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Species-specific identification and differentiation of *Arcobacter*, *Helicobacter* and *Campylobacter* by full-spectral matrix-associated laser desorption/ionization time of flight mass spectrometry analysis

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Rapid and reliable identification of *Arcobacter* and *Helicobacter* species, and their distinction from phenotypically similar *Campylobacter* species, has become increasingly important, since many of them are now recognized as human and/or animal pathogens. Matrix-associated laser desorption/ionization–time of flight (MALDI-TOF) MS has been shown to be a rapid and sensitive method for characterization of micro-organisms. In this study, we therefore established a reference database of selected *Arcobacter*, *Helicobacter* and *Campylobacter* species for MALDI-TOF MS identification. Besides the species with significance as food-borne pathogens – