Identification of *Clostridium* spp. derived from a sheep and cattle slaughterhouse by matrix-assisted laser desorption and ionization-time of flight mass spectrometry (MALDI-TOF MS) and 16S rDNA sequencing

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Abstract Clostridia are widespread and some of them are serious human pathogens. Identification of *Clostridium* spp. is important for managing microbiological risks in the food industry. Samples derived from sheep and cattle carcasses from a slaughterhouse in Iran were analyzed by MALDI-TOF MS using direct transfer and extended direct transfer sample preparation methods and 16S rDNA sequencing. MALDI-TOF MS could identify ten species in 224 out of 240 *Clostridium* isolates. In comparison to the 16S rDNA sequencing, correct identification rate of the *Clostridium* spp. at the species level by MALDI-TOF MS technique was 93.3%. 16 isolates were not identified by MALDI-TOF MS but 16s rDNA sequencing identified them as *C. estertheticum*, *C. frigidicarnis*, and *C. gasigenes* species. The most frequently identified *Clostridium* species were: *C. sporogenes* (13%), *C. cadaveris* (12.5%), *C. cochlearium* (12%) and *C. perfringens* (10%). Extended direct transfer method [2.26 ± 0.18 log (score)] in comparison to direct transfer method [2.15 ± 0.23 log (score)] improved *Clostridium* spp. identification. Using a cut-off score of 1.7 was sufficient for accurate identification of *Clostridium* species. MALDI-TOF MS identification scores for *Clostridium* spp. decreased with longer incubation time. *Clostridium* species predominantly were isolated from

carcasses after skinning and evisceration steps in the slaughterhouse. MALDI-TOF MS could be an accurate way to identify *Clostridium* species. Moreover, continuous improvement of the database and MALDI-TOF MS instrument enhance its performance in food control laboratories.

Keywords *Clostridium* spp. · Slaughterhouse · 16S rDNA · MALDI-TOF MS

Introduction

Clostridia are anaerobic spore-forming bacteria which are found in the environment and also in the intestinal tract of humans and animals (Wiegel et al. 2006). Some *Clostridium* species such as *C. perfringens*, *C. botulinum*, *C. tetani*, and *C. difficile* cause infections in humans and produce lethal toxins (Doulgeraki et al. 2012; Lee and Pascall 2012; Wiegel et al. 2006). The most important *Clostridium* species involved in food spoilage are *Clostridium sporogenes*, *Clostridium butyricum*, *Clostridium putrefaciens*, *Clostridium tyrobutyricum*, and *Clostridium pasteurianum* (Naim et al. 2008). *Clostridium* species contaminate the abattoirs mainly through attached soil particles to the hide and feces of animals (Silva et al. 2012). Bacterial contamination on the hide could transfer to carcasses during the slaughtering process (Narvaez-Bravo et al. 2013). The meat industry is concerned about *Clostridium* species on meat surfaces and equipment in slaughterhouses. Meat and meat products are the favored medium for growth and toxin production of *Clostridium* species (Húngaro et al. 2016). Although heat processing and sanitizing treatment in food processing facilities destroy the vegetative cells of *Clostridium* species, their spores can survive and

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proliferate in prepared food stored at room temperature (Lee and Pascall 2012). Temperature abuse during cooling, distribution, storage and reheating of cooked products is an important factor negatively impacting shelf-life and quality of meat products resulting in spore germination and rapid growth of bacteria such as *Clostridium* species. Subsequently, this leads to spoilage of meat and thus increases the risk of food poisoning (Redondo-Solano et al. 2013). Therefore, fast and reliable detection and identification of *Clostridium* spp. are crucial to adequately manage related microbiological risks. Phenotypic identification is limited to a few specific laboratory characteristics and the restricted species in databases of commercial kits (Chean et al. 2014). The correct and reproducible identification of *Clostridium* species by use of biochemistry, fermentation profiles, and gas–liquid chromatography are not easy to manage for inexperienced users and restricted to reference laboratories (Chean et al. 2014; Grosse-Herrenthey et al. 2008). In addition, 16S ribosomal DNA (rDNA) sequencing has been widely used to identify and differentiate *Clostridium* species. However, for the routine diagnostic application, it's not suitable because it's time consuming, costly and needs sequence interpretation (Chean et al. 2014).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been developed as a routine microbiological diagnosis tool with dependable results (Gutiérrez et al. 2017). This method is based on generating complex fingerprint spectra of biomarker molecules by measuring the mass/charge (m/z) of peptides and proteins, which represent high-abundant proteins with housekeeping functions, such as ribosomal proteins (Alisphahic et al. 2011). Accordingly, the mass spectra patterns are stable and can be used for finding similarities and phylogenetic relationships of the microorganisms (Angeletti 2017).

