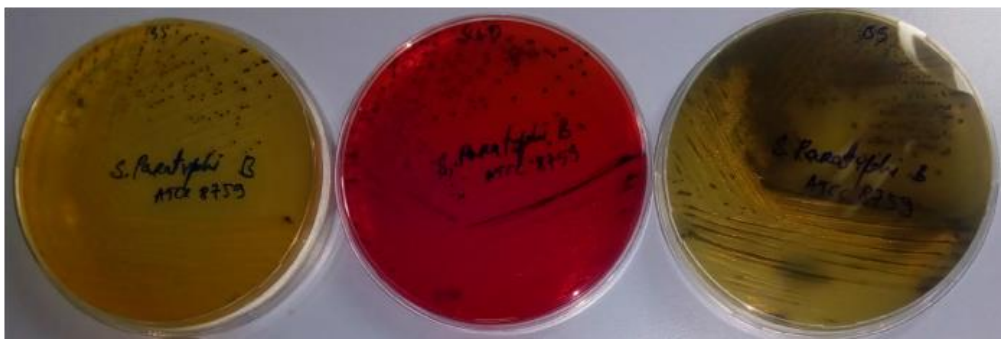

Figure 1. Growth of *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (ATCC 8759) on solid media (SS agar, XLD agar and BS agar)

The results obtained by plating the bacterial suspension on PCA showed 1×10^8 CFU/mL, and based on this count, the number of *Salmonella* bacteria at the different contamination levels was estimated. The results of the detection of *Salmonella* spp. for zero level (negative control) and four levels of artificial contamination are presented in Table 9.

Table 9. The results of the detection of *Salmonella* spp. for every level of artificial contamination

Level of artificial contamination	Estimated artificial contamination (CFU in 1g)	Estimated artificial contamination (CFU in 25g)	Number of tested samples	Number of samples with detected *Salmonella spp.*
0 (negative control)	0	0	5	0
I	0.04	1.0	5	4
II	0.032	0.8	5	2
III	0.020	0.5	20	8
IV	0.012	0.3	10	2

The ISO 6579-1:2017 [12] standard provides a horizontal method for the detection of *Salmonella* spp. in food, feed, and samples from primary production. While the standard itself does not explicitly define a numerical limit of detection (LOD), various validation studies have assessed its sensitivity in different food matrices. Cahyaningsih et al. [31] investigated the limit of detection (LOD) for *Salmonella* spp. in processed Pindang eggs using the ISO 6579-1:2017 [12, 13] method, following the guidelines of ISO 16140-3:2021. The study evaluated inoculated samples at contamination levels of 11, 3.67, 1.22, and 0.41 CFU per 25 g. All samples at 11, 3.67, and 1.22 CFU/25 g were detected as positive using pre-enrichment and selective enrichment media, followed by isolation on XLD agar and confirmation with API 20E tests. For the lowest contamination level (0.41 CFU/25 g), the detection rate was 75%, with one out of four replicates yielding a negative result. Based on these findings, the authors concluded that the reliable LOD for *Salmonella* spp. detection in Egg Pindang was 1.22 CFU/25 g, whereas lower levels showed reduced detection reliability.

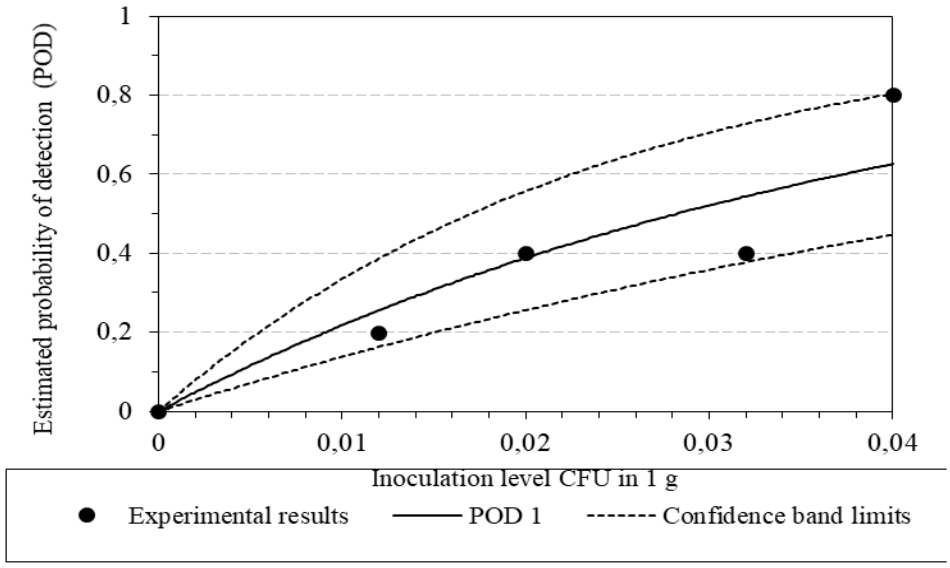

Figure 2. Experimental results and the estimated probability of detection (POD) curve and its 95% confidence band for artificially contaminated chicken paste with bacterial suspension of *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (ATCC 8759)

