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Influence of milk product type and its initial contamination on the efficiency of different methods for detection of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Escherichia coli* O157:H7

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Abstract

This paper investigates differences in efficacy of isolating pathogenic bacteria *Salmonella* Enteritidis, *Listeria monocytogenes* and *Escherichia coli* O157:H7 between conventional cultivation (ISO method) and immunomagnetic separation (IMS method) related to the types of dairy products and initial numbers of bacteria. Different milk products (dairy pudding- vanilla or chocolate; a mixture of yoghurt and pudding; solid, liquid and fruit yoghurt; AB culture - with or without fruit; cheese spread) were intentionally contaminated with different numbers (≈ 10 and ≈ 30) of live cells of the observed bacteria per mL. The obtained results showed that the classical ISO procedure still represents an equally adequate method for the detection of *S. Enteritidis* and *L. monocytogenes* in dairy products as well as the IMS method. However, the ISO method was found to be inefficient for determination of *E. coli* O157:H7 when the initial contamination was low (≈ 10 live cells per mL). In such cases, even the IMS method appeared to be inefficient when used for fermented dairy products analysis. Fermented dairy products in contrast to the non-fermented ones, still represent a challenge for the development of routine detection methods, especially for *S. Enteritidis*, whilst the detection of *L. monocytogenes* and *E. coli* O157:H7 has improved by introducing the IMS method. The largest difference in the ability to detect bacteria in dairy product samples with reference to the initial number of bacteria by both methods was in the detection of *E. coli* O157:H7. The choice of broth (non-selective fluid broth vs. selective fluid broth) did not matter in the detection of *S. Enteritidis* and *L. monocytogenes* by applying the IMS procedure. However, for the detection of *E. coli* O157:H7 the application of modified tripton-soya broth with novobiocin (mTSB+Nb) has proved to be superior when compared to using the buffered peptone water (BPW). The presented results may be of importance as a scientific basis for future determination of standard methods related to laboratory detection of pathogens in dairy products.

Key words: milk, dairy products, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Escherichia coli* O157:H7, immunomagnetic separation

Introduction

Milk, as an extraordinarily nutritious food, is an aqueous solution of protein, fat and carbohydrates, containing a large number of vitamins and minerals. Due to its high water content, almost neutral pH and nutrient richness, it is an ideal basis for growth and transmission of bacteria, especially pathogenic bacteria. Good microbiological quality of fresh raw milk is a prerequisite for good microbiological quality of milk and dairy products intended for consumption. In addition, bacterial contamination of milk can significantly increase the total number of microorganisms in milk and thus reduce its quality.

In food microbiology, the term “food safe for consumption” implies that it does not contain pathogenic bacteria, or it does contain them only to the extent that cannot cause health problems in humans. The pathogenic bacteria in food are mainly determined by qualitative methods to prove their presence in a given sample mass. Since no large number of pathogenic bacteria is expected in food, isolation methods are based on inoculating the specimens into different liquid broths that allow recovery and cultivation of sub-lethally damaged bacteria, followed by inoculation onto solid substrates (Reinders et al., 2003), after which bacteria still have to be confirmed biochemically and/or serologically. Such a method of isolation requires large amounts of different broths and is time-consuming, hence the aim is to find faster, more effective and more sensitive methods for isolation and identification of pathogenic bacteria, which will produce results about the microbiological safety of food in a short time.

Immunomagnetic separation (IMS) is a method of concentration and separation of microorganisms based on the specific binding of bacteria to microscopic, magnetized beads with adsorbed antibodies. This results in selective concentration of targeted bacteria in the investigated sample. The method is a substitute for a standard cultivation of bacteria and is relatively inexpensive, fast and easy to use (Kohn, 2000). When performing the IMS method, polystyrene beads are used, the surfaces of which are magnetized and hydrophobic, allowing binding of different molecules. The advantage of the magnetized surface is reflected in the magnetic field under which the pellets are collected at the nearest point

to the magnet. Outside the magnetic field, pellets are easily dispersed into a homogeneous suspension (Fratamico and Crawford, 2000; Park et al., 2013; He et al., 2014).

The aim of this paper was to investigate differences in efficacy of isolating pathogenic bacterial strains of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Escherichia coli* O157:H7 between the conventional ISO procedure and immunomagnetic separation (IMS) in relation to the different types of dairy products and initial numbers of bacteria.