MALDI-TOF MS is a user friendly, fast and inexpensive identification method that makes it possible to identify a wide range of microorganisms in routine clinical microbiology laboratories (Grosse-Herrenthey et al. 2008; Veloo et al. 2011a, b, c; Agustini et al. 2014; Barba et al. 2014; Hsu and Burnham 2014; Randall et al. 2015; Seng et al. 2009).

The objective of this study was to apply MALDI-TOF MS to identify isolates of *Clostridium* spp. derived from sheep and cattle carcasses at different steps of the slaughtering process in Iran. Two sample preparation methods were applied in parallel for each isolate: direct transfer and extended direct transfer. The second objective was to verify MALDI-TOF MS results with 16S rDNA gene sequencing. Finally, the third objective was to evaluate the reproducibility using the different state of bacteria after cultivation.

Materials and methods

Sample collection and bacteriological analysis

60 samples were taken from one small slaughterhouse of sheep and cattle as a representative of slaughterhouses located in the province of Tehran, Iran, with respect to slaughtering capacity (approximately 25–30 sheep and 15–20 cattle per hour), utensils, equipment, and pre-slaughter holding pen conditions. The animals were received from traditional livestock farms. Slaughtering procedures were without physical division between clean and unclean areas, but cutting and deboning for retail were performed in a separate room. In the sheep slaughter line, bleeding and skinning were done on the floor while in the cattle slaughter line, all slaughtering processes were performed on a production line and vertical rail dressing and automatic hide pullers were used.

Sterile gauzes were moistened with sterile phosphate-buffered saline (PBS) (Merck, Germany) and used for swab sampling. Five carcasses were selected in each slaughter line by simple random sampling methods. Swab samples were taken from carcasses after skinning, evisceration, trimming, and final washing at four different points: neck, brisket, flank, and rump in cattle, and flank, thorax lateral, brisket, and breast in sheep according to Salmela et al. (2013). Rectal swab samples were taken from carcasses immediately after evisceration. Swab samples were also taken from meat after cutting and deboning of selected carcasses (Salmela et al. 2013). After swabbing, the gauze was immediately transferred to 20 ml of Thioglycollate broth (Oxoid, UK). Isolation of psychrotrophic *Clostridium* was done according to Silva et al. (2012). For isolation of spores of mesophilic *Clostridium*, Thioglycollate broth tubes containing swab samples were incubated anaerobically at 37 °C for a period of 10 days. The tubes were heated to 80 °C for ten minutes for inactivation of vegetative cells. The pelleted spores were washed with 100 ml sterile distilled water and then centrifuged. The washing process was done according to Brunt et al. (2015) and repeated three times (Brunt et al. 2015). Finally, sediments were plated on Columbia agar supplemented with 5% (v/v) blood sheep (BioMerieux, France). Plates were incubated for 24 h at 37 °C using self-contained gas generating system AnaeroGenTM (Oxoid, UK) (Grosse-Herrenthey et al. 2008; Silva et al. 2012). Primary identification of pure colonies were done by their microscopic and colonial appearances, gram staining test and presence or absence of β -hemolysis according to the British standards for microbiology investigations, identification of *Clostridium* species from Public Health England. Probable *Clostridium* bacteria

were subjected to MALDI-TOF MS and 16S rDNA sequence analysis.

Identification with MALDI-TOF MS

For sample preparation direct transfer (DT) and extended direct transfer (EDT) methods were used in parallel.

In the DT method, one single colony was spread onto a MALDI target plate (MSP 96 target polished steel) with a toothpick. Each spot was overlaid with 1 μ l matrix (3 mg/mL solution of α -cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany) in 50% (v/v) acetonitrile (Sigma-Aldrich), and 2.5% [v/v] trifluoroacetic acid (Sigma-Aldrich), and allowed to dry prior to analysis at room temperature according to Chean et al. (2014).

In the EDT method, one single colony was similarly smeared directly on the MALDI-TOF MS target plate and overlaid with 1 μ l of formic acid (70%) (Sigma-Aldrich) and dried off before adding the matrix solution (Chean et al. 2014).

