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Establishing a MALDI-TOF-TOF-MS method for rapid identification of three common Gram-positive bacteria (*Bacillus cereus, Listeria monocytogenes*, and *Micrococcus luteus*) associated with foodborne diseases

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Abstract

We aimed to establish a method for the rapid identification of three common Gram-positive bacteria (*Bacillus cereus*, *Listeria monocytogenes*, and *Micrococcus luteus*) associated with foodborne diseases. MALDI-TOF-MS was used to determine the effects of sample pretreatment, culture medium, and culture time on the identification results. Then, MALDI-TOF-TOF-MS was used to establish an optimized method and further explore the effects of culture time on secondary proteins. Among the three sample pretreatment methods, formic acid extraction outperformed direct transfer and extended direct transfer, resulting in more protein peaks and higher peak intensity in *B. cereus* culture samples. However, with different culture times (1–7 d), ion peaks of all bacterial proteins were relatively stable according to MALDI-TOF-TOF-MS data. The protein peaks of *B. cereus* (1430 m/z), *L. monocytogenes* (2100 m/z), and *M. luteus* (2140 m/z) were split into small ion peaks at 1162, 1465, and 1625 m/z, respectively. After 7 d of culture, the secondary spectra, peak intensities, and peak values of formic acid-treated samples were relatively stable, indicating that secondary protein peaks were less affected by culture time. It provides a new approach for the routine identification and market supervision of food safety in China.

Keywords: culture time; characteristic peak; pathogenic bacteria; MALDI-TOF-MS; MALDI-TOF-TOF-MS.

Practical Application: [The research will help to establish the MALDI-TOF-MS method for the standard operation of strain identification and improve the identification level. MALDI-TOF-TOF-MS is used to establish a validation method and identify bacteria based on secondary protein peaks, helping to support the application of this method, improve the identification of foodborne pathogens, and regulate the operation of food safety market supervision.]

1 Introduction

Diseases caused by the presence of bacteria in food, is the main food safety concerns of many food manufacturers, consumers, researchers and regulatory agencies around the world.According to data released by WHO in 2020, an estimated 600 million people (nearly one-tenth of the world's people) get sick from eating contaminated food, and 420,000 people die every year, resulting in the loss of 33 million healthy life years (DALYs).In particular, bacterial food poisoning incidents that occur in China every year account for 30%–90% of the total number of food poisoning incidents worldwide (Tang et al., 2011; Shan et al., 2012). Food contamination by pathogenic microorganisms poses a huge and growing global public health problem (Liu et al., 2004). Therefore, monitoring foodborne diseases is imminent, and it is imperative to strengthen the detection and identification of pertinent pathogens.

There are many types of common foodborne pathogens, including Gram-positive and Gram-negative bacteria. *Bacillus cereus*, *Listeria monocytogenes*, and *Micrococcus luteus* are common Gram-positive bacteria that cause foodborne diseases. *B. cereus* is a facultative aerobic bacterium belonging to the Bacillaceae family. It is widely found in water, air, and soil, and it is easy to detect in food (Yu et al., 2019; Guo et al., 2020; Jessberger et al., 2020; Yu et al., 2020; Sbhatu et al., 2021; Zhao et al., 2021). *B. cereus* is a conditional pathogen that can cause foodborne poisoning, in addition to traumatic endophthalmitis, orthopedic wound infections, and multiple tissue infections in people with low immune levels (Gaur et al., 2001; Yagishita et al., 2011; Safri et al., 2014; Lotte et al., 2017; Wang et al., 2017).

L. monocytogenes (Silva et al., 2021), a facultative anaerobe, is the main pathogenic bacterium in the Listeriaceae family. It can grow at a wide range of temperatures and shows strong vitality. *L. monocytogenes* occurs widely in nature and is one of the most lethal foodborne pathogens. If *L. monocytogenes*-contaminated food is carelessly consumed, it can lead to meningitis, abortion, and sepsis in humans as well as animals, and the mortality rate is high at 20%–30% (Kathariou, 2002; Chiang et al., 2012; Razei et al., 2017; Uematsu et al., 2021). Examples include the contamination of ready-to-eat meat with *Listeria monocytogenes* in South Africa in 2017/18, resulting in 1060 cases of listeriosis and 216 deaths,which comes from WHO statistics . Further,

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M. luteus is an obligate aerobic bacterium belonging to the Micrococcaceae family. It is widely distributed in air, water, and soil, and it is found in the skin, pharynx, eyes, and other parts of the body. *M. luteus* is also a conditional pathogen. In humans or animals with low immunity levels, *M. luteus* can grow in the plasma and normal tissues, competing for nutrition and secreting toxins, leading to extensive tissue damage (Zhang et al., 2016; Erbasan, 2018; Guerra et al., 2019).