The experimental results and the estimated probability of detection (POD curve) and its 95% confidence band are presented in Figure 2. The results of our study were as follows: the matrix effect was 0.982; the 50% detection level (LOD_{50}) was 0.028 [0.017; 0.047] CFU/g, and the 95% detection level (LOD_{95}) was 0.122 [0.074; 0.202] CFU/g. The matrix effect statistic IzI was 0.073. Sensitivity was 80% for Level I inoculation (1.0 CFU/25 g), 40% for Level II (0.8 CFU/25 g), 20% for Level III (0.5 CFU/25 g), and 20% for Level IV (0.3 CFU/25 g). Specificity was 100%. Based on the results, it can be concluded that the matrix did not affect the detection of *Salmonella* bacteria, and the obtained detection limits indicate the reliability of the applied method for this matrix.

Conclusions

Due to their impact on human and animal health, the detection of *Salmonella* spp. in food represents a significant challenge. Special attention should be given to foods of animal origin, which are frequent sources of salmonellosis. The conducted study on determining detection limits and the matrix effect, using artificially contaminated chicken paste samples, demonstrated that the tested method is reliable for detecting *Salmonella* spp without interference from the matrix.

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PROCJENA LIMITA DETEKCIJE *SALMONELLA* SPP. U VJEŠTAČKO KONTAMINIRANOJ PILEĆOJ PAŠTETI

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Sažetak. *Salmoneloza je bolest koju izaziva bakterija salmonela. Uobičajeni simptomi infekcije salmonelom su dijareja, groznica, grčevi u stomaku, glavobolja, mučnina ili povrćanje. Ovi simptomi se obično javljaju nakon konzumiranja kontaminirane hrane. Efikasna izolacija salmonele iz hrane omogućava preventivno djelovanje ili identifikaciju uzročnika bolesti. Cilj ove studije bio je da odredi granice detekcije Salmonella spp. u vještački kontaminiranim uzorcima pileće paštete. Za kontaminaciju je korišćena bakterijska suspenzija Salmonella enterica subsp. enterica serovar Paratyphi B (ATCC 8759). Detekcija Salmonella spp. provedena je prema SRPS EN 6579-1:2017+A1:2020, uključujući tri selektivna bujona za bogaćenje (Muller Kauffmann tetrationat-novobiocin bujon, Rappaport-Vassiliadis bujon, selenit cistein bujon) i tri čvrste podloge za izolaciju (salmonela šigela agar, ksiloza lizin deoksiholatni agar, bizmut sulfitni agar). Pripremljene su četiri koncentracije bakterijske suspenzije i uzorci pileće paštete su kontaminirani na četiri nivoa. Testirano je pet uzoraka koji nisu kontaminirani, kao nulti nivo kontaminacije. Broj pozitivnih nalaza Salmonella spp. i ukupan broj uzoraka po inokulacionom nivou obrađeni su u PODLOD_ver12.xls EXCEL programu od Wirlich i Wilrich. Ovim programom se procenjuje funkcija vjerovatnoća detekcije (POD) i limit detekcije (LOD) kvalitativnih mikrobioloških metoda. Rezultati detekcije Salmonella spp. pokazali su* $LOD_{50\%}$ *0.028 [0.017; 0.047] CFU u 1 g, i* $LOD_{95\%}$ *0.122 [0.074; 0.202] CFU u 1 g. Rezultati detekcije Salmonella spp. izraženi po testiranoj porciji su za* $LOD_{50\%}$ *0.706 [0.426; 1.171] CFU u 25 g, i za* $LOD_{95\%}$ *3.051 [1.839; 5.062] u 25 g. Kombinacija primjenjenih selektivnih bujona za bogaćenje i čvrstih podloga za izolaciju nije pokazala razliku u rezultatima detekcije.*

Ključne riječi: *limit detekcije, mikrobiološko ispitivanje, pileća paštета, Salmonella spp.*