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Mass spectrometric identification of pathogens in foods using a zirconium hydroxide immobilization approach

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Dedicated to Professor Catherine Fenselau in honor of her outstanding contributions to biological mass spectrometry.

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ABSTRACT

The analysis of bacteria in foods is a challenge because the food matrices might severely interfere with bacterial detection. Pathogens in milk/pudding/coffee were isolated with magnetized zirconium hydroxide, directly cultivated without colony isolation, and then analyzed using MALDI and LC–MS/MS. The analysis was less time-consuming than conventional biological methods because a colony isolation procedure was not required. Further, the MS analysis of bacteria was broad-spectrum detection that did not need prior knowledge of the target information such as DNA sequences, as required in PCR detection. The results showed that the bacteria stayed alive when attached to the synthesized magnetic metal hydroxide. We could concentrate and identify both Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Vibrio parahaemolyticus*) bacterial cells using the magnetized zirconium hydroxide immobilization approach. The detection limit of *E. faecalis* spiked into milk was down to a level as low as 32 CFU/mL when cultivation of the isolated bacteria was performed prior to MS analysis. The immobilization-based MS approach is simple and allows analysis of microorganisms in complex food samples.

1. Introduction

Foodborne pathogens, pose a major threat to public health. Preventing foodborne illness/pathogens is difficult because of the multiple stages in the manufacture and preparation of food. Therefore, the ability to carry out epidemiological analysis and to determine the primary sources of microbial contamination is important to improve public health. Establishing a new strategic approach is a key step in preventing future disease outbreaks worldwide. Therefore, rapid detection and identification of microbial pathogens in food is crucial to prevent/recognize problems related to health and safety [1,2]. Conventional culture/colony-based methods remain the most reliable and precise techniques in identifying the foodborne pathogens [3]. However, the microbiological methods are laborious and time-consuming because they often require 2–3 days for initial results, and up to 7–10 days for confirmation. This causes delay in preventing or resolving the pathogen-related problems. Immunology-based methods such as enzyme immunoassay (EIA) [4], enzyme-linked immunosorbent assay (ELISA) [5], enzyme-linked immunomagnetic chemiluminescence (ELIMCL) [6], immunochromatography (ICG) strip test [7], agglutination test [8], and Western blots [9] have been successfully employed for the detection of bacterial cells, spores, viruses and toxins [10]. Although, the immunology-based detection methods are not as accurate and sensitive as nucleic acid-based methods, they are faster, more robust and have the ability to directly detect microbial toxins [10]. Further, compared to traditional culture techniques these techniques have much less assay time, but the task to detect microorganisms in real-time is still challenging. PCR-based techniques can detect a single copy of a target DNA sequence, and thus, less prone to produce false-positive results. The methods detect a microorganism by amplifying the target rather than the signal, and might be employed to detect a single pathogenic bacterium in food. Although rapid methods to detect pathogens based on PCR have been developed, some problems remain in the identification of atypical strains whose sequences are unknown.In recent years, mass spectrometry (MS) has become a valuable tool for the analysis of pathogenic microorganisms [11,12]. The bacterial classification and identification have been greatly enhanced by the development of two critical soft ionization techniques namely matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). Mass spectrometry with both techniques has emerged as a rapid and sensitive method for accurate pathogen identification at the genus, species or sub-species level. The MS methods are based on either the detection of proteins and peptides or the detection

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of nucleic acids [12]. MALDI-MS has been applied to distinguish between pathogenic and nonpathogenic contaminants in foods. For instance, Mazzeo and co-workers [13] used MALDI-MS to identify 24 bacterial species and to discriminate the foodborne pathogenic *Escherichia coli* O157:H7 and the nonpathogenic *E. coli* ATCC 25922. Other foodborne pathogens including *Salmonella species* [14] and *Listeria species* [15] that causes serious illnesses were successfully identified using MALDI-MS. Alternatively, ESI-MS/MS is a highly selective and sensitive analytical technique for characterizing the complex microbial mixtures. In earlier studies we successfully identified multiple bacterial species-specific proteins in clinical specimens by performing CE–MS or LC–MS analyses on the proteolytic digests of cell extracts and monitored only the selected marker peptide masses using MS/MS and subsequent database searches [16–19].

Matrixes in real samples often suppress the signals of protein ions obtained from microbial cells, making the direct identification of bacteria difficult or infeasible. Therefore, a simple and rapid bacterial separation and concentration method is necessary for the detection of pathogens in food prior to MS analysis. An affinity separation method has been used to trap bacterial cells from complex biological samples. Magnetic or non-magnetic surfaces that carry charges, chemical, or biochemical groups might interact with target bacterial cells in samples [20–26]. The target bacterial cells might be separated from the sample and subjected to further analyses. Immunomagnetic separation (IMS) is a popular affinity separation technology and has been widely used to reduce the detection time/suppression effect and to improve detection sensitivity [27,28]. For example, Ochoa and Harrington investigated the rapid isolation and identification of the foodborne pathogen, *E. coli* O157:H7, from ground beef using an immunomagnetic isolation technique combined with MALDI-MS [28]. Although the IMS technique has been applied to pathogen isolation, it allows enrichment of specific target bacteria only, rather than a group of bacteria. In addition, a disadvantage of using antibodies is their limited stability. Another promising bacterial concentration approach is immobilization of bacterial cells with metal hydroxides. Kennedy et al. [29] first demonstrated the use of titaneous/zirconium hydroxide to immobilize bacterial cells and later several groups applied the method to the concentration of cells from culture media, clinical samples, and foods [30,31]. Lucore et al. [32] used metal hydroxide immobilization to concentrate foodborne bacteria (pathogenic and non-pathogenic) from samples of nonfat dry milk (NFDM). Cullison and Jaykus [33] simplified the method using magnetized metal hydroxide to concentrate bacteria in NFDM. PCR amplification was performed after the microorganisms were captured.