Materials and methods

The study included bacterial cultures of *Salmonella* Enteritidis ATCC strain 13076, *Listeria monocytogenes* ATCC strain 7644 and *Escherichia coli* O157: H7 serotype 35150, purchased from the Oxoid Ltd. company as Culti-Loops®. The samples of following dairy products were examined: dairy pudding (vanilla, chocolate); a mixture of yoghurt and pudding; solid, liquid and fruit yoghurt; AB culture (with and without fruit); cheese spread. Sampling and sample preparation were carried out according to the standard recommendations (British Standard BS 4285, 1993). As a control procedure, each sample was examined in parallel triplicates in order to confirm the same density of bacterial colonies each time of testing the same sample.

Using a densitometer, three bacterial suspensions were prepared in a saline solution containing approximately 3.0×10^8 bacterial cells (*S. Enteritidis*, *L. monocytogenes* or *E. coli* O157:H7). Subsequently, two decimal dilutions of each of the basic bacterial suspensions were prepared with saline (3×10^3 CFU/mL and 10^3 CFU/mL). For the purpose of intentional contamination, 1 mL of selected decimal dilution (3×10^3 CFU/mL, or 10^3 CFU/mL) was added to 100 mL of each investigated sample of the milk product, resulting with ≈ 10 or ≈ 30 live bacteria cells (*S. Enteritidis*, *L. monocytogenes* or *E. coli* O157:H7) per mL of each sample, respectively. Subsequently, samples were homogenized and incubated for 24 hours at 37° C. Isolation of the bacteria was performed by the classical isolation procedure and by the IMS. The procedure was carried out using a non-selective fluid broth for cultivation in

buffering peptone water (BPW) and selective fluid broth for cultivation as follows; for *Salmonella* Enteritidis Rappaport-Vassiliadis (RV), for *Listeria monocytogenes* Half Fraser, and for *Escherichia coli* O157: H7 modified tripton soya broth with novobiocin (m-TSB + Nb). For the control, in triplicate, the same samples of milk and milk products were prepared with 1 mL of saline solution, then homogenized, incubated for 24 hours at 37 °C. Isolation of *Salmonella* Enteritidis was performed according to the international standard ISO 6579: 2002, *Listeria monocytogenes* according to the international standard ISO 10560: 1993, BS 4285: 1993, and isolation of *Escherichia coli* O157: H7 according to the method described by Batt (2000).

The IMS isolation method of these bacteria was performed as follows. Microcentrifugation tubes were punctured with 20 µL of Dynabeads (Dyna) pellets with adsorbed antibodies of selected bacteria and 1mL suspension of the investigated sample

was incubated for 24 hours in broth for cultivation. The contents of the test tubes were lightly mixed on a rotary mixer at a rate of about 12 x per minute at room temperature for 3 minutes. After that, the tubes were removed from the mixer and a magnet was placed on them for a period of 1-3 minutes. The magnet served to attract the material that was attached to the Dynabeads pellets onto the walls of the test tubes. The liquid part was carefully separated and the pellet-bearing material was retained on the wall by the magnetic field. Afterwards 1 mL of PBS-Tween flushing buffer was added to the tubes. The procedure was repeated 2-4 times, depending on the time required for the pellet-bound bacteria to rinse off the excess. In the final step, 100 µL of PBS-Tween flushing buffer was added to each of the tubes and the contents of the tubes were homogenized. After the IMS procedure, 50 µL of Dynabeads bacterial complex was inoculated onto solid broth.

Table 1. Comparison of density of *Salmonella* Enteritidis colonies isolated by different methods from samples of investigated dairy products after intentional contamination with ≈10 and ≈30 live bacterial cells per mL

Sample	Intentional inoculation with ≈10 live cells of <i>S. Enteritidis</i> per mL			Intentional inoculation with ≈30 live cells of <i>S. Enteritidis</i> per mL		
	Isolation method					
	Classical ISO procedure	IMS on non-selective fluid broth (BPW)	IMS on selective fluid broth (RV)	Classical ISO procedure	IMS on non-selective fluid broth (BPW)	IMS on selective fluid broth (RV)
Dairy pudding (vanilla)	4+	4+	4+	4+	4+	4+
Dairy pudding (chocolate)	4+	4+	4+	4+	4+	4+
Yoghurt + pudding	2+	1+	1+	3+	1+	1+
Solid yoghurt	0	0	0	0	0	0
Liquid yoghurt	0	0	0	0	0	0
Fruit yoghurt	0	0	0	2+	1+	1+
AB culture	0	0	0	0	0	0
AB culture (with fruit)	0	0	0	1+	1+	1+
Cheese spread	1+	1+	1+	3+	2+	2+

Density of bacterial colonies: (0) = no colonies growth; (1+) = 1-100 colonies; (2+) = 101-1000 colonies; (3+) = > 1000 colonies; (4+) = fused colonies

IMS - immunomagnetic separation; BPW - buffered peptone water; RV - Rappaport-Vassiliadis broth

Note: As an internal check, each sample was examined in parallel triplicates in order to confirm the same density of bacterial colonies each time of testing the same sample

Results and discussion

The obtained results revealed differences in the efficacy of different methods of isolation of pathogenic bacteria *S. Enteritidis*, *L. monocytogenes* and *E. coli* O157:H7 from dairy product samples that were intentionally contaminated with lower (≈ 10) and higher (≈ 30) numbers of live bacterial cells per mL.

As shown in Table 1, the number of colonies of *S. Enteritidis* increased to the greatest extent in samples of dairy puddings, from which they were easily isolated by all investigated methods, even when there was little initial contamination, so with ≈ 10 living bacterial cells mL.

After smaller and larger initial contamination, *S. Enteritidis* was successfully extracted from a mixture of yoghurt and pudding, and from cheese spread, indicating that these products being a good media for its growth and reproduction. After 24 hours' incubation, *S. Enteritidis* was not isolated at all from solid and liquid yoghurt, or from AB culture, while from fruit yoghurt and fruit AB culture it was

extracted only at higher initial contamination levels of samples (≈ 30 live bacterial cells per mL). The obtained results were in agreement with the results of Nissen and Holck (1998), who explained the small number of *S. Kentucky* bacteria in fermented cured meat products by the action of bacteriocine derived from lactic acid bacteria. Porto-Fett et al. (2008), and Hwang et al. (2009) also confirmed that fermentation and drying, i.e. low pH value and water activity, as well as growth of competitive microorganisms (lactic acid bacteria), reduce the number of *Salmonella* bacteria in fermented sausages. As quoted by Odumer and León-Velarde (2012), methods based on isolation of bacteria by nutrient broths are still most widely used and represent the standard for detection of bacteria from *Salmonella* genus due to their selectivity and sensitivity. Özer and Kimiran-Erdem (2013) have proved that the classical ISO method of growing bacteria was more sensitive than the IMS method for determining the presence of bacteria of the *Salmonella* genus in meat samples. Similarly, Eriksson and Aspan (2007)

Table 2. Comparison of density of *Listeria monocytogenes* colonies isolated by different methods from samples of investigated dairy products after intentional contamination with ≈ 10 and ≈ 30 live bacterial cells per mL

Sample	Intentional inoculation with ≈ 10 live cells of <i>L. monocytogenes</i> per mL			Intentional inoculation with ≈ 30 live cells of <i>L. monocytogenes</i> per mL		
	Isolation method					
	Classical ISO procedure	IMS on non-selective fluid broth (BPW)	IMS on selec- tive fluid broth (Fraser broth)	Classical ISO procedure	IMS on non-selective fluid broth (BPW)	IMS on selec- tive fluid broth (Fraser broth)
Dairy pudding (vanilla)	2+	2+	2+	2+	2+	1+
Dairy pudding (chocolate)	2+	2+	2+	2+	2+	1+
Yoghurt + pudding	1+	1+	1+	2+	1+	1+
Solid yoghurt	0	0	0	1+	1+	1+
Liquid yoghurt	0	0	0	1+	1+	1+
Fruit yoghurt	0	0	0	2+	1+	1+
AB culture	0	0	0	0	1+	1+
AB culture (with fruit)	0	0	0	1+	1+	1+
Cheese spread	2+	0	0	2+	1+	1+

Density of bacterial colonies: (0) = no colonies growth; (1+) = 1-100 colonies; (2+) = 101-1000 colonies; (3+) = > 1000 colonies; (4+) = fused colonies