Prior to sample measurement, the MicroFlex LT mass spectrometer (Bruker Daltonics) was calibrated using IVD BTS bacterial test standard (Bruker Daltonics, Germany) containing *E. coli* DH5 α ribosomal proteins and two additional proteins (Doern 2013). The spectra were analyzed using Bruker Biotyper 3.3 software and Biotyper database version V3.3.1.0-4613.

The MALDI Biotyper output is a log (score) in the range 0–3.0, computed by comparison of the peak list for an unknown isolate with the reference spectra in the database. A BioTyper log (score) ≥ 2.3 indicates highly probable identification at the species level. A log (score) between 2.0 and 2.3 indicates highly probable identification at the genus level and probable identification at the species level. A log (score) between 1.7 and 2.0 indicates probable genus identification, while a log (score) < 1.700 means no reliable identification.

To test if identification was possible after anaerobic incubation of bacteria for 1, 3 and 5 days, bacterial colonies were subjected to MALDI-TOF MS analysis using the DT and EDT sample preparation methods in parallel. Analysis were replicated three times for each isolate.

16S rDNA sequence analysis

A-purified single colony was transferred to TE buffer for molecular analysis. After DNA extraction using Qiagen Stool kit (Qiagen, Germany), fragments of the 16S rDNA were amplified using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' and 518r 5'-ATT ACC GCG GCT GCT GG-3' in a concentration of 0.5 pM per reaction volume with a ready-to-use GoTaq[®] Green Master Mix (Promega, USA) according to Remely et al. (2013). After

purification using QIAquick PCR Purification Kit (Qiagen, Germany) samples were sent for sequencing to LGC Genomics GmbH (Germany). Nucleotide sequences were analyzed with RDP 10.32 (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Statistics

The Shapiro–Wilk test was used to evaluate the distribution of MALDI-TOF MS results. The Mann–Whitney test was used to compare the means of log scores of MALDI-TOF MS for *Clostridium* identification obtained by two different sample preparation methods. The effect of incubation time on the identification of species was analyzed by the Friedman test. For all comparisons, p values ≤ 0.05 were considered significant. All statistical tests were conducted using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA).

Results

240 *Clostridium* species were isolated from 60 samples. All 240 *Clostridium* isolates were identified by 16S rDNA sequencing and assigned to 13 different *Clostridium* species. MALDI-TOF MS identified ten different *Clostridium* species in 224 isolates: *C. sporogenes* (n = 32), *C. cadaveris* (n = 30), *C. cochlearium* (n = 29), *C. perfringens* (n = 25), *C. halophilum* (n = 22), *C. tertium* (n = 20), *C. lundense* (n = 19), *C. sordellii* (n = 18), *C. tetani* (n = 15), and *C. algidixylanolyticum* (n = 14). For 16 isolates, the log score values were lower than 1.7 hence no identification was possible by MALDI-TOF MS. These strains were identified via 16S rDNA as *C. estertheticum* (n = 8), *C. frigidicarnis* (n = 5), and *C. gasigenes* (n = 3) species (Fig. 1). The correlation between 16S rDNA sequencing and MALDI-TOF MS results was 93.3%.

The EDT method provides better quality mass spectra and higher identification log score values compared to the DT method ($p < 0.05$). The EDT method had an average log score of 2.26 ± 0.18 in comparison to an average log score of 2.15 ± 0.23 for the DT method after 1-day anaerobic incubation (Fig. 2).

Highly probable species identification was obtained for 101/224 (45%) and 125/224 (56%), secure genus identification and probable species identification for 47/224 (21%) and 77/224 (34%), and probable genus identification for 76/224 (34%) and 22/224 (10%) isolates by the DT and the EDT methods respectively after 1 day anaerobic incubation.

The averages of MALDI-TOF MS log scores for *Clostridium* spp. identification by the EDT method on day three and day five were 2.17 ± 0.16 and 2.02 ± 0.17 , in comparison to 1.96 ± 0.21 and 1.78 ± 0.14 by the DT

Fig. 1 Overview of Bruker MALDI-TOF MS identification of *Clostridium* isolates at the species level. MALDI-TOF MS identified 94% isolates while 6% of isolates were not identified by MALDI-TOF MS. These isolates were identified via 16S rDNA as *C. estertheticum* (n = 8), *C. frigidicarnis* (n = 5), and *C. gasigenes* (n = 3) species

Fig. 2 Comparing direct transfer and extended direct transfer sample preparation methods for identification of *Clostridium* species by MALDI-TOF MS at different days of anaerobic incubation (1, 3, and 5). Each isolate was tested three times for each method of sample preparation at each day

method respectively. However, the isolates with a log score value of ≥ 1.7 were correctly identified at the species level regardless of sample preparation method and incubation time.