Conventional biological analysis of microorganisms in food requires cultivation of the microorganisms in medium and subsequent colony isolation. The process is time-consuming. Immobilization methods are convenient but often require PCR amplification of the captured bacteria using target-specific primers. In this work, we combined a microbial immobilization method with MS analyses. Immobilization of bacterial cells using zirconium hydroxides was easy, rapid, efficient, and nonspecific. Also, zirconium hydroxide-immobilized bacteria remained viable. Analysis of bacteria viability in food products/real samples is of economic and clinical significance because only viable pathogens might cause medical concerns. The immobilization method isolates bacteria from complex food matrices and the MS offer the ability of broad-spectrum detection of bacterial species. To improve the detection limit for the immobilization approach, bacteria spiked in NFDM, pudding, and coffee were captured using magnetized zirconium hydroxide and cultivated for a relatively short period of time prior to MALDI-MS and LC–MS/MS analyses.

2. Materials and methods

2.1. Materials

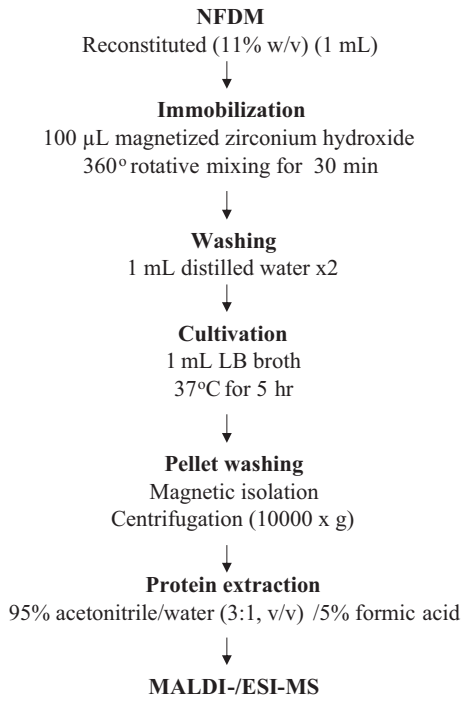
Zirconium (IV) chloride, carbonyl iron, α-cyano-4-hydroxycinnamic acid (CHCA) were acquired from Sigma–Aldrich (Saint Louis, MO, USA) and used without further purification. Organic solvents were of HPLC grade. Water was purified with a Millipore water purification system Milipore Milli-Q (Bedford, MA, USA). Porcine trypsin (sequence grade) was provided by Promega (Madison, WI, USA). Acetonitrile, acetic acid, and methanol were purchased from JT Backer (Phillipsburg, NJ, USA). Granulated agar was obtained from Conda Laboratories (Pronadisa, Spain). Ammonium hydroxide solution was supplied by Wako Pure Chemical Industries (Osaka, Japan). Ammonium bicarbonate and Luria-Bertani (LB) broth were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NFDM and trifluoroacetic acid (TFA) were obtained from Fluka (Buchs, SG, Switzerland). The bacteria used in this study included *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *E. coli*, and *Enterococcus faecalis*. All the bacterial cells were prepared in a BSL-2 safety cabinet at National Dong Hwa University.

2.2. Preparation of magnetized zirconium hydroxide

Magnetized metal hydroxide suspensions were prepared as previously reported with minor modification [32,33]. A 50 mL volume of distilled water was added to 1.0 g of zirconium (IV) chloride and 1.0 g of carbonyl iron. The solution mixture (pH is approximately 2.0) was adjusted to neutral by the dropwise addition of ammonium hydroxide (15%) with continuous stirring and resulted in the production of zirconium hydroxide (floc) suspension. After the floc-like product appeared, the solution was washed three times with 200 mL of 0.9% sodium chloride to remove excess ammonium ions. The metal hydroxide was concentrated by applying an external magnetic field and the supernatant was decanted carefully. The metal hydroxide was washed two times with 50 mL of distilled water and re-suspended in 30 mL of water. The final suspension was stored in the dark at room temperature.

2.3. Immobilization and cultivation studies

One milliliter of 11% (w/v) NFDM (Fluka, Buchs, SG, Switzerland) or 0.5 g of pudding/coffee samples in 0.5 mL of distilled water were seeded with various concentrations of bacteria and were mixed with 100 μL magnetized zirconium hydroxide suspension. The concentration was not estimated because of the floc nature of the hydroxide. The suspension was agitated by 360° rotative mixing for 30 min to facilitate cell immobilization. After incubation, the bacterial cells immobilized on zirconium hydroxide were collected from suspension with a magnet and the pellets were washed two times with 1 mL of distilled water. The metal hydroxide–bacteria complex was re-suspended in 1 mL of LB broth at 37 °C for 5–8 h. The metal pellets were separated from the solution with a magnet and the bacterial cells in the supernatant were collected into an eppendorf tube. The cultivated bacterial cells were centrifuged at 10,000 × *g* for 10 min and the supernatant was decanted. The bacteria were washed two times with 1 mL of distilled water and centrifuged at 10,000 × *g* for 10 min. An appropriate amount of a solution containing 95% acetonitrile/water (3:1, v/v) and 5% formic acid was used to extract proteins from the cell pellets by sonication. The entire immobilization process is outlined in Scheme 1. In direct analyses of bacteria in food, 5×10^5 CFU of bacteria were spiked into 0.5 mL of NFDM or 0.5 g of pudding/coffee and the samples were mixed with 0.5 mL of LB broth. The suspensions were incubated for 8 h and centrifuged. The pellets were washed two times with 1 mL of



