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Short communication

# Immunomagnetic nanobeads based on a streptavidin-biotin system for the highly efficient and specific separation of *Listeria monocytogenes*

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ABSTRACT

Immunomagnetic separation was used to decrease the assay time for *Listeria monocytogenes* (*LM*) isolation. The coupling conditions and immunomagnetic bead (IMB) capture process were optimized. Results showed that the incubation time and separation time for 0.1 mg of 180 nm immunomagnetic nanobeads (IMB-S-180) coupled with monoclonal antibody using a streptavidin-biotin system was 45 and 3 min, respectively. Magnetic separation was affected by the separation medium and magnetic density of the magnetic separator. Capture efficiency (CE) was 94.12% when the magnetic density was 1.5 T, using 1 ml of PBS as separation medium. The IMBs exhibited highly specific binding with *LM* strains (CE > 90%) but low binding with non-target bacteria (CE < 5%). The CE of IMBs (0.1 mg/ml) against *LM* in ground beef and milk samples were 25.1% and 91.2%, respectively. These results can serve as a guide for the appropriate selection of IMBs for developing isolation processes for pathogens.



## 1. Introduction

Food contamination caused by pathogens has attracted increasing attention worldwide. It is extremely important to set up a rapid and sensitive method for detecting pathogens in food products. Thus, rapid and sensitive detection methods, aside from methods for controlling food contamination, should be developed. Traditional methods for detecting food-borne pathogens include enrichment, plating on selective and/or differential agar, and biochemical or serological confirmation. However, obtaining positive identification using these processes is time-consuming. Over the last 20 years, immunomagnetic separation (IMS) using immunomagnetic beads (IMBs) have had an increasingly important function in the isolation and concentration of target organisms in food sample preparation, and this process is more efficient than conventional centrifugation or filtration methods (Radisic, Iyer, & Murthy, 2006; Valdés, Gonzále, Calzón, & García, 2009). Several advantages of the use of magnetic beads have been reported, such as the concentration of target bacteria from the sample, removal of inhibitory agents, and significant reduction of background microflora (Stevens & Jaykus, 2004). IMS has also been applied in conjunction with a chromogenic medium (Wadud, Leon-Velarde, Larson, & Odumeru, 2010), enzyme-linked immunosorbent assay (Cheng, Lai, & Ko, 2012; Xu et al. 2012), polymerase chain reaction (Tram, Cao, Høgberg, Wolff, & Bang, 2012; Yoshitomi, Jinneman, Zapata, Weagant, & Fedio, 2012), flow cytometry (Fuchslin, Kotzsch, Keserue, & Egli, 2010), and biosensors (Jiang et al. 2011; Kim, Cho, Seo, Jeon, & Paek, 2012) to decrease the total assay time.

The most commonly used magnetic beads in IMS assay are Dynabeads (Dynal, Oslo, Norway), which are polystyrene-based microbeads ranging from 2.8 μm to 4.5 μm. However, microbeads have the disadvantages of low capture and separation efficiencies (Madhukar, Yang, Su, & Li, 2005). In recent years, magnetic nanobeads have gained increasing attention in improving the capture efficiency (Jin, Liu, Shan, Tong, & Hou, 2014). Magnetic nanobeads can effectively adhere to the surface of bacterial pathogens and improve the efficiency of capturing bacterial pathogens in food samples (Wang, Li, Wang, & Slavik, 2011). Compared with magnetic microbeads, magnetic nanobeads are more mobile in complex samples and have faster reaction kinetics, lower mass, higher surface-to-volume ratio, and multiple binding on the target surface (Huang et al. 2011).

A commonly used method for immobilizing antibodies on magnetic beads is covalent coupling (Cao et al. 2012). Although this method is simple and gentle, the adsorbed antibody is easily affected by minor changes in pH, monodispersity, and cross-link

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time. Streptavidin-biotin system has increasingly gained widespread attention because of its good reaction specificity and high affinity. Streptavidin is a tetrameric protein that can conjugate four biotin molecules. The hydrogen bonds and van der Waals forces also contribute to the stability of the system (Yuan, Chen, Kolb, & Moy, 2000).