IMS - immunomagnetic separation; BPW - buffered peptone water

Note: As an internal check, each sample was examined in parallel triplicates in order to confirm the same density of bacterial colonies each time of testing the same sample

found that the standard ISO procedure is the most reliable and most sensitive method for detecting different strains of *Salmonella* in faecal samples compared to other tested methods. Based on their results, the IMS method seems to be less sensitive for the isolation of certain strains of *Salmonella*, which has been linked with lower binding of antibodies present on magnetic pellets. However, according to our results, the IMS method, in most cases, had the same sensitivity for the detection of *S. Enteritidis* in dairy products as the traditional ISO procedure. The types of broths did not play a role in the efficiency of isolation, as the results were identical by usage of non-selective fluid broth (BPW) and selective fluid broth (RV).

Similarly, *L. monocytogenes* has also been successfully isolated by all the three methods from dairy pudding (vanilla and chocolate) and pudding with the addition of yoghurt at higher and lower initial levels of contamination (Table 2).

On contrary, *L. monocytogenes* colonies were isolated from fermented dairy products (solid yoghurt, liquid yoghurt, fruit yoghurt, AB culture and AB culture with fruit) only at higher initial contamination of the samples (≈ 30 live cells). Isolation from cheese spread was more successful by the classical ISO procedure, whilst IMS was found to be inefficient for lower initial contamination (≈ 10 live cells). These findings can be explained by a large number of live lactic acid bacteria in fermented dairy products and the growth of bacteriocin, which inhibits the reproduction of *L. monocytogenes* (Djenane et al., 2005, Liu et al., 2008). Wadud et al. (2010) compared the success of three different isolation and identification methods for *L. monocytogenes* in ready-to-eat foods. The results of their research showed that IMS was equally sensitive as the traditional isolation method, but could be more favourable since it allows small number of bacteria to be identified after 18 to 24 hours, which reduces the volume of work

Table 3. Comparison of density of *Escherichia coli* O157:H7 colonies isolated by different methods from samples of investigated dairy products after intentional contamination with ≈ 10 and ≈ 30 live bacterial cells per mL

Sample	Intentional inoculation with ≈ 10 live cells of <i>E. coli</i> O157:H7 per mL			Intentional inoculation with ≈ 30 live cells of <i>E. coli</i> O157:H7 per mL		
	Isolation method					
	Classical ISO procedure	IMS on non-selective fluid broth (BPW)	IMS on selec- tive fluid broth (mTSB+Nb)	Classical ISO procedure	IMS on non-selective fluid broth (BPW)	IMS on selec- tive fluid broth (mTSB+Nb)
Dairy pudding (vanilla)	0	1+	1+	3+	3+	3+
Dairy pudding (chocolate)	0	1+	1+	3+	3+	3+
Yoghurt + pudding	0	1+	1+	1+	1+	2+
Solid yoghurt	0	0	0	0	1+	2+
Liquid yoghurt	0	0	0	0	1+	2+
Fruit yoghurt	0	0	1+	1+	1+	2+
AB culture	0	0	0	0	1+	2+
AB culture with fruit	0	0	0	0	1+	2+
Cheese spread	0	0	1+	1+	1+	2+

Density of bacterial colonies: (0) = no colonies growth; (1+) = 1-100 colonies; (2+) = 101-1000 colonies; (3+) = >1000 colonies; (4+) = fused colonies

IMS - immunomagnetic separation; BPW - buffered peptone water
mTSB+Nb - modified tripton-soya broth with novobiocin

Note: As an internal check, each sample was examined in parallel triplicates in order to confirm the same density of bacterial colonies each time of testing the same sample

and time needed to determine the presence of bacteria in the samples. The same has been confirmed by results of the present research, except for the cheese spread with low initial contamination. Non-selective broths (BPW) were slightly more efficient than non-selective fluid broths (BPW) in the case of IMS applied to the pudding samples.

Regarding the *E. coli* O157:H7 isolation, in the cases of higher initial sample contamination (≈ 30 live cells), it was successfully isolated by the usage of all the methods and broths from dairy pudding, yoghurt with pudding, fruit yoghurt and cheese spread. However, there was no success in isolation of this bacteria by standard ISO procedure from fermented products (solid yoghurt, liquid yogurt, AB culture, AB culture with fruit) (Table 3).