Scheme 1. Flow chart of cell-immobilization approach for analysis of bacteria in nonfat dry milk.

distilled water. The microbial proteins were extracted by mixing the pellets containing bacteria and solid food matrices with a solution containing 95% acetonitrile/water (3:1, v/v) and 5% formic acid. The supernatant of suspensions was subjected to MS analysis.

Viable bacteria may be counted by diluting samples with a PBS buffer (pH = 7.4), plating the dilutions on a solid medium, and counting the colonies. Bacterial concentrations were expressed as colony-forming units (CFU). Only plates with 30–300 colonies were counted. To evaluate the recovery rate of bacteria using magnetized zirconium hydroxide, 100 μL of the magnetized zirconium hydroxide suspension was mixed with 1 mL of *E. coli* (10^7 CFU) suspension in water. The bacteria-zirconium hydroxide complexes were collected from the suspension using a magnet after the suspension was incubated for 30 min. The supernatant was serially diluted and subsequent inoculation on agar was performed. Average recovery percentage for three replicate samples based on loss to supernatant was calculated according to the following formula: percent recovery = [total population in sample before concentration − total population in supernatant after immobilization] × 100/[total population in sample before concentration].

2.4. Characterization

MALDI spectra were acquired using an Autoflex time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337-nm nitrogen laser (10 Hz, 3-ns pulse width). Spectral data were obtained in the linear mode with an acceleration voltage of 20 kV. Each mass spectrum was derived from 150 summed scans. A saturated matrix solution was prepared by dissolving excess α-cyano-4-hydroxycinnamic acid in a solution of 50% acetonitrile/water containing 0.1% TFA. A 0.5 μL aliquot of matrix solution was used to overlay the sample spot and allowed to air-dry. LC–MS acquisition was conducted on a LCQ Duo MS system (Thermofinnigan, San Jose, CA, USA). This instrument was operated in the positive mode under the control of an Xcalibur program. Chromatographic separation was performed on an Agilent 1100 series capillary pump (Agilent, Palo Alto, CA) equipped with a home-made reverse phase C18 column (300 Å, 5 μm, 320 μm × 150 mm) with a flow rate of 4 μL/min. The mobile phase A was 5% acetonitrile in water containing 0.1% trifluoroacetic acid; mobile phase B was 5% water in acetonitrile containing 0.1% trifluoroacetic acid. Proteins were digested with 1 μg trypsin in a 55 mM (pH = 8.5) NH_4HCO_3 solution. The digestion was carried out under the irradiation of a domestic microwave for 10 min [34]. Tryptic digests of cell extracts were subjected to LC–MS/MS analysis. FT-IR spectra were measured in an FT-720 FT-IR instrument (Horiba, Japan). Spectra were recorded in the wave number interval between 4000 and 400 cm^{-1}. The morphology of the magnetized zirconium hydroxide was examined by field emission scanning electron microscopy (FE-SEM) using a JSM-6500F model microscope (JEOL, Japan).

2.5. Database search

Tentative identification of proteins was carried out by matching the MALDI mass spectral data to protein masses in the UniProtKB/TrEMBL database using the Sequence Retrieval System (SRS). A ±3 Da tolerance in molecular weight was used. The MS/MS spectra were searched against the NCBInr database using SEQUEST (ThermoFinnigan Inc.). In the data-dependent experiments, the mass tolerances of the precursor and fragment ions were set to 2.0. A full MS scan was acquired from m/z 400 to 2000, followed by three MS/MS scans between m/z 400 and 2000 of the most-, second-most-, and third-most-intense ions of the full MS scan. Only those peptides that gave cross-correlation scores (Xcorr) > 1.8 for singly charged, >2.0 for doubly charged peptides, and >3.3 for triply charged peptides [each with delta-correlation scores (DelCn) > 0.1] were considered as legitimate identifications.

3. Results and discussion

Microbial immobilization using metal hydroxides is one of the promising methods for bacterial capture/separation. In the present study, magnetized zirconium hydroxide floc was synthesized and characterized by using energy-dispersive X-ray spectroscopy (EDS) and FT-IR. EDS was used for the elemental analysis of magnetized zirconium hydroxide. The peaks in the EDS spectrum represent characteristic emission from particular elements including carbon, oxygen, iron, and zirconium (see supporting information Fig. S1). The chemical structure of magnetized zirconium hydroxide floc was further examined by FT-IR spectroscopy. Fig. S2 in supporting information presents an infrared spectrum of magnetized zirconium hydroxide floc. In the FT-IR spectrum, the broad band centered upon 3406 cm^{-1} might result from the hydroxyl groups of the zirconium hydroxide. The intense band at 2298 cm^{-1} is attributable to the metal carbonyl stretching vibrations. The band at 1633 cm^{-1} might be related to the scissor bending mode of coordinated water. In addition, a strong absorption at 467 cm^{-1} is due to the Zr–O vibration. The above results provide support for the successful synthesis of magnetized zirconium hydroxide floc.