*Listeria monocytogenes* (*LM*) is a Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped, and intracellular pathogenic bacterium that causes a food-borne disease called listeriosis (Jadhav, Bhave, & Palombo, 2012). *LM* can be found in various raw and processed foods. Soft cheese, raw and undercooked seafood, coleslaw, lettuce, poultry, and ready-to-eat meat products have been associated with *LM* contamination. Such contamination has raised great concern to the food industry because of the bacterium's ability to grow at refrigerated temperatures and processing environments, making it potentially hazardous to refrigerated products (Farber & Peterkin, 1991). IMS has been applied in conjunction with conventional plating (Wadud et al. 2010), polymerase chain reaction (Yang et al. 2007), and flow cytometry methods (Hibi et al. 2006) for *LM* detection.

In this paper, the IMS based on streptavidin-biotin system for capturing *LM* was systematically studied. A highly efficient and specific separation of *LM* from whole milk and ground beef were developed.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*LM* (CMCC 54007, ATCC 13932) was grown in brain heart infusion (BHI; BD Company, Sparks, MD, USA) to stationary phase (20 h–24 h) at 37 °C for further applications. *Escherichia coli* (ATCC 25922), *E. coli* O157:H7 (CMCC 44828), *Micrococcus luteus* (CMCC 28001), *Proteus vulgaris* (CMCC 49027), *Bacillus subtilis* (BD 366), *Salmonella paratyphi* (ATCC 9150), *Cronobacter sakazakii* (CMCC 4540), and *Bacillus cereus* (CMCC 63303) were cultured in Luria–Bertani (LB) medium (Oxoid, Basingstoke, UK) at 37 °C for 20 h before use. Serial dilutions of cultures in PBS (0.01 M, pH 7.4) were produced and plated on trypticase soy agar (TSA; BD Company, Sparks, MD, USA) to determine the number of viable cells. The plates were then incubated at 37 °C for 24 h.

### 2.2. Preparation of IMBs

#### 2.2.1. Preparation of the directly bound IMBs (IMB-D) (Cui, Xiong, Xiong, Shan, & Lai, 2014)

Magnetic beads (180 nm, 10 mg, Allrun Nano Science & Technology Co., Ltd., Shanghai, China) were concentrated in a microcentrifuge with a magnetic separator (Zodolabs Biotech Co., Ltd., Wuxi, China) for 3 min, and the storage buffer (0.01 mol/l Morpholine ethanesulfonic acid hemisodium salt, pH 5.5) was then removed. The magnetic beads were resuspended in 3 ml of freshly made ethyl (dimethylaminopropyl) carbodiimide (EDC)–N-hydroxysuccinimide sodium salt (NHSS) (Sigma–Aldrich, St. Louis, MO, USA) solution (0.4% EDC, 0.5% NHS in 0.01 M 2-morpholinoethanesulfonic acid buffer), and then mixed by a rotator (Ningbo Scientz Biotechnology Company, Ningbo, China) at 15 rpm for 60 min. After incubation, the EDC–NHSS solution was removed, and beads containing activated carboxyl group surfaces were resuspended with 10 ml of 0.01 M borate saline (BS) buffer (pH 8.5). Afterward, 800 μg of anti-*LM* monoclonal antibody (Meridian Life Science Inc., Memphis, TN, USA) was immediately added into 1 ml of the solution by thorough mixing, and then placed on the rotator at 15 rpm at room temperature for 4 h. The antibody-conjugated magnetic beads (IMB-D-180) were blocked in 1 ml of BS containing 120 μl of ethanolamin at room temperature for 1 h, washed four times with 0.01 M PBS (pH 7.4), and resuspended at a final concentration of 1 mg/ml in 0.01 M PBS (0.2‰ $NaN_3$ and 0.2% bovine serum albumin, pH 7.4).

#### 2.2.2. Preparation of IMBs by streptavidin-biotin (SA-biotin) interactions (IMB-S)

One milligram of carboxyl-modified magnetic beads (diameter = 180, 350, and 1180 nm) were covalently bound with 40 μg of SA (Thermo Fisher Scientific Inc., Rockford, USA) by the EDC–NHSS method as previously described. The antibody and biotin (Thermo Fisher Scientific Inc., Rockford, USA) were mixed at a volume ratio of 1:10 and placed on the rotator at 15 rpm at room temperature for 4 h. An aliquot of 1 mg of SA-coated magnetic beads were coupled with 80 μg of the biotinylated antibody by mixing, and then placed on the rotator at 15 rpm at room temperature for 30 min. The beads were then washed three times with 1 ml of 0.01 M PBS solution. After the final wash, the beads were resuspended at a final concentration of 1 mg/ml in 0.01 M PBS (0.2‰ $NaN_3$ and 0.2% BSA). The IMB through SA-biotin interactions with different magnetic beads (180, 350, and 1180 nm) was described as IMB-S-180, IMB-S-350, and IMB-S-1150.