At higher initial contamination of the samples, the best isolation method was IMS, preceded by the cultivation in selective liquid broth with mTSB and novobiocin added, except for dairy puddings, where all the observed methods were equally effective in the isolation of *E. coli* O157:H7. In case of minor bacterial contamination (≈ 10 live cells), *E. coli* O157:H7 was not isolated by ISO method from none of the investigated dairy products, whilst IMS method was successful in extraction from dairy pudding, yoghurt with pudding, fruit yoghurt and cheese spread (the latest two only on selective fluid broth mTSB+Nb). In general, the most sensitive method, same as it was at the higher bacterial contamination, was the IMS method after multiplication in selective liquid broth mTSB with novobiocin. This is in accordance with Saab Khudhir (2013), who investigated the effectiveness of IMS and conventional isolation methods for isolation of *E. coli* from samples of fresh milk and soft cheese. Their results showed that IMS method was significantly more efficient and more sensitive method for detecting small number of *E. coli* than the conventional inoculation method on solid substrates, which was consistent with our results.

The *E. coli* O157:H7 was isolated in all samples intentionally contaminated with ≈ 30 live bacterial cells/mL by IMS method, suggesting that this strain may survive under unfavourable conditions, such as the presence of lactic acid bacteria (Nissen and Holck, 1998; Lahti et al., 2001). *E. coli* O157:H7 is obviously able to survive very low pH

values, such as in stomach conditions (pH = 1-2), and has been shown to utilize various mechanisms to adapt to acidic stress (Richard and Foster, 2003; Stincone et al., 2011; Kanjee and Houry, 2013). In the presented results, the difference in the density of bacterial colony growth was more obvious after contamination of dairy products with small number (≈ 10) of live bacteria *E. coli* O157. The IMS method carried out with the addition of novobiocin in the selective liquid medium of mTSB enabled successful isolation of *E. coli* O157:H7 from products with smaller initial number of bacteria (≈ 10 live cells) such as dairy puddings, yoghurt with pudding, fruit yoghurt and cheese spread. After carrying out the IMS method with the prior cultivation in nonselective liquid broth, i.e. BPW and low initial contamination (≈ 10 live cells), *E. coli* O157:H7 was successfully isolated only from dairy puddings and yoghurts with pudding, and was not isolated by traditional ISO method, at all.

The presented results suggest that such a small initial concentration as ≈ 10 live cells could be well detectable at the same level as the initial concentration of ≈ 30 live cells in the cases of contamination of non-fermented products (dairy puddings) by *S. Enteritidis* and *L. monocytogenes* (Tables 1 and 2). The detectability was especially efficient for detection of *S. Enteritidis* in pudding, as all the colonies were even fused after both initial bacterial concentrations (≈ 10 and ≈ 30 live cells, Table 1). In contrast to that, we found obvious differences for *E. coli* O157:H7 detection depending on initial contamination, with no colony growth detected, or 1-100 colonies detected, after initial contamination with ≈ 10 live cells using classical ISO procedure, or IMS procedure (both on selective and non-selective broths; BPW and mTSB+Nb), respectively. At the same time, the intentional inoculation by ≈ 30 live cells of *E. coli* O157:H7 resulted in > 1000 colonies detected both by classical ISO procedure, or IMS procedure (both on selective and non-selective broths; BPW and mTSB+Nb). Lower growth rate of *E. coli* O157:H7 after inoculation by such a small concentration as ≈ 10 live cells compared to ≈ 30 live cells, as well as compared to the growth rates of *S. Enteritidis* and *L. monocytogenes*, is in accordance with the results of Kim and Bhunia (2008). They found

no growth of *E. coli* O157:H7 inoculated by ≈ 10 live cells into mEC+n (modified *E. coli* broth with 20 mg of novobiocin/L), whilst the same level of intentional contamination was detectable in the cases of *S. Enteritidis* and *L. monocytogenes* (on respective broths), where the small level of initial contamination did not play such important role. According to the same authors, the initial contamination by ≈ 30 live cells was sufficient for growth and detection for all of the examined bacterial strains.