The analysis of bacteria in NFDM/pudding/coffee is a challenging task because the bacteria may be dispersed at low concentrations in foods. Consequently, there is a need for a preconcentration step, which separates the target bacteria from the complex background of food products [2,35]. This study used a metal hydroxide immobilization approach to isolate and concentrate the bacteria from complex food matrices. The immobilization method has been applied to the analysis of *S. aureus*, *V. parahaemolyticus*, *E. coli* and *E. faecalis* artificially spiked in foods. After magnetized zirconium hydroxide was added to bacteria-spiked samples, the suspensions were agitated by rotative mixing. The magnetized zirconium hydroxide floc interacted with and efficiently immobilized the pathogens. The immobilized bacterial cell aggregates were sep-

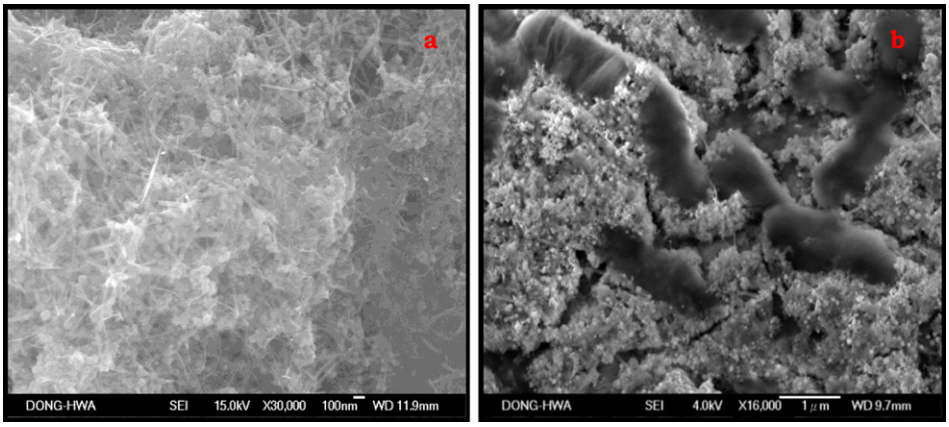


Fig. 1. Field emission scanning electron microscope (FE-SEM) images of (a) pure magnetized zirconium hydroxide; (b) *E. coli* immobilized on magnetized zirconium hydroxide.

arated from the complex food background by applying a magnet to the sample vial. Fig. 1 represents FE-SEM images of magnetized zirconium hydroxide floc, before and after bacterial immobilization. We first evaluated the ability of the magnetized zirconium hydroxide floc to immobilize bacterial cells using *E. coli* as a model. A 100-μL volume of the magnetized zirconium hydroxide suspension was mixed with 1 mL of water containing 10^7 CFU of *E. coli*. After the bacterial cells were isolated from the bacterial suspension by the magnetic zirconium hydroxide, the bacterial loss to the supernatant was determined through the serial dilution of the supernatant and subsequent plating. *E. coli* was readily immobilized by the magnetized zirconium hydroxide. Insignificant (<1%) bacterial loss to the supernatant (99.97% recovery) was observed. The results indicate that 100μL of magnetized metal hydroxide suspension is sufficient to capture bacteria up to 10^7 cells. The immobilization of bacteria by zirconium hydroxide might involve covalent interaction. Kennedy et al. [29] hypothesized that covalent bonding might occur between the metal hydroxide and ligands (free amino, hydroxyl, and carboxyl residues) from the bacterial cell.

When the bacteria in NFDM were isolated with magnetized zirconium hydroxide and analyzed by MALDI-MS without microbial culturing, the lowest detectable concentration of bacteria spiked in the complex NFDM was ~5.0×10^4 CFU/mL (data not shown). Functionalized particles have been used to analyze various bacteria spiked in water or urine samples with detection limits of $\geq 10^3$ CFU/mL [22,23,25]. Hence, to improve the detection limit for the zirconium hydroxide approach, we cultivated bacteria in foods (NFDM, pudding, and coffee) after the bacteria were captured by the hydroxide. Panels (a)–(d) in Fig. 2 show MALDI spectra of *S. aureus*, *E. faecalis*, *V. parahaemolyticus*, and *E. coli* spiked in NFDM samples at a concentration of 5.0×10^3 CFU/mL. The important protein biomarker peaks representing these bacteria appear in the region from *m/z* 5000 to 10,000. For instance, the ion peaks at *m/z* 5509, 5877, 5936, 6357, 6427, 6555, 6849, 6892, 7174, 7573, 8095, 8895, and 9182 shown in Fig. 2a represent specific proteins of *S. aureus*. All these biomarker ions can be identified through the protein database searching [36]. Table 1 lists the corresponding proteins that we identified from the protein database within a mass tolerance of ±3 Da. These spectra indicate that the magnetized zirconium hydroxide floc is capable of capturing bacteria in complicated NFDM and that these target bacteria captured can be characterized by MALDI-MS. The spectrum obtained from a NFDM