### 2.3. IMS of LM by IMBs

#### 2.3.1. Comparison of the CE of different IMBs

One milliliter of PBS containing $10^4$ CFU of *LM* was mixed with IMB-D-180, IMB-S-180, IMB-S-350, and IMB-S-1180 by a rotator at 15 rpm for 45 min, respectively. Five amounts of IMBs (0.05, 0.1, 0.2, 0.4, and 0.6 mg) were used. The magnetic bead-bacteria complexes were captured on the side of the tube by a magnetic separator for 3 min. An aliquot of 0.1 ml from the supernatant was spread on PALCAM (Land Bridge Technology Co., Ltd., Beijing, China) agar for bacterial enumeration after appropriate dilution. The beads were then washed three times with 1 ml of PBST (0.01 M PBS containing 0.05% Tween 20, pH7.4). An aliquot of 0.1 ml of washing solution was spread on PALCAM agar for bacterial enumeration after appropriate dilution. All the PALCAM agar plates were incubated at 37 °C for 24 h for bacterial enumeration. All enumeration experiments were performed in triplicate.

The IMB-S-180, IMB-S-350, and IMB-S-1180 capturing *LM* were scanned with transmission electron microscope (Malvern Instruments Ltd., Worcestershire, U.K.) to characterize the IMBs-bacteria complexes.

#### 2.3.2. Immunoreaction time and separation time of IMS with IMB-S-180

An aliquot of 0.1 mg of IMB-S-180 was added into 1 ml of PBS containing $10^4$ CFU of *LM* and mixed by a rotator at 15 rpm at different immunoreaction times (15, 30, 45, and 60 min). The bead-bacteria complexes were concentrated on the side of the tube by a magnetic separator for five magnetic separation times (1, 2, 3, 5, and 10 min). The beads were then washed three times with 1 ml of PBST. An aliquot of 0.1 ml of supernatant and washing solution was spread on PALCAM agar, respectively, for bacterial enumeration after appropriate dilution.

#### 2.3.3. CE With different separation medium and magnetic intensity

Approximately $10^4$ CFU/ml of *LM* was spiked to separation medium including PBS, *Listeria* enrichment broth base (LEB, Beijing Land Bridge Technology Co. Ltd., Beijing, China) and 10% LEB in PBS buffer. Approximately 0.1 mg of IMB-S-180 was mixed with 1 ml of the separation medium by a rotator at 15 rpm for 45 min. The bead-bacteria complexes were concentrated on the side of the tube by a magnetic separator (the magnetic intensity was 0.4, 0.8, and 1.5 T,

respectively) when the bead-bacteria complexes were completely magnetically separated. The beads were then washed three times with 1 ml of PBST. An aliquot of 0.1 ml of supernatant and washing solution was spread on PALCAM agar, respectively, for bacterial enumeration after appropriate dilution.

### 2.4. Specificity test

The specificity of the IMBs was carried out using non-target bacteria. *E. coli* (ATCC 25922), *E. coli* O157:H7 (CMCC 44828), *M. luteus* (CMCC 28001), *P. vulgaris* (CMCC 49027), *B. subtilis* (BD 366), *S. paratyphi* (ATCC 9150), *C. sakazakii* (CMCC 4540), and *B. cereus* (CMCC 63303) were cultured in LB at 37 °C for 12 h, and then serially diluted to $10^4$ CFU/ml in PBS. *LM* was cultured to $10^4$ CFU/ml. The *LM* and non-target bacteria were not mixing and respectively captured by the IMS to determinate the specificity of the method.