Apart from the initial contamination, the type of the broth used for the IMS methods in detection of *E. coli* O157:H7 plays the role in sensitivity, as well, which is in favour of using selective fluid broth (mTSB+Nb).

Conclusions

The classical ISO procedure still represents an equally adequate method for detection of *S. Enteritidis* and *L. monocytogenes* in dairy products as the IMS method. However, the ISO method was found to be inefficient for determination of *E. coli* O157:H7 at low initial contamination (≈ 10 live cells per mL). In such cases, even the IMS method appeared to be inefficient when used on fermented dairy products. Fermented dairy products still represent a challenge for development of routine detection methods, especially for *S. Enteritidis*, whilst the detection of *L. monocytogenes* and *E. coli* O157:H7 has improved by the introduction of the IMS method. The largest difference in the ability to detect bacteria in dairy product samples related to the initial number of bacteria by both methods was observed in the detection of *E. coli* O157:H7. Whilst choice of broth (non-selective fluid broth vs. selective fluid broth) did not matter for applying the IMS procedure in the detection of *S. Enteritidis* and *L. monocytogenes*, in the cases of detection of *E. coli* O157:H7 the usage of modified tripton-soya broth with novobiocin (mTSB+Nb) has proved to be superior compared to the usage of buffered peptone water (BPW). The above results may be of importance as a scientific basis for future determination of standard methods related to laboratory detection of important pathogens in dairy products.

Utjecaj vrste mliječnih proizvoda i početne kontaminacije na učinkovitost različitih metoda dokazivanja bakterija Salmonella Enteritidis, Listeria monocytogenes i Escherichia coli O157:H7

Sažetak

U radu se istražuju razlike u učinkovitosti dokazivanja patogenih bakterija *Salmonella Enteritidis*, *Listeria monocytogenes* i *Escherichia coli* O157:H7 između konvencionalne kultivacije bakterija (ISO metoda) i imunomagnetne separacije (IMS metoda) u različitim vrstama mliječnih proizvoda s inicijalnim brojem bakterija. Različiti uzorci mliječnih proizvoda (mliječni puding - vanilija ili čokolada; mješavina jogurta i pudinga; čvrsti, tekući i voćni jogurt, AB kultura - sa ili bez voća; sirni namaz) namjerno su kontaminirani različitim brojem istraživanih bakterija (≈ 10 i ≈ 30 živih stanica po mL). Klasični ISO postupak pokazao se jednako adekvatnom metodom za uporabu u svrhu otkrivanja *S. Enteritidis* i *L. monocytogenes* u mliječnim proizvodima kao i IMS metoda. Međutim, ISO postupak pokazao se neučinkovitim za dokazivanje *E. coli* O157:H7 u slučajevima kada je početna kontaminacija bila niska (≈ 10 živih stanica po mL). U takvim slučajevima IMS metoda nije bila učinkovita niti na fermentiranim mliječnim proizvodima. Nasuprot nefermentiranim mliječnim proizvodima, fermentirani proizvodi još uvijek predstavljaju izazov za razvoj rutinske metode detekcije, osobito za *S. Enteritidis*, dok je detekcija *L. monocytogenes* i *E. coli* O157:H7 poboljšana uvođenjem IMS metode. Najveća razlika u efikasnosti dokazivanja patogenih bakterija u uzorcima mliječnih proizvoda, povezana s niskim početnim brojem bakterija u uzorku, pokazala se pri detekciji *E. coli* O157:H7. Dok izbor hranjive podloge (neselektivna tekuća hranjiva podloga, nasuprot selektivnoj tekućoj hranjivoj podlozi) ne igra važnu ulogu u primjeni IMS postupka pri otkrivanju *S. Enteritidis* i *L. monocytogenes*. U slučajevima dokazivanja *E. coli* O157:H7, uporaba modificiranog tripton-sojinog bujona s novobiocinom (mTSB + Nb) pokazala se učinkovitijom u usporedbi s uporabom puferirane peptonske vode (PPV). Dobiveni rezultati mogu

biti od značaja kao znanstvena osnova za buduće određivanje standardnih metoda vezanih za laboratorijsko otkrivanje važnih patogena u mliječnim proizvodima.

Ključne riječi: mlijeko, mliječni proizvodi, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Escherichia coli* O157: H7, imunomagnetna separacija

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