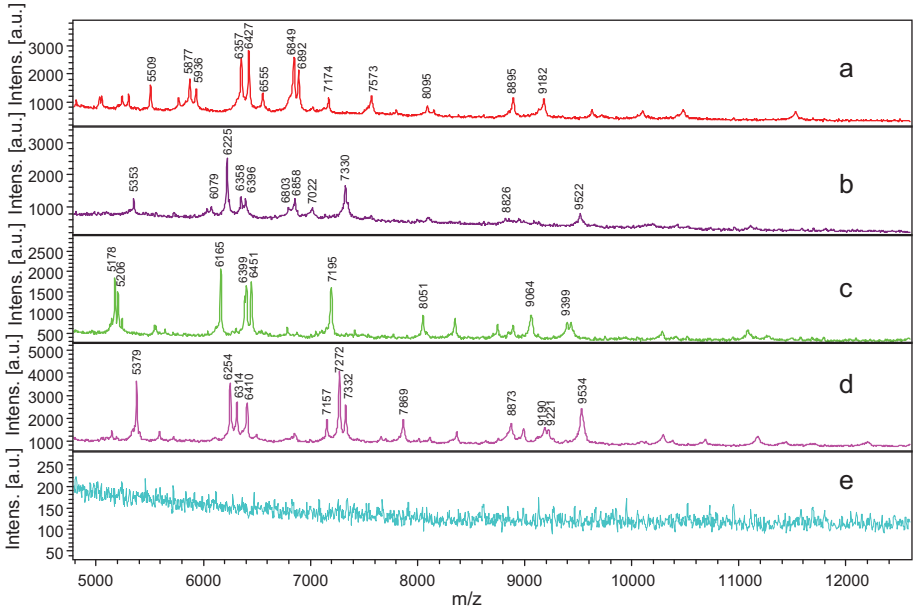


Fig. 2. MALDI mass spectra of (a) *S. aureus*, (b) *E. faecalis*, (c) *V. parahaemolyticus*, and (d) *E. coli* spiked in NFDM samples at a concentration of 5.0×10^3 CFU/mL. (e) MALDI mass spectrum obtained from a NFDM blank (without bacteria) using the immobilization approach. All the samples were mixed with magnetized zirconium hydroxide and the hydroxide pellets were incubated in LB broth for 5 h prior to MALDI analysis.

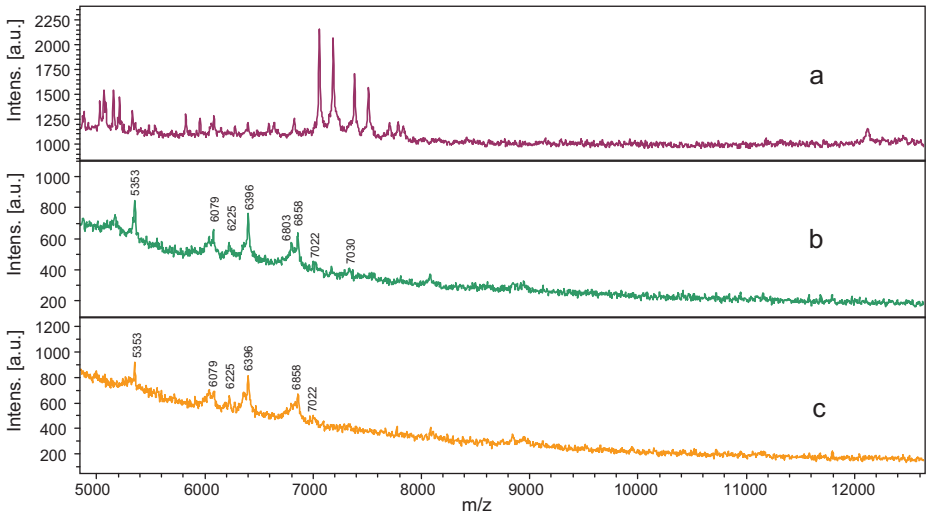


Fig. 3. MALDI mass spectra of *E. faecalis* obtained from *E. faecalis*-spiked NFDM samples at a concentration of (a) 5.0×10^5 CFU/mL (direct culturing without magnetized zirconium hydroxide immobilization) (b) 5.0×10^2 CFU/mL (with hydroxide immobilization) (c) 32 CFU/mL (with hydroxide immobilization). The bacteria in the sample were cultivated for 8 h with or without magnetized zirconium hydroxide immobilization before MALDI-MS analysis.

Table 1
Biomarkers observed in the MALDI mass spectra of bacteria and their matched proteins *in the SwissProt/TrEMBL database*.