Many methods are commonly used for microbial detection and identification, but most have specific disadvantages. For example, traditional identification methods have a long detection cycle; moreover, they are cumbersome to perform and show poor specificity and low sensitivity (Chon et al., 2012). Molecular identification methods mainly include PCR, loop-mediated isothermal amplification, and 16S rRNA sequencing. However, for PCR identification, there is no uniform target gene, which makes standardization challenging (Cao et al., 2016). Loop-mediated isothermal amplification is sensitive but prone to contamination, resulting in false-positive results. Further, with 16S rRNA sequencing, it is difficult to achieve interspecific differentiation within microbial groups (Welker & Moore, 2011). Given the limitations of these methods, there is an urgent need to develop rapid, accurate, and high-throughput microbial identification methods. Consequently, fast, low-power, specific, simple, sensitive, and suitable methods have emerged, including immunological, metabolic, molecular biological, and mass spectrometry-based methods.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a new bacterial protein-based method for microbial identification (2–20 ku). Based on a certain ratio, microbial samples and matrix solution are transferred onto a target plate. After solvent volatilization and laser bombardment, the matrix that has absorbed energy is transferred to the microbial sample for desorption. Ions with different mass-to-charge ratio (*m/z*) are then separated by a TOF detector, and identification results are obtained by collecting mass spectra and comparing with a database (Murray, 2012; Branquinho et al., 2014). In comparison with traditional biochemical phenotyping and molecular biological methods, MALDI-TOF-MS is simple, rapid, accurate, economical, and high-throughput, and thus suitable to detect and monitor pathogenic microorganisms. However, the identification results of MALDI-TOF-MS are affected by culture conditions and sample preparation. Evidence suggests that multiple factors, including culture medium, culture time, sample purity, pretreatment method, and database completeness, affect the emergence of biomarkers and lead to changes in mass spectra (Monedeiro et al., 2021). Therefore, different influencing factors need to be optimized for accuracy of MALDI-TOF-MS identification results.

Unlike MALDI-TOF-MS that contains only a single TOF analyzer, MALDI-tandem TOF-MS (MALDI-TOF-TOF-MS) consists of a tandem structure of TOF/TOF. In MALDI-TOF-MS, ion cloud signal is collected by the TOF analyzer directly in the linear or reflection mode, while in MALDI-TOF-TOF-MS, the ion cloud first forms smaller fragments via collision-induced dissociation and then conducts a new accelerated flight; data are then collected by the secondary TOF analyzer. In comparison with first-order MS, second-order MS shows higher resolution and higher sensitivity, plus a wider range of molecular weights can be determined (Wang, 2018).

Herein a new method was established for rapid identification of the three common Gram-positive bacteria, namely *B. cereus*, *L. monocytogenes*, and *M. luteus*, associated with foodborne diseases. MALDI-TOF-MS-based microbial identification was optimized in terms of sample pretreatment, culture media, and culture time. Further, MALDI-TOF-TOF-MS was used to establish a verification method and identify bacteria based on secondary protein peaks. Our results should be helpful to support the application of the proposed method, improve the identification of foodborne pathogens, and standardize the operation for market supervision of food safety.

2 Materials and methods

2.1 Bacterial strains

B. cereus CMCC 63312, *L. monocytogenes* CICC 21583, *M. luteus* CICC 24814, and *Escherichia coli* ATCC 8739 were purchased from China Institute for Food and Drug Control (Beijing, China).

2.2 Reagents and equipment

Blood plate, tryptone soy agar (TSA), nutrient agar (NA), mannitol yolk polymyxin (MYP) agar, and PALCAM agar were purchased from Beijing Luqiao Technology Co., Ltd. (Beijing, China). Matrix α-cyano-4-hydroxy-cinnamic acid (HCCA) and peptide protein standards were from Bruker (Germany). Matrix and standard preparation solvent (50% acetonitrile, 2.5% trifluoroacetate, 47.5% water), as well as formic acid, acetonitrile, and absolute ethanol, were obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China).

MALDI-TOF-MS was performed on a Microflex™ MALDI-TOF mass spectrometer (Bruker) and MALDI-TOF/TOF-MS on a MALDI-7090 mass spectrometer (Shimadzu Corp., Kyoto, Japan). Other equipment included a constant temperature incubator (MIR-54; Samyo, Japan), biological safety cabinet (BHC-300IIA/B3; Suzhou Purification Equipment Co., Ltd., China), and desktop-refrigerated centrifuge (3K15; Sigma, German).

2.3 Selection of sample pretreatment method

A 1-d-old culture of *B. cereus* CMCC 63312 grown on blood plates was pretreated with three different methods: direct transfer, extended direct transfer, and formic acid extraction. The effects of these methods on MALDI-TOF-MS identification results were analyzed to select the optimal pretreatment method.