### 2.5. Capture of LM from contaminated food sample

Whole milk (3.25% milk fat) and ground beef were purchased from a local grocery store and tested as negative samples for *LM*. Twenty five grams of whole milk spiked with *LM* at concentrations of $10^3$, $10^4$, and $10^5$ CFU/g was added into 225 ml PBS. An aliquot of 0.1 mg of IMB-S-180 was added into 1 ml of the PBS. The mixture was rotated at 30 rpm for 45 min at room temperature and separated for 3 min by magnetic separator. The beads were then washed three times with 1 ml of PBST. Twenty five grams of ground beef was spiked with *LM* at concentrations of $10^3$, $10^4$, and $10^5$ CFU/g and stored at 4 °C overnight. Then 25 g of the ground beef was added into 225 ml of PBS. An aliquot of 0.1 mg of IMB-S-180 was added into 1 ml of the PBS. The mixture was rotated at 30 rpm for 45 min at room temperature and separated for 3 min by magnetic separator. The beads were then washed three times with 1 ml of PBST. The treatments were performed in triplicate. An aliquot of 0.1 ml of supernatant and washing solution was spread on PALCAM agar, respectively, for bacterial enumeration after appropriate dilution. All enumeration experiments were performed in triplicate.

### 2.6. Calculation of CE and statistical analysis

CE is defined as the percentage fraction of the total bacteria retained on the surface of IMBs. CE was calculated using the following equation: $CE\ (\%) = (1 - C_b/C_0) \times 100\%$ (Xiong et al. 2014).

Where $C_0$ is the total number of cells present in a sample (CFU/ml) and $C_b$ is the estimated number of cells unbound to IMBs (CFU/ml). Standard deviations were calculated from the mean results of the triplicate experiments. One-way ANOVA was used to compare the mean among the groups, statistical significance threshold was determined as $P < 0.05$.

## 3. Results and Discussion

### 3.1. CE of different immunomagnetic nanobeads

The difference in bead diameter resulted in a significantly different coverage on the bacterial cells. For IMB-S-1150 (Fig. 1C), bacterial cells were bound to few magnetic beads. In this study, the high CE of IMB-S-180 may also be attributed to the large surface area of the nanoparticles available for immunoreaction. The surface area of the nanoparticles available for the capture of bacteria was larger than that of the microbeads used in the tests. Fig. 1A clearly indicates that many magnetic beads bound to the surface of a single cell. For biological binding reaction, small beads with high surface/volume ratio and high mobility have many opportunities to interact with bacterial cells in a solution, resulting in high CE (Yavuz et al. 2006).

The results showed that the size and amount of IMBs are important factors that affect the CE (Table 1). The CE increased as the size of the magnetic beads decreased. Meanwhile, CE increased when the amount of the IMBs increased. CE was greater than 90% when 0.1 mg or more IMB-S-180 was added to 1 ml of bacterial suspensions for immunomagnetic separation.

IMB-D-180 (antibody directly binding IMBs) and IMB-S-180 (antibody binding IMBs based on a Streptavidin-Biotin System) were compared (Table 1). When 0.05, 0.1, 0.2, 0.4, 0.6 mg of IMB-D-180 and IMB-S-180 was added to 1 ml of bacterial suspensions for immunomagnetic separation, the CE with IMB-D-180 was 15.66%, 28.01%, 37.33%, 50.76%, and 63.44%, respectively while the CE with IMB-S-180 was 66.31%, 93.91%, 97.02%, 96.87%, and 99.01%, respectively. Biotinylated antibody was conjugated onto the beads containing a layer of SA. The layer of SA coating may have enhanced the orientation and conformation of the antibodies bound to the beads and affected how well the antibodies bound to the surface antigens on the bacteria. In addition, the affinity of SA for biotin represented a strong noncovalent interaction with a dissociation constant of $10^{-15}$ M (Yuan et al. 2000), which rivals that of covalent bonds. This interaction effectively reduces the probability that the antibody falls off the magnetic nanobead surface in subsequent experiments.

### 3.2. Selection of immunoreaction time and separation time

The IMBs were incubated at four immunoreaction times (15, 30, 45, and 60 min) with $10^4$ CFU/ml of *LM*. The CE increased to 90.79% with a 45 min reaction time, and longer reaction times did not significantly increase the CE. CE was close when separation times were 3, 5, and 10 min. To achieve the desired results and reduce the total processing time, 45 and 3 min were selected as the immunoreaction time and magnetic separation times, respectively.