Species	Observed mass (Da)	Expected mass (Da)	Accession number	Description	p*I*
S. aureus	5509	5509	D6SD74	Putative uncharacterized protein	9.84
	5877	5874	C5Q1Z0	Putative uncharacterized protein	9.69
	5936	5936	C5N6T4	Putative uncharacterized protein	4.14
	6357	6354	D8HCM8	DNA helicase, phage-associated	4.73
	6427	6424	C5N0H5	Putative uncharacterized protein	5.47
	6555	6554	A7X5D6	50S Ribosomal protein L30	10.11
	6849	6848	C5N087	Putative uncharacterized protein	9.8
	6892	6894	C7ZVR6	Glutamic acid specific protease prepropeptide	9.4
	7174	7173	C8KPP0	Putative uncharacterized protein	9.52
	7573	7570	C5N093	Putative uncharacterized protein	4.75
	8095	8095	C7ZWR3	Predicted protein	9.71
	8895	8893	D2FL44	Putative uncharacterized protein	8.93
	9182	9181	A0EWH6	Transcription regulator	6.26
E. faecalis	5353	5353	Q82YU9	50S Ribosomal protein L34	12.66
	6079	6077	C0X731	Putative uncharacterized protein	8.68
	6225	6223	C7UZU2	Transcriptional repressor	9.46
	6358	6355	Q839E6	50S Ribosomal protein L30	9.99
	6396	6398	C0X223	Putative uncharacterized protein	9.51
	6803	6801	C7CX33	Predicted protein	3.99
	6858	6861	C7VHG5	Predicted protein	8.84
	7022	7022	C0X5X5	Putative uncharacterized protein	9.84
	7330	7330	Q839F6	50S Ribosomal protein L29	9.6
	8826	8823	C2D9Z3	Putative uncharacterized protein	6.31
	9522	9523	C7VWG8	Predicted protein	10.09
V. parahaemolyticus	5178	5180	Q87TR3	50S Ribosomal protein L34	12.96
	5206	5205	A6AXV5	Putative uncharacterized protein	8.98
	6165	6163	E1CVD8	Putative lipoprotein	5.99
	6399	6402	E1CWE9	Putative uncharacterized protein	10.53
	6451	6452	A6B1U6	Putative uncharacterized protein	4.37
	7195	7193	Q87T05	50S Ribosomal protein L29	9.81
	8051	8050	B8K9I1	Putative uncharacterized protein	10.00
	9064	9063	Q87LS8	30S ribosomal protein S16	10.42
	9399	9400	Q87TP4	Sulfurtransferase tusA homolog	5.35
E. coli	5379	5380	A7ZTQ9	50S Ribosomal protein L34	13.00
	6254	6251	Q0TFN3	Uncharacterized protein yojO	9.74
	6314	6315	B0FIL1	Putative uncharacterized protein	5.14
	6410	6407	B3I765	Putative uncharacterized protein	9.10
	7157	7158	B0FIJ4	Putative uncharacterized protein	9.45
	7272	7273	A7ZSK1	50S Ribosomal protein L29	9.98
	7332	7331	Q1RBS2	Two-component-system connector protein SafA	6.71
	7869	7871	A7ZUF1	50S Ribosomal protein L31	9.46
	8873	8874	P76521	Uncharacterized protein yfdY	8.61
	8990	8989	B0LT93	Truncated outer membrane protein C	4.83
	9190	9191	A7ZQ49	30S ribosomal protein S16	10.54
	9221	9219	P27193	Protein traD	3.67
	9534	9535	P0ACF2	DNA-binding protein HU-alpha	9.57

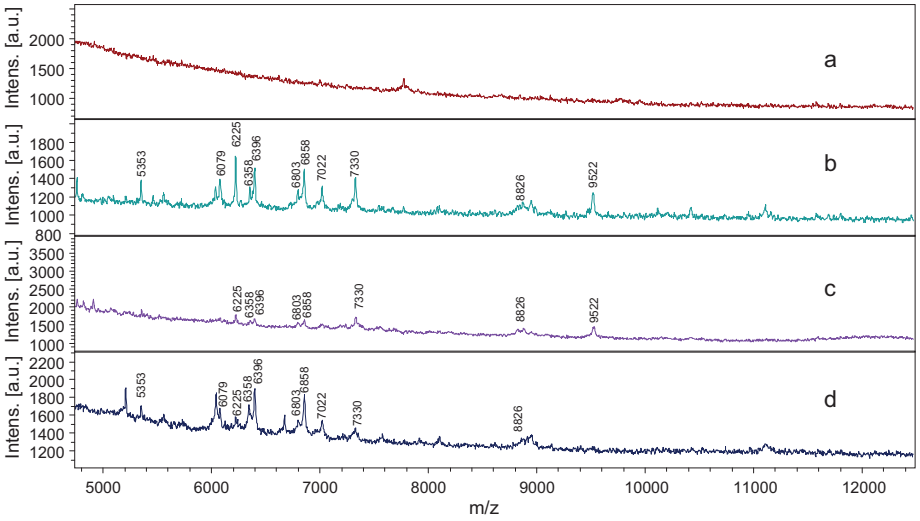


Fig. 4. MALDI mass spectra of *E. faecalis* obtained from *E. faecalis*-spiked pudding and coffee samples at a concentration of (a) 5.0×10^5 CFU/mL (pudding, direct culturing) (b) 5.0×10^4 CFU/mL (pudding, with hydroxide immobilization) (c) 5.0×10^5 CFU/mL (coffee, direct culturing) (d) 5.0×10^4 CFU/mL (coffee, with hydroxide immobilization). The bacteria in the samples were incubated for 5 h with or without magnetized zirconium hydroxide immobilization before MALDI-MS analysis.