(a) Direct transfer: A single colony of *B. cereus* was spread on the target plate to form a thin layer, and then covered with 1 μL HCCA solution and air-dried;

(b) Extended direct transfer: A single colony of *B. cereus* was spread on the target plate to form a thin layer, and then

covered with 1 μL 70% formic acid and air-dried. It was then covered with 1 μL HCCA solution and air-dried again;

(c) Formic acid extraction: A single colony of *B. cereus* was transferred into a centrifuge tube containing 300 μL deionized water. After mixing well, 900 μL absolute ethanol was added, followed by thorough mixing. The mixture was centrifuged at 14,650 ×*g* for 2 min, and the supernatant was decanted. After re-centrifugation, the supernatant was discarded with a pipette. The precipitate was dried at room temperature for 3 min, and 30 μL 70% formic acid was then added to the tube and mixed well with a pipette. Further, 30 μL pure acetonitrile was added to the tube and mixed well with a pipette. The mixture was centrifuged at 14,650 × *g* for 2 min. Subsequently, 1 μL supernatant was added to the target plate and air-dried, and then covered with 1 μL HCCA solution and air-dried again.

Each pretreatment group was repeated five times, and the optimal pretreatment method was selected in terms of high reliability score, more protein peaks, high peak intensity, smooth baseline, and high signal-to-noise ratio.

2.4 Protein profile analysis

Protein profile analysis was performed to determine the effects of culture time and media on microbial identification results. Briefly, an appropriate volume of cryopreserved cell culture was streaked on target plates (*B. cereus*: blood, TSA, NA, and MYP agar plates; *L. monocytogenes*: blood, TSA, NA, and PALCAM plates; and *M. luteus*: blood, TSA, and NA plates). The inoculated plates were incubated for 1, 2, 3, 5, and 7 d. The samples were then subjected to instrumental analysis. Each group had five replicates.

2.5 MALDI-TOF-MS data acquisition and evaluation

A MALDI Biotyper (Bruker, Germany) was used to calibrate the instrument with a peptide/protein standard solution. FlexControl was used to collect sample data under the following conditions: instrument parameter = smartbeam laser; laser frequency = 200 Hz; laser pulse signals accumulated for each sample spectrum = 200; mass range = 2–20 ku. In this manner, the mass spectrum of the sample was obtained. The Biotyper software was used to compare the spectra of samples with the standard spectra in the database, and results were evaluated according to the score.

The score indicates the degree of agreement between the spectra of the test and standard strains in the MS database. The higher the score, the closer the spectrum of the test strain is to that of the standard strain in the database (quality and peak height). A score of 2.300–3.000 indicates that the identification of bacterial species has high reliability, 2.000–2.299 indicates the identification of conservative genus or possible species, 1.700–1.999 indicates the identification of possible genus, and 0.000–1.699 indicates that the identification result is unreliable (Kiehntopf et al., 2011).

2.6 MALDI-TOF-TOF-MS secondary spectrum acquisition

E. coli ATCC 8739 was used as the standard strain before the detection of each test sample. Data were collected using the MALDI Solutions Data Acquisition software under the following conditions: instrument parameter = solid-state UV laser and maximum laser repetition frequency = 2000 Hz. One mass spectrum was obtained by every 100 times of laser sampling, and multiple positions were selected at each target for laser shock; the final mass spectrum was obtained after averaging and peak type processing. Reflection MS-MS was selected as the instrument program, with the mass range being 0–3000 Da.

2.7 Statistical analysis

SPSS for Windows 19.0 (SPSS Inc., Chicago, IL, USA) was used to perform one-way analysis of variance on experimental results. Values represent mean ± standard deviation (SD). $P < 0.05$ was considered to indicate significant differences.

3 Results and discussion

3.1 Comparison of different sample pretreatment methods

Three different methods were used to pretreat a culture of *B. cereus*; The reagents involved in these pretreatment methods were sterile water, ethanol, formic acid, and acetonitrile. Sterile water and ethanol were mainly used to wash away metabolites, pigments, agar, and other impurities on the surface of a single colony. These impurities are known to interfere with cell lysis and co-junction crystals are formed between microorganisms and matrix, thus affecting identification results (Wang, 2018). Formic acid was used to destruct the bacterial cell wall to a certain extent. The acidic environment provided by formic acid protects detected sample molecules and improves protonation efficiency (Zhou Yuexia & Yingjian, 2016). Acetonitrile was used to alter the intra- and intermolecular hydrogen bonds of proteins to condense and precipitate them. Because it is highly volatile, acetonitrile has little impact on subsequent identification (Li et al., 2016). In addition, acetonitrile can reduce the influence of heavy hydrogen in the protonation process and improve the resolution of the spectrum (Bernardo et al., 2002).

Protein profiles of the pretreated samples are shown in Figure 1. Among the three groups, the samples pretreated by formic acid extraction showed more protein peaks, with smooth baseline, high peak intensity, and high peak score. The mean reliability score was >1.900 in all cases (Table 1), indicating that the identification results were reliable (Xu et al., 2019). Therefore, formic acid extraction was considered to be the optimal method for sample pretreatment.

3.2 Effects of culture media on bacterial protein profiles

The 1-d-old cultures of *B. cereus*, *L. monocytogenes*, and *M. luteus* grown in different media were pretreated with formic acid extraction; protein profiles of the pretreated samples are shown in Figure 2. There was no significant difference in protein spectrum score among the groups (Table 2). For example, *B. cereus* samples grown in four different culture media showed

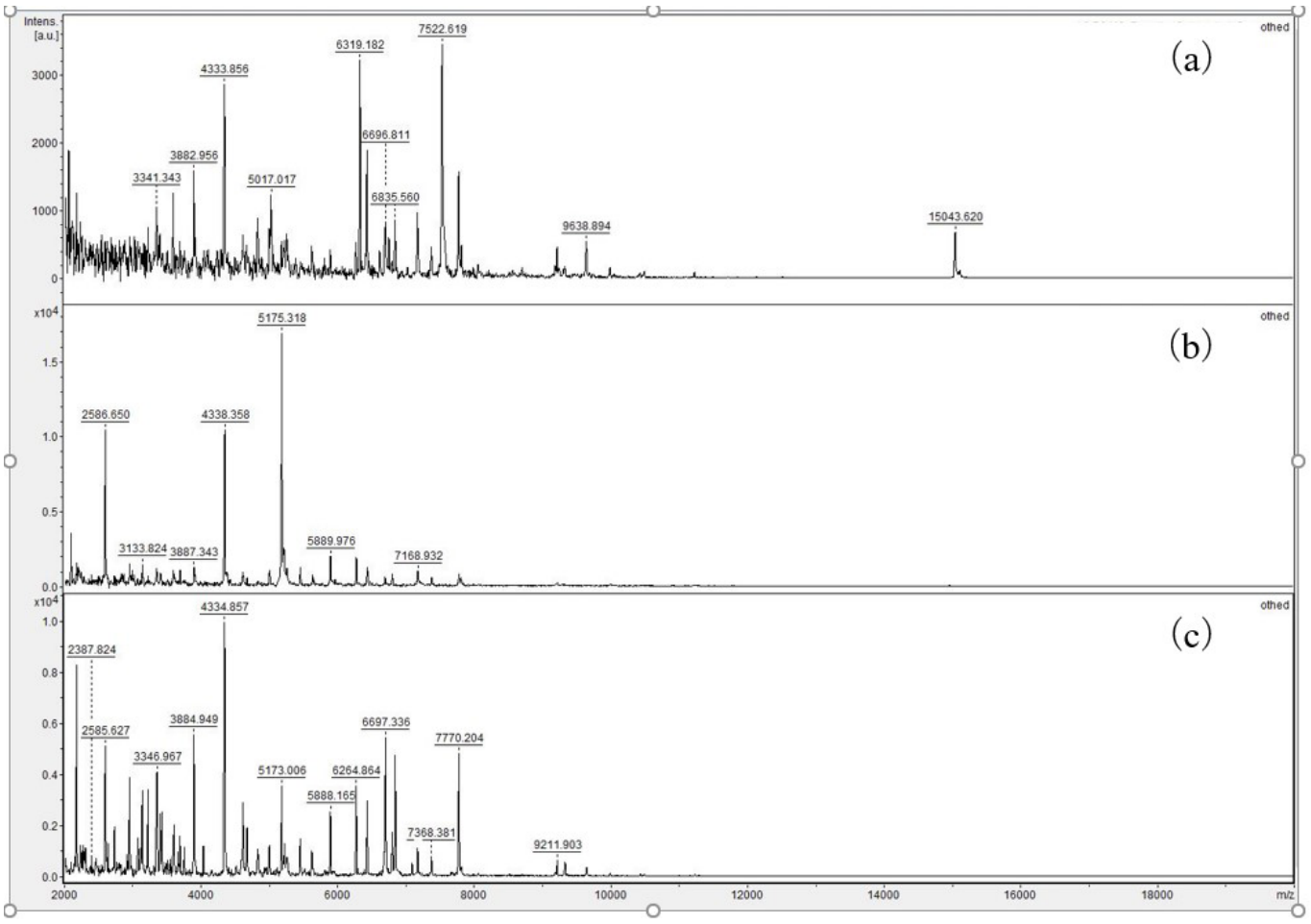

Figure 1.Comparative protein profiles of *B. cereus* samples pretreated with three different methods. (a) Direct transfer, (b) extended direct transfer, and (c) formic acid extraction. The abscissa represents the mass-to-charge ratio (*m/z*) of the ion, and the ordinate represents the intensity (%) of the ion peak.

Table 1. Protein spectrum score and peak number of *B. cereus* samples pretreated using three different methods.

	Direct transfer	Extended direct transfer	Formic acid extraction
Protein spectrum score	1.904 ± 0.037[b]	2.036 ± 0.038 [b]	2.307 ± 0.050[a]
Protein peak number	26.00 ± 2.915 [b]	41.60 ± 3.209 [b]	68.20 ± 4.207 [a]

Values are means ± SD. Different lower case letters in the same row indicate a significant difference ($P < 0.05$).

relatively high peak spectrum scores and reliability scores were >2.0. The protein spectrum scores were similar, and no significant differences were detected among the four groups.