Clostridium species were isolated mainly from carcasses after skinning and evisceration steps and were isolated also from carcasses after final washing and from meat in the slaughterhouse (Fig. 3).

Discussion

In this study, we compared the identification of *Clostridium* spp. isolated from sheep and cattle carcasses at different stages of slaughter in an abattoir using MALDI-TOF MS against 16S rDNA sequencing. 16S rDNA sequencing is a reliable method for accurate identification of anaerobic bacteria, including *Clostridium* species, but this technique remains difficult to implement in routine microbiology laboratories (Cheat et al. 2014; AlMogbel 2016). The MALDI-TOF MS method supplements the phenotypic methods for fast, cost-effective, robust, and reliable identification in the first step of characterization of the cultivable microbial community (AlMogbel 2016; Nacef et al. 2016). Accuracy and reliability of MALDI-TOF MS identification highly depend on the database which is used to identify microorganisms such as the existence of the mass spectra of the species in the database (Doern 2013; Veloo et al. 2011a, b, c). Reference data should include a spectrum of many individual species and large numbers of reference spectra for each species because not every single spectrum shows all the species' biodiversity (Cheat et al.

Fig. 3 Results of identification of the *Clostridium* species according to sampling site **a** sheep slaughter line, **b** cattle slaughter line

2014). Alternatively, it is possible to have acceptable identification outcome for species with a low number of spectra in the database when the organisms produce the same mass spectrometry patterns as those in the database (Chean et al. 2014).

In our study, MALDI-TOF MS could not identify some of *Clostridium* species such as *C. estertheticum* whose spectra were not in the database. However, identification of those bacteria having a low number of spectra in the database, for example, *C. algidixylanolyticum* was possible. *C. botulinum* was not in the IVD MALDI Biotyper Database. Some strains of *C. botulinum* group I probably are neurotoxic strains of *Clostridium* sporogenes (Bano et al. 2017). Obviously also 16S rDNA gene sequencing cannot differentiate between *C. sporogenes* and *C. botulinum*. Therefore additional tests for neurotoxin or the presence of neurotoxin genes are needed. Grosse-Herrenthey et al. (2008) reported MALDI-TOF MS could differentiate between some species which are difficult to differentiate by 16S rDNA due to high sequence similarities such as *C. chauvoei* and *C. septicum*.

In this study thirteen *Clostridium* species were identified in 240 *Clostridium* isolates derived from 60 samples by 16S rDNA sequencing. MALDI-TOF MS identified 224 of

isolates belonging to ten species but could not identify 16 isolates, including three species whose spectra were not in the database. Likewise, in the study conducted by AIMogbel (2016), MALDI-TOF-MS identified 88.8% of the *Clostridium* spp. isolates at the species level. Similar results for non-*difficile* *Clostridium* spp. and excellent identification of *C. difficile* were obtained in a study conducted by Kim et al. (2016) who recommended using MALDI-TOF MS in routine microbiology laboratories in order to have successful and rapid identification of *Clostridium* species. Also, Grosse-Herrenthey et al. (2008) suggested MALDI-TOF MS as a powerful tool for dependable identification of *Clostridium* species. Veloo et al. (2011a, b, c) compared the performance of two MALDI-TOF MS systems, Bruker MS and Shimadzu MS, with 16S rDNA gene sequencing as the reference standard. They concluded that the composition and quality of the database are extremely important for an accurate identification. Further, the databases of both systems need to be optimized for routine identification of anaerobic bacteria.

In this study, the number of spectra and the number of *Clostridium* species of Biotyper database version V3.3.1.0-4613 were 169 and 81, respectively. The ratio of the

number of reference spectra to the number of species in the database was 2.07 for *Clostridium* species.

Correct identification using the Bruker's recommended cut-off score (a Biotyper score of > 2.0 for—species-level identification) was obtained for 148/224 (65%) and 202/224 (89%) of the analyzed isolates with DT and EDT preparation methods, respectively. Using a cut-off value of 1.7 allowed accurate identification of *Clostridium* spp. derived from slaughterhouse regardless of sample preparation methods and incubation time. Chean et al. (2014), Fournier et al. (2012), and Hsu and Burnham (2014) have reported the similar cut-off score for correct identification of anaerobic bacteria including *Clostridium* spp.