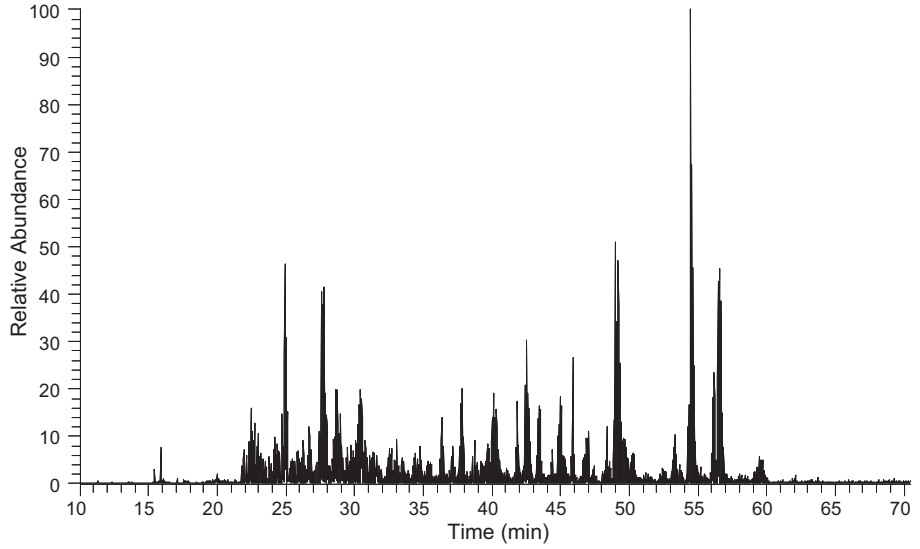


Fig. 5. Data-dependent base-peak ion chromatogram obtained from the LC–MS/MS analysis of the tryptic digest of the *E. faecalis* cell extract.

blank (without bacteria) using the immobilization approach was also shown in Fig. 2(e). No signal was observed in the spectrum. The result implies that the NFDM was bacteria-free or the number of bacteria cells in NFDM was too few to interfere with our experiments. Furthermore, analysis of target bacteria from reconstituted milk failed when using a filtration or centrifugation method (data not shown). The high abundance of milk matrix rendered the analysis impossible.

We further examined the lowest detectable concentration of bacteria using the immobilization approach. *E. faecalis* was

Table 2
SEQUEST search results for the top 10 identified peptides generated from the LC–MS/MS analysis of the proteolytic digest of the *E. faecalis* cell extract.

Xcorr	Peptide mass (charge)	Peptide	P (pep)	Protein	Microbial source
5.717	2654.3 (+3)	R.KVDYIAANHIEYIDYKDTELLK.R	3.07E−10	Ribosomal protein S18	*Enterococcus faecalis*
5.082	2807.4 (+3)	K.DYAVVNLDTLNRFEDGTEVTPVVLK.E[a]	1.57E−07	50S Ribosomal protein L15	*Enterococcus faecalis*
4.807	1858.8 (+2)	R.NMTSSDFAEITTSTPEK.S	3.35E−08	50S Ribosomal protein L2	*Enterococcus faecalis*
4.590	1854.9 (+2)	-.MKEGQEQFLTFILDR.T	7.89E−06	Hypothetical protein EF0857	*Enterococcus faecalis*
4.412	2324.1 (+2)	K.KFEEAVAAGADNVDALYNEAVK.A	1.32E−06	Ribosomal protein S20	*Enterococcus faecalis*
4.305	2972.4 (+3)	R.HEGDTGSPEVQIAVLTEDINQLNEHAR.T	7.20E−06	Ribosomal protein S15	*Enterococcus faecalis*
4.239	2843.4 (+3)	K.EAVEATVGRPAFVSFDTEKLEGSFTR.L	5.53E−04	30S Ribosomal protein S4	*Enterococcus faecalis*
4.208	2972.4 (+2)	R.HEGDTGSPEVQIAVLTEDINQLNEHAR.T	1.91E−09	Ribosomal protein S15	*Enterococcus faecalis*
4.042	1515.7 (+2)	R.SANIALVHYEDGVK.A	1.10E−04	50S Ribosomal protein L2	*Enterococcus faecalis*
3.962	2296.3 (+3)	-.NLDKAVEELTVISGQKPLITK.-	8.86E−01	50S Ribosomal protein L5	*Carnobacterium* sp.

[a] The unique peptide sequences are underlined.

relatively difficult to culture (smaller OD values at 600 nm) compared to *S. aureus*, *V. parahaemolyticus* and *E. coli* (see supporting information Fig. S3) and, therefore, was used for the study of detection limit. The bacteria in NFDM were isolated with magnetized zirconium hydroxide and cultivated for 8 h prior to MALDI analysis. The bacteria in NFDM were also directly cultured without separating the bacteria from the milk matrix with zirconium hydroxide. Fig. 3a presents a MALDI mass spectrum obtained from direct culturing of an *E. faecalis*-spiked NFDM sample (5.0×10^5 CFU/mL before culturing). No specific protein signals of *E. faecalis* appear in the spectrum. The dominant peaks are likely associated with casein fragments in NFDM [37]. After the direct bacterial culturing of the milk sample, the pellet obtained by centrifugation contained bacteria and the solid matrix of milk. Part of the solid matrix is water-insoluble protein aggregates [38]. When the pellet was mixed with the extraction solvent (95% acetonitrile/water (3:1, v/v) and 5% formic acid), some proteins other than microbial proteins were dissolved into the solvent. These proteins suppressed the signals of microbial proteins and were detected by MS. Fig. 3b represents a MALDI mass spectrum of *E. faecalis* obtained from cultivation of the bacteria that were isolated from the *E. faecalis*-spiked NFDM sample using magnetized zirconium hydroxide. The bacterial concentration used to obtain Fig. 3b was 5.0×10^2 CFU/mL before culturing. The ions at m/z 5353, 6079, 6225, 6396, 6803, 6858, 7022, and 7330 represent proteins of *E. faecalis*. All these ions match proteins in the protein database (Table 1). When the concentration of *E. faecalis* was decreased to a level as low as 32 CFU/mL, the ions at m/z 5353, 6079, 6225, 6358, 6396, 6858, and 7022 still appear in the mass spectrum (Fig. 3c). The concentration at 32 CFU/mL was considered as the detection limit for *E. faecalis* because a further decrease of concentration to 15 CFU/mL did not show any signals. These results indicate that the magnetized zirconium hydroxide suspension is capable of capturing the traces of bacteria from complex food materials such as NFDM.