However, the number of protein peaks in the MYP group was significantly higher than that of those in the other three groups ($P < 0.01$). Therefore, for subsequent experiments, MYP medium was selected as the culture medium for *B. cereus* (Zhou et al., 2009), PALCAM for *L. monocytogenes* (Chang-yun, 2016), and TSA for *M. luteus*. This shows that in comparison with non-selective media (blood, TSA, and NA agar plates), selective culture media are superior considering the addition of carbon and nitrogen sources, vitamins, and mineral elements, which are essential for microbial growth. Besides, selective culture media are supplemented with ingredients that support the growth of specific microorganisms and inhibit that of other microorganisms. For *M. luteus*, a non-selective medium was used in this experiment; the difference in its composition is bound to affect the number of protein peaks.

3.3 Effects of culture time on bacterial protein profiles

The samples of *B. cereus* cultured in MYP, *L. monocytogenes* in PALCAM, and *M. luteus* in TSA media for different durations were treated with formic acid. Protein profiles and protein spectrum score of the pretreated samples are shown in Figure 3 and Table 3. With an increase in culture time, the spectrum scores

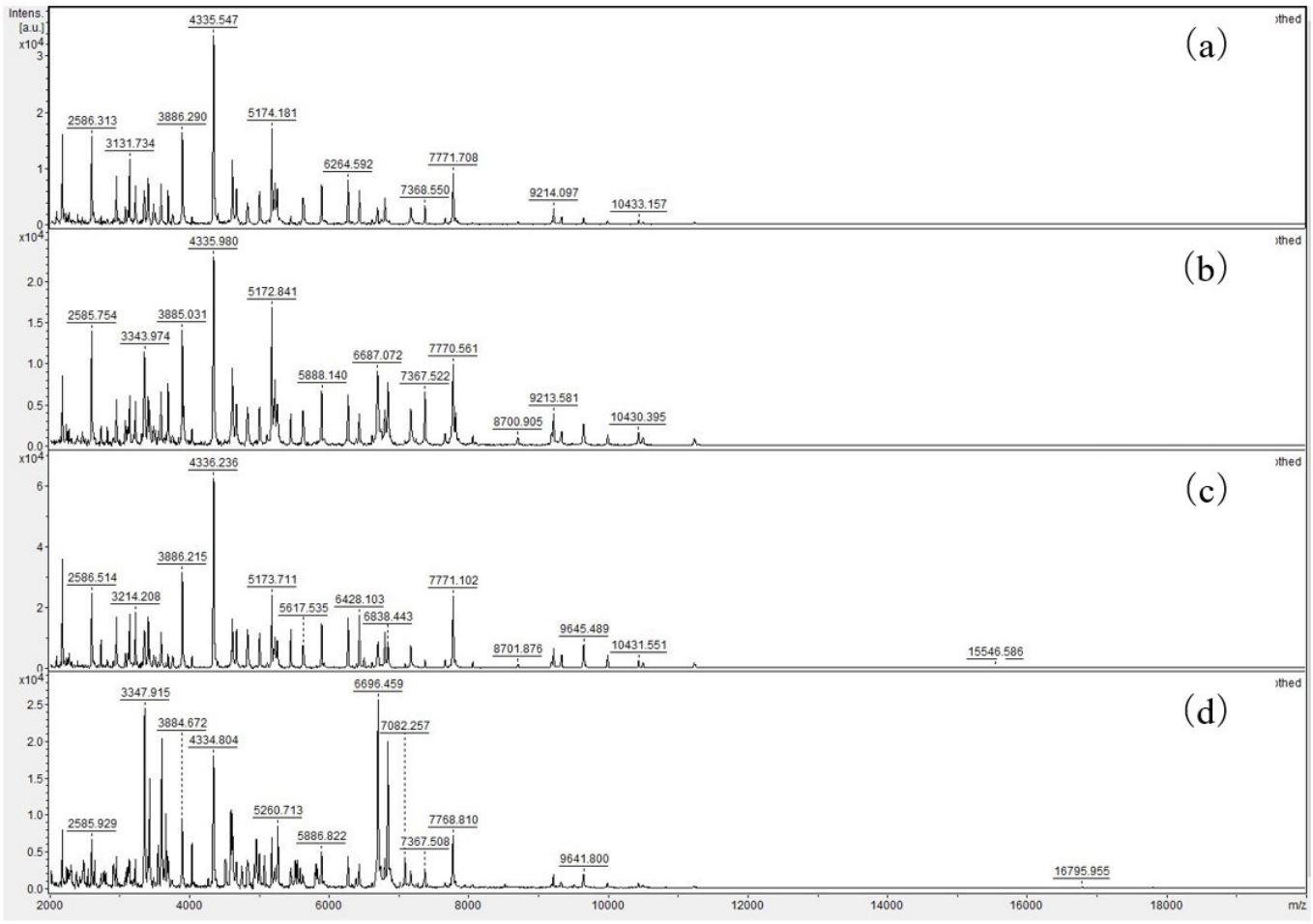

Figure 2. Comparative protein profiles of *B. cereus* grown in four different culture media (a: blood plate, b: tryptone soy agar, c: nutrient agar, and d: mannitol yolk polymyxin agar).

of the three bacterial strains exhibited a downward trend; both the peak intensity and number of protein peaks also decreased.