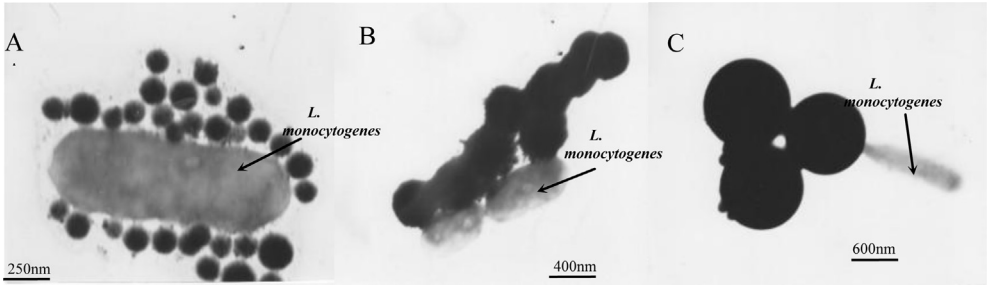


**Fig. 1.** Transmission electron microscope observations of *LM* interacting with different sizes of magnetic beads: (A) 180 nm (30,000 ×), (B) 350 nm (20,000 ×), and (C) 1150 nm (10,000 ×).

**Table 1**
CE of IMB for *LM* with different conjugation methods, sizes, and amount of IMBs.

| | Capture efficiency (%) | | | | |
|---|---|---|---|---|---|
| | Amount of IMBs (mg) | | | | |
| | 0.05 | 0.1 | 0.2 | 0.4 | 0.6 |
| IMB-D-180 | $15.66 \pm 9.41^{BFHd}$ | $28.01 \pm 3.88^{BFc}$ | $37.33 \pm 3.47^{BbG}$ | $50.76 \pm 4.64^{DEa}$ | $63.44 \pm 5.66^{AC}$ |
| IMB-S-180 | $66.31 \pm 9.86^{B}$ | $93.91 \pm 1.77^{A}$ | $97.02 \pm 1.97^{A}$ | $96.87 \pm 3.27^{A}$ | $99.01 \pm 1.44^{A}$ |
| IMB-S-350 | $11.26 \pm 2.62^{BDd}$ | $15.66 \pm 6.59^{BD}$ | $21.00 \pm 7.36^{Bbc}$ | $32.35 \pm 3.66^{Ca}$ | $36.48 \pm 2.65^{A}$ |
| IMB-S-1150 | $3.54 \pm 2.73^{Bb}$ | $7.40 \pm 5.22^{B}$ | $11.83 \pm 4.36^{Ba}$ | $24.73 \pm 2.66^{A}$ | $25.90 \pm 3.89^{A}$ |

Values are LSM ± Std. Mean with different capital or little character on superscript within the same row in the same IMB are significantly different ($p < 0.01$ or $P < 0.05$).

**Table 2**
CE of IMB-S-180 from magnetic intensity and various separation media, including PBS buffer (A), LEB (B), and 10% LEB in PBS buffer (C).

| Magnetic intensity (T) | Volume of separation medium | Capture efficiency (%) |
|---|---|---|
| 0.4 | 1 ml (A) | $87.56 \pm 3.21^{A}$ |
| | 1 ml (B) | $35.77 \pm 16.74^{B}$ |
| | 1 ml (C) | $73.16 \pm 5.83^{A}$ |
| 0.8 | 1 ml (A) | $89.71 \pm 3.81^{A}$ |
| | 1 ml (B) | $46.31 \pm 13.21^{B}$ |
| | 1 ml (C) | $77.27 \pm 6.81^{A}$ |
| 1.5 | 1 ml (A) | $94.12 \pm 2.47^{Aa}$ |
| | 1 ml (B) | $49.21 \pm 6.87^{BD}$ |
| | 1 ml (C) | $81.26 \pm 9.01^{bC}$ |

Values are LSM ± Std. Mean with different capital or little character on superscript within the same column in the same magnetic intensity are significantly different ($p < 0.01$ or $P < 0.05$).

### 3.3. CE With IMB-S-180 from various separation media and magnetic intensities

The rapid magnetic separation of bacteria by IMBs must overcome a significant amount of resistance force as it moves through the separation solution toward the magnet (Kelland, 1998). The CE was affected by magnetic intensity and the various separation media, including PBS buffer, LEB, and 10% LEB in PBS buffer. The results showed that the more magnetic field force was used, the more CE was obtained. The CE was the largest when PBS buffer was selected as separation medium with the same magnetic force (Table 2). The reason was that there was no matrix interference in PBS buffer. The CE was 94.12% when the magnetic density was 1.5 T and 1 ml of PBS was used as the separation medium.