The successful cultivation of immobilized *E. faecalis* at the trace level shows that the bacterial species remained viable after exposure to the synthesized magnetized zirconium hydroxide. Our results are consistent with previous reports [29,32,33,39]. Jaykus and coworkers evaluated the effects of zirconium hydroxides on the viability of *Salmonella enteritidis* and *Listeria monocytogenes*. They concluded that the zirconium hydroxides were reasonably nontoxic after 2 days of exposure. Analysis of bacteria viability has clinical significance because only viable pathogens might cause medical concerns.

To test the applicability of the proposed approach, we spiked *E. faecalis* in other complex food products such as pudding and coffee. *E. faecalis* in the pudding/coffee were cultivated for 5 h with/without magnetized zirconium hydroxide immobilization prior to MALDI analysis. Fig. 4a and c shows MALDI mass spectra of *E. faecalis* obtained from direct culturing (for 5 h without magnetized zirconium hydroxide separation) of bacteria-spiked pudding and coffee samples at a concentration of 5.0×10^5 CFU/mL. We did not observe significant ion signals in the spectrum displayed in Fig. 4a. The pudding matrix seemed to cause poor crystallization of the MALDI matrix and to degrade the quality of mass spectra. We observed some weak signals of bacterial proteins from the coffee sample (Fig. 4c). When we used magnetized zirconium hydroxide to isolate and concentrate *E. faecalis* from the pudding/coffee samples (5.0×10^4 CFU/mL) before cultivation was performed, the ion abundances of bacterial proteins in mass spectra were greatly enhanced. Fig. 4b and d represents the MALDI mass spectra of *E. faecalis* obtained from the pudding and coffee samples, respectively, using the hydroxide immobilization approach.

The challenges in bacterial identification using MALDI-MS include maintaining the spectral reproducibility and differentiating different species when a bacterial mixture is present in samples. Many statistical algorithms have been developed to match acquired spectra to reference spectra, or to extract and compare bacterial fingerprints from samples containing more than one bacterial species [40,41]. In this study, we also used LC–MS/MS analysis and database searches to identify *E. faecalis* in NFDM (1.7×10^5 CFU/mL). The bacteria in NFDM was isolated with magnetized zirconium hydroxide and cultivated for 5 h. After the *E. faecalis* proteins were extracted and digested, the peptide digests were analyzed with LC–MS/MS. The MS/MS spectra were searched against an NCBInr protein database using the SEQUEST application to identify the proteins, and to deduce the source of the microorganism. Fig. 5 shows a data-dependent base-peak ion chromatogram for *E. faecalis*. The base-peak ion chromatogram includes many full MS scans of the separated peptides, with every full scan followed by MS/MS scans of the three most-intense precursor ions. Hundreds of product ion mass spectra of peptide ions were produced during the LC-MS/MS analysis of the tryptic digest of the *E. faecalis* cell extract. Table 2 summarizes the top 10 identified peptides (nine different peptide sequences) that gave cross-correlation (Xcorr) scores and DelCn values higher than the set values described in the experimental section as well as the microbial sources of the matched proteins. Eight out of these 9 peptide sequences lead to the identification of the proteins associated with the spiked bacteria, *E. faecalis*. Only one peptide identified a protein of *Carnobacterium* sp. The mismatch might result from the uncertainty of MS/MS data and was revealed by the large peptide probability (88.6%), P (pep), shown in Table 2. The probability value represents the statistical likelihood of finding an equally good peptide match by chance. The overall results clearly indicate that *E. faecalis* is in the NFDM sample. We note that five of the eight peptides that are associated with *E. faecalis* are unique peptide biomarkers. The other three peptides are not unique biomarkers because the matched proteins are shared by several bacterial sources. Nevertheless, the identification of the five unique peptides leads to reliable characterization of *E. faecalis*.

4. Conclusions

In this study, the suspension of magnetized zirconium hydroxide successfully isolated foodborne pathogens from foods including NFDM, pudding, and coffee. We combined the microbial immobilization method with MS analyses to identify both Gram-positive and Gram-negative bacteria in foods. To improve the detection limit for the immobilization approach, bacteria spiked in foods were captured using magnetized zirconium hydroxide and directly cultivated for 5–8 h. Because a colony isolation procedure was not required, the analysis is simpler and less time-consuming than conventional methods. The peptide digests obtained from protein extracts of bacteria were also analyzed with LC–MS/MS. The MS/MS spectra were searched against an NCBInr protein database to identify the proteins, and to deduce the source of the microorganism. The database searching method identifies peptide sequences related to proteins of bacteria. The MS-based approach using direct concentration and amplification of bacteria from foods should provide an efficient means of identifying pathogens in complex food matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.05.014.

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