These results indicated that longer culture times caused bacteria to enter the decline stage. At this stage, bacterial cells are easily deformed or autolyzed, which may cause changes in the protein composition. Consequently, it is difficult to obtain an accurate MS spectrum, which affects microbial identification results . In summary, when using MADLI-TOF-MS to quickly identify *B. cereus*, *L. monocytogenes*, or *M. luteus*, culture time has a large influence on the identification results, and it is recommended to preferentially use 1–2-d-old culture for identification purposes.

3.4 MALDI-TOF-TOF-MS protein profiles

MALDI-TOF-TOF-MS was used to study the secondary spectra of *B. cereus*, *L. monocytogenes*, and *M. luteus* at different culture times. When the 1430 *m/z* protein peak of *B. cereus* was shocked in the ion gate range of 1400–1460 *m/z*, the ion peak fragmented into many small peaks. After 7 d of continuous culture, MS data showed that the ion peaks at 1162 *m/z* appeared to be stable (Figure 4a). This result indicates that the 1430 *m/z* protein peak of *B. cereus* is relatively stable and after formic acid treatment, it can be fragmented into small peaks at 1162 *m/z*, which are relatively stable and exist for a long time. Accordingly, these ion peaks have the possibility of being marker ion peaks for *B. cereus*.

A similar phenomenon was observed for the 2100 *m/z* protein peak of *L. monocytogenes*. When the ion gate range was 2050–2150 *m/z*, a relatively stable ion peak appeared at 1465 *m/z*, and there was no remarkable attenuation with an increase in culture time. This result indicates that the 2100 *m/z* protein peak of *L. monocytogenes* is stable and can be fragmented into relatively stable small ion peaks at 1465 *m/z* after formic acid treatment. Therefore, these ion peaks may be used as marker ion peaks for *L. monocytogenes*. Likewise, the culture samples of *M. luteus* produced a relatively stable protein peak at 2140 *m/z*. When the ion gate range was 2110–2170 *m/z*, the ion peak was fragmented into relatively stable ion peaks at 1625 *m/z*, which may serve as marker ion peaks for *M. luteus*.

MALDI-TOF-TOF-MS protein profiles indicated that the protein peaks of the three pathogenic bacteria can be fragmented into small ion peaks at specific location; these were relatively

Table 2. Protein spectrum score and peak number of three gram-negative bacteria grown in different culture media.

Strain	Index	Blood plate	TSA	NA	MYP	PALCAM Agar
B. cereus	Protein spectrum score	2.135 ± 0.148[b]	2.090 ± 0.099[b]	2.314 ± 0.024[a]	2.337 ± 0.025[a]	–
	Protein peak number	65.2 ± 6.261[c]	72.8 ± 3.834[b]	78.2 ± 5.263[b]	90.2 ± 5.848[a]	–
L. monocytogenes	Protein spectrum score	2.189 ± 0.105[a]	2.181 ± 0.060[b]	2.278 ± 0.034[a]	–	2.247 ± 0.069[a]
	Protein peak number	42.4 ± 1.817[b]	43.8 ± 1.924[b]	44.0 ± 3.391[b]	–	49.2 ± 2.387[a]
M. luteus	Protein spectrum score	2.199 ± 0.038[a]	2.213 ± 0.066[a]	2.224 ± 0.070[a]	–	–
	Protein peak number	63.2 ± 5.495[b]	68.8 ± 2.168[a]	63.0 ± 2.646[c]	–	–

Values are means ± SD. Different lower case letters in the same row indicate a significant difference ($P < 0.05$). TSA, tryptone soy agar; NA, nutrient agar; and MYP, mannitol yolk polymyxin. PALCAM medium for selective isolation of Listeria monocytogenes (FDA standard).

Table 3. Protein spectrum score and peak number of three gram-negative bacteria with different culture times.