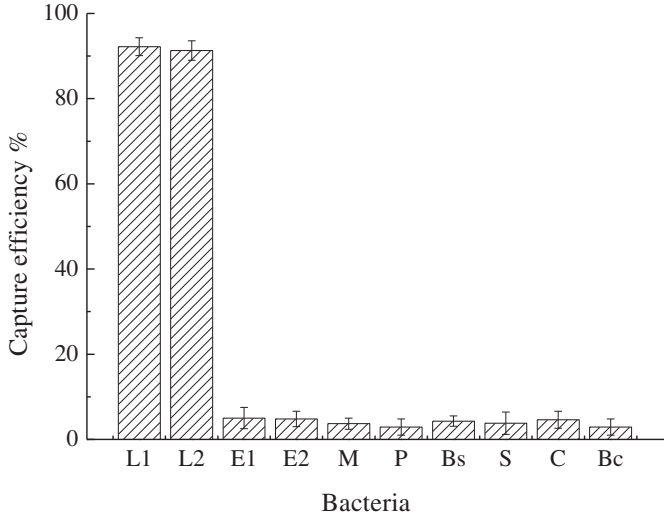


**Fig. 2.** Specificity of IMBs with selected bacterial strains. L1 represents *LM* (CMCC 54007), L2 represents *LM* (ATCC 13932), E1 represents *E. coli* (ATCC 25922), E2 represents *E. coli* (CMCC 44828), M represents *M. luteus* (CMCC 28001), P represents *P. vulgaris* (CMCC 49027), Bs represents *B. subtilis* (BD366), S represents *S. paratyphi* (ATCC 9150), C represents *C. sakazakii* (CMCC 4540), and Bc represents *B. cereus* (CMCC 63303).

### 3.4. Specificity test with selected bacterial strains

When $10^4$ CFU/mL of *LM* (CMCC 54007, ATCC 13932), *E. coli*, *M. luteus*, *P. vulgaris*, *B. subtilis*, *S. paratyphi*, *C. sakazakii*, and *B. cereus* were separated by IMS, the CE of the two *LM*s were 92% and 91%, respectively; the CE of the other eight bacteria were all lower than 5% (Fig. 2). The result shows that IMBs exhibited only sufficient specificity with *LM* strains.

### 3.5. Food analysis

The application of IMBs to capture *LM* in whole milk and ground beef was presented in Table 3. When $10^4$ CFU/mL of *LM* was captured by the IMS method, the CE with ground beef and whole milk was 36.54% and 88.68%, respectively. The result showed that the CE with ground beef was much lower than that with whole milk. Similarly result was reported that approximately 50% of cells in PBS were captured by IMBs while only 15% of *E. coli* O157:H7 cells in ground beef were captured by IMBs (Fu, Rogelj, & Kieft, 2005). There are more solid content and fat in ground beef than in whole milk. The solid content and fat might interfere with the contact between IMBs and *LM*, prevent IMBs and *LM* from binding together, and stop IMBs-*LM* complex from concentrating by magnetic separator. So the capture efficiency of *LM* in ground beef was lower than in milk.

## 4. Conclusions

The results of this investigation highlight several important characteristics of the magnetic separation of target biological cells from the separation medium. Beads modified by SA and biotinylated mAb can capture *LM* with high CE and high specificity. Magnetic separation was significantly influenced by magnetic bead sizes, magnetic intensity, immunoreaction time, separation time,

**Table 3**
CE with IMB-S-180 in ground beef and whole milk.

| Food sample | Capture efficiency (%) | | |
|---|---|---|---|
| | *LM* concentration (CFU/g) | | |
| | $10^3$ | $10^4$ | $10^5$ |
| Ground beef | $25.46 \pm 4.97^{Bb}$ | $36.54 \pm 4.32^{Ba}$ | $51.23 \pm 4.56^{A}$ |
| Whole milk | $84.91 \pm 3.78$ | $88.68 \pm 1.87^{a}$ | $81.13 \pm 3.81^{b}$ |

Values are LSM ± Std. Mean with different capital or little character on superscript within the same row in the same food sample are significantly different ($p < 0.01$ or $P < 0.05$).

and separation medium. The IMBs method can be used to effectively capture *LM* in food matrix.

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