The results of this study on the comparison of the two different sample preparation methods, DT and EDT, suggest that using the EDT sample preparation method improves the quality of the mass spectra and, as a result, improves identification at the species level, which is also supported by Randall et al. (2015) findings. Hsu and Burnham (2014) reported that the EDT method has a better identification rate compared to the DT method even when the isolates undergo multiple sub-culturing and incubation in undesirable conditions. The EDT method reduces the background signal that could affect the recognition of bacterial peaks, especially in Gram-positive bacteria due to their cell wall structure (Chean et al. 2014; Randall et al. 2015).

The value of MALDI-TOF MS for the identification of *Clostridium* spp. derived from slaughterhouse significantly decreased during longer incubation time. On the first day of incubation, the MALDI-TOF MS identification scores of the colonies were higher compared to day three and five. These findings coincide with several studies which found that the length of the incubation time affects protein expression and mass spectra (Chean et al. 2014; TeKippe et al. 2013). In contrast, Veloo et al. (2014) reported that incubation time has no influence on the quality of anaerobic Gram-positive bacteria spectra. The authors have suggested performing MALDI-TOF MS when a sufficient growth is attained which could be after 24 h of incubation. However, spores have undesirable effects on accurate identification by showing slight deviations in fingerprint patterns. This is because spores have high amounts of proteins to survive in suboptimal conditions (Grosse-Herrenthey et al. 2008).

We did not investigate how culture media and time of exposure to oxygen impact mass spectrometry results. However, previous studies have shown that culturing the bacteria on different media and time of exposure to oxygen have no influence on the quality of the spectra of anaerobic bacteria for identification at the species level (Chean et al. 2014; Veloo et al. 2014).

Recently, Shafer et al. (2017) reported that the number of smeared cells on the target plate is important to have an acceptable identification outcome. They suggested 10^7 – 10^8 bacterial cells provide the acceptable spectral resolution. They also made clear that uniformity and smoothness of smeared cells on the target plates are important to have good results.

Meat is one of the most perishable foods owing to its high water activity, suitable pH and its composition (Húngaro et al. 2016). Meat spoilage involves different chemical and biological activities, leading to undesirable odors and tastes, discoloration, and unsafe foods for human consumption as well as economic issues due to food losses (Húngaro et al. 2016; de Koster and Brul 2016). Some of *Clostridium* species isolated in this study are not considered as food spoilage or food-borne pathogens, rather they are known as human and animal pathogens. One prominent example for such species is *Clostridium tetani*, which produces a potent neurotoxin and causes life-threatening diseases like tetanus (Bruggemann et al. 2015; Wiegel et al. 2006). In this study, we identified *C. tetani* in 6.25% of isolates. *Clostridium sordellii* is an uncommon human pathogen but virulent strains cause fatal infections in a wide range of animals, such as cattle and sheep (Kozma-Sipos et al. 2010; Vidor et al. 2015). In this study, we found *C. sordellii* in 7.5% of isolates. There are some reports of bacteremia and other infections in immunocompromised individuals from non-toxigenic species such as *C. cadaveris* and *C. tertium* (Salvador et al. 2013; Shinha 2015; You et al. 2015). In our study, the abundance of *C. cadaveris* and *C. tertium* in the isolates were 12.5 and 8.33%, respectively. *C. perfringens* is a common cause of food-borne gastrointestinal diseases in humans. According to the production of four major toxins of alpha (α), beta (β), epsilon (ϵ), and iota (ι), strains are classified into five toxin types (A–E). Important human illnesses caused by *C. perfringens* are gas gangrene and food poisoning (Aras and Hadimli 2015). In the present study, *C. perfringens* was found in 10.4% of isolates.

Psychrotolerant clostridia, such as *C. algidilylanolyticum*, *C. estertheticum*, *C. frigidicarnis*, and *C. gasigenes*, were found in 12.5% of isolates in this study. These bacteria cause blown pack spoilage in vacuum-packed meat resulting in high amounts of drip, gas, unpleasant odor, and discolored and tender meat (Adam et al. 2013). Some authors have shown that even the existence of as few as four spores/cm² on the meat surface significantly decreased the shelf life of chilled, vacuum-packed meats (Húngaro et al. 2016).

The main sources of *Clostridium* species are soil and the intestinal tracts of humans and animals. Since the animals being studied in the slaughterhouse, representative of slaughterhouses located in Tehran, were never washed or

cleaned before arriving at the plant or before skinning, it is proposed that *Clostridium* species could enter the abattoir via the hides of animals and are spread through the skinning process throughout the production line and environment of the cattle and sheep slaughterhouse. In general, contamination in slaughterhouses occurs due to inadequate hygienic conditions especially in handling of carcasses (Bakhtiary et al. 2016). Carcasses can be contaminated microbiologically during slaughtering process by different routes such as contact with the hides, equipment, and facilities, workers' hands, as well as rupture of the viscera and spread of gut contents on the carcasses (Salmela et al. 2013). Consequently, the viscera must be removed intact and the anal sphincter with rectum also must be carefully separated from the carcass.

In this study the *Clostridium* species were mainly isolated from carcasses after skinning and evisceration in the slaughterhouse. Our preference was identification of different isolates from each sample, rather than different samples per slaughterhouse.

After evisceration, animal carcasses were physically decontaminated by trimming and washing using high-pressure cold water before entering the cooling section. The main purpose of carcass washing is to remove visible contamination such as blood stains and improve carcass appearance after chilling. Suspended carcasses should be washed thoroughly by jet water with appropriate pressure from the top of the carcass in a downward direction in adequate time. Madden et al. (2004) confirmed that washing with cold water is not effective to reduce bacterial load on carcasses, while Koohdar (2013) reported carcass washing with cold water has a significant effect on decreasing microbial populations. In our study, *Clostridium* species were found in sheep and cattle carcasses after washing. Additionally, they were detected in meat samples. In general, the load of bacteria from live animals entering the slaughterhouses decreases when slaughtering processes and sanitary programs work effectively (Narvaez-Bravo et al. 2013). Based on our results, adequate sanitary procedures need to be implemented to reduce cross contaminations in the slaughterhouse. These include a better availability of knife sterilizers with hot water (82 °C), performing the bleeding cut at the neck when the carcass is hanging on the bleeding rail, using automatic hide removal systems in sheep slaughter lines, cleaning the animal before slaughtering, no exposure of skinned carcass surfaces to the hide as well as to the hands of workers and furthermore, training the meat handlers in the concepts and requirements of personal hygiene. Food safety management system (FSMS) implementation should be mandatory for all stages of the meat supply chain in Iran. All meat companies and slaughterhouses should have a FSMS based on good manufacturing practices (GMPs) and the hazard analysis

critical control points (HACCPs) principles in order to minimize carcass contamination with soil, feces and intestinal contents to avoid contamination with *Clostridium* bacteria and effectively manage microbial hazards. Höll et al. (2016) found the type of breed and farm practices could have significant roles in developing the initial bacterial contamination and resulting in further spoilage after slaughtering.

In addition to good hygiene practice in slaughterhouses and meat plants, a strict control of temperature during the cold chain is important to prevent food spoilage and food-borne diseases by *Clostridium* species. For example, storage of vacuum-packed meat at -1.5 °C increases shelf life and reduces microbial spoilage (Húngaro et al. 2016; Prevost et al. 2013).

To the author's knowledge, the present study is the first report of the identification of *Clostridium* species derived from the slaughterhouse by MALDI-TOF in Iran. Our study provides information regarding *Clostridium* spp. contamination in slaughterhouses and underlines possible sources of contamination. In the present study, we investigated the identification of different isolates of *Clostridium* spp. derived from slaughterhouse samples by comparing MALDI-Biotyper and 16S. However, it was not possible to differentiate *Clostridium* species down to subspecies level. Therefore, the number of different strains in the isolates remains uncertain. Further work will focus on a deeper molecular characterization for differentiation of isolates in order to track down sources of *Clostridium* species contamination in slaughterhouses. Our study suggests the addition of further *Clostridium* species including Psychrotolerant clostridia to the database which may then allow a better identification of *Clostridium* spp. in the meat industry.

Conclusion

Our results demonstrated that MALDI-TOF MS is an accurate method to identify *Clostridium* species derived from slaughterhouses, at the species level. However, continuous improvement of the MALDI-TOF MS database with food spoilage bacteria and food-borne pathogens and standardization of culture conditions will develop its application in the food industry for identification and tracking sources of microbial contamination along the food chain.

In MALDI-TOF MS identification using EDT sample preparation method from fresh cultures could improve *Clostridium* spp. identification in comparison to the DT method. Moreover, 16S rDNA gene sequencing analysis could be used as a complementary method for MALDI-TOF MS to verify the identification of the organism. As our

results are based on samples taken from one slaughterhouse, investigating the *Clostridium* species contamination in several slaughterhouses located in different areas will provide more information about *Clostridium* contaminations. In general, implementation of hazard analysis critical control point (HACCP) system at all stages of the meat supply chain will help to adequately manage microbial hazards.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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