Strain	Index	1 d	2 d	3 d	5 d	7 d
B. cereus	Protein spectrum score	2.320 ± 0.029[a]	2.279 ± 0.025[a]	2.134 ± 0.192[b]	1.846 ± 0.056[c]	1.530 ± 0.090[d]
	Protein peak number	89.60 ± 3.78[a]	74.2 ± 5.54[b]	57.20 ± 4.49[c]	50.40 ± 6.19[c]	23.20 ± 3.27[d]
L. monocytogenes	Protein spectrum score	2.257 ± 0.060[a]	2.287 ± 0.035[a]	2.096 ± 0.067[b]	2.037 ± 0.059[b]	2.028 ± 0.062[b]
	Protein peak number	49.40 ± 4.159[a]	58.60 ± 2.811[b]	37.40 ± 2.881[c]	27.40 ± 2.408[d]	24.40 ± 1.140[d]
M. luteus	Protein spectrum score	2.223 ± 0.075[a]	2.132 ± 0.078[b]	2.030 ± 0.047[c]	1.963 ± 0.055[c]	1.913 ± 0.067[c]
	Protein peak number	63.80 ± 4.324[a]	68.80 ± 2.588[a]	61.20 ± 5.718[a]	45.80 ± 4.868[b]	40.00 ± 3.162[c]

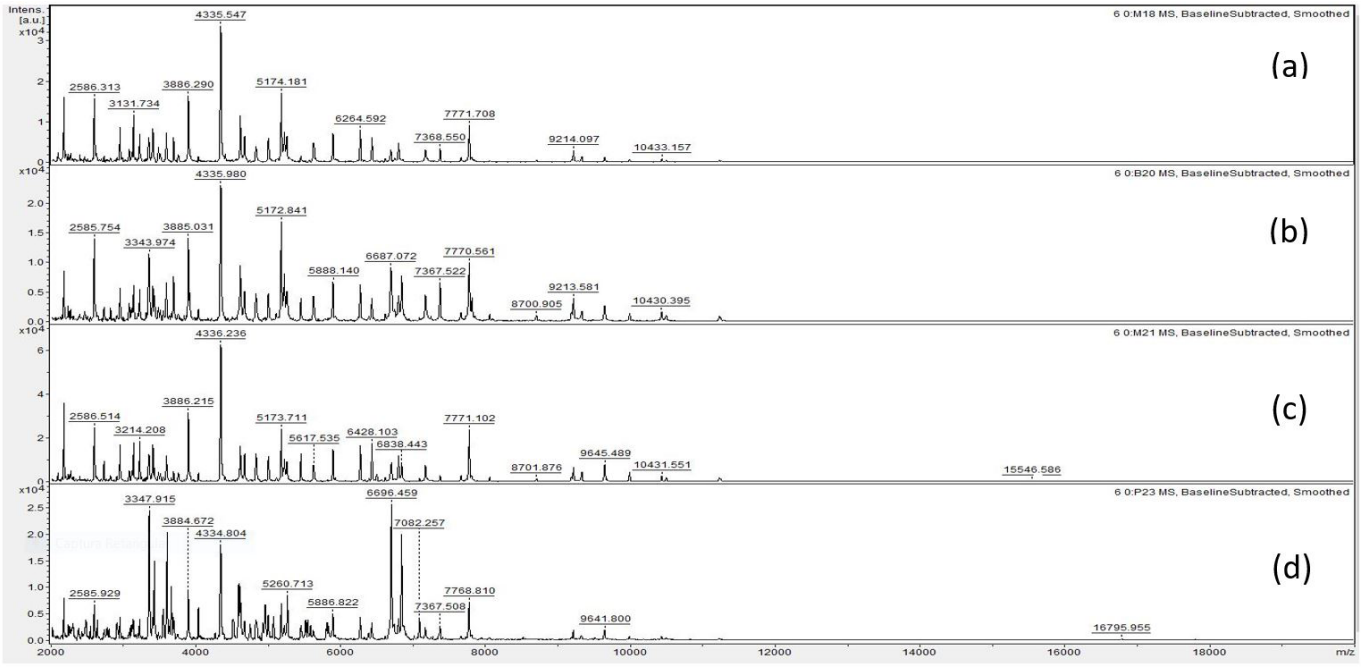

Figure 3. Comparative bacterial protein profiles at different culture times (a) *B. cereus*, (b) *L. monocytogenes*, and (c) *M. luteus*.

stable and did not decay with time. This implies that marker ion peaks can be used for the rapid identification of *B. cereus*, *L. monocytogenes*, and *M. luteus* based on MALDI-TOF-TOF-MS. In addition, unlike the correlation between culture time and peak intensity and peak number in the first-order mass spectrum, secondary protein peaks do not attenuate with an increase in culture time. Therefore, these protein peaks can be used as a new MALDI-TOF-TOF-MS-based method to identify *B. cereus*, *L. monocytogenes*, and *M. luteus*. This is also a new and valuable attempt to identify bacteria based on MALDI-TOF-TOF-MS.

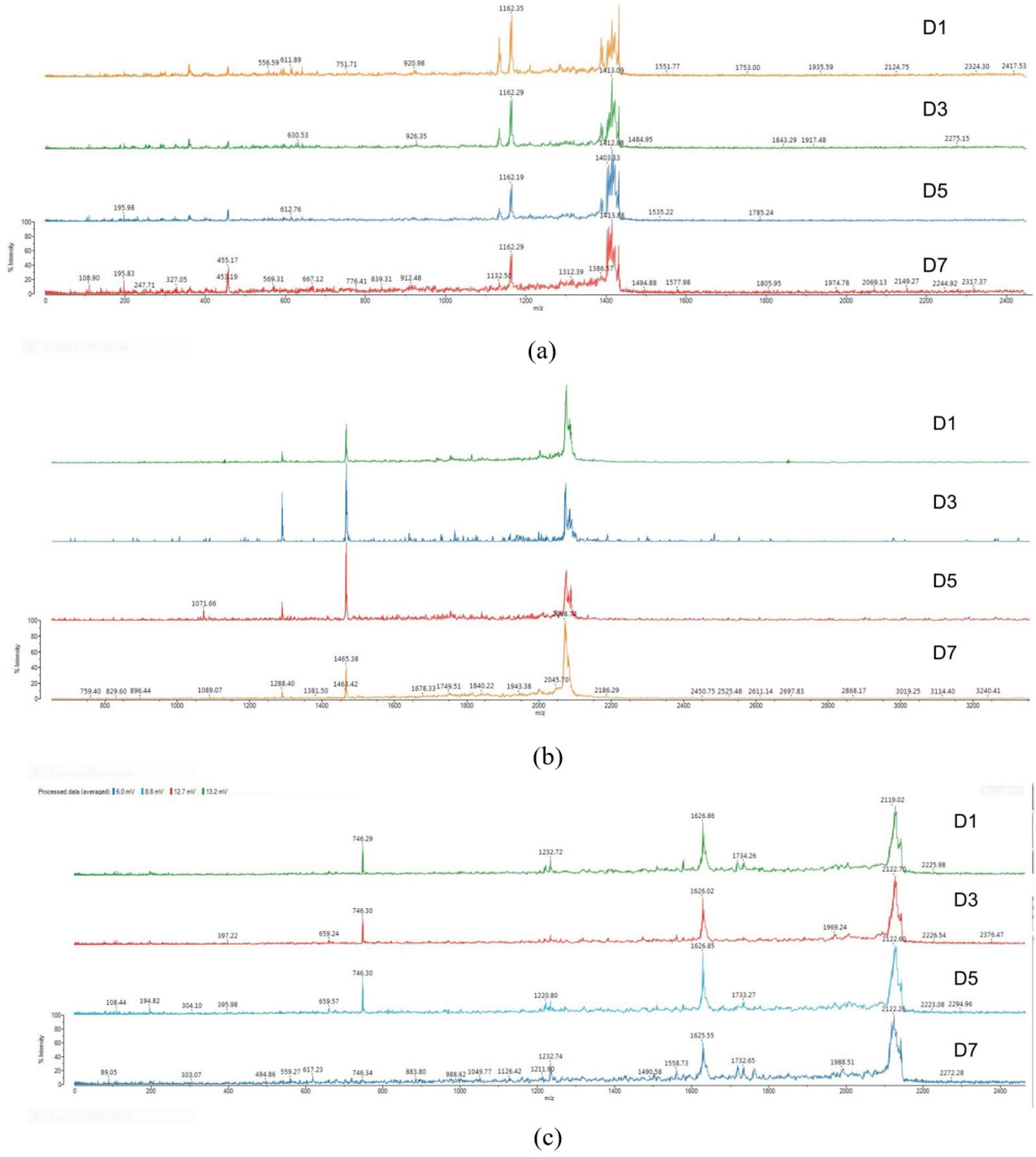

Figure 4. Comparison of secondary protein profiles of three Gram-negative bacteria at different culture times (a) *B. cereus*, (b) *L. monocytogenes*, and (c) *M. luteus*.

4 Conclusions

To summarize, we herein used *B. cereus* as an example to evaluate the effects of three different sample pretreatment methods on MALDI-TOF-MS identification of common foodborne bacteria. We found that formic acid extraction was better than direct transfer and extended direct transfer methods. In addition, culture media had little effect on MALDI-TOF-MS protein profiles of *B. cereus*, *L. monocytogenes*, and *M. luteus* samples pretreated with formic acid. In terms of protein peak number, MYP, PALCAM, and TSA were selected as the culture media for

the three pathogenic bacteria, respectively. Further, culture time had a profound effect on the identification results; 1–2-d-old cultures were preferred for MALDI-TOF-MS identification. Based on MALDI-TOF-TOF-MS, secondary protein peaks of the three bacteria were relatively stable and did not change with culture time. The characteristic peaks of different bacteria could be used as marker ion peaks for MALDI-TOF-TOF-MS identification, which is a novel method. MALDI-TOF-TOF-MS is advantageous over routine inspection and even MALDI-TOF-MS for identifying *B. cereus*, *L. monocytogenes*, and *M. luteus*. The proposed method is suitable for the rapid identification of these Gram-negative bacteria after continuous culture for a long time.

Conflicts of interest

The manuscript has not been submitted to more than one journal for simultaneous consideration. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Data Availability

The data used to support the findings of this study are included within the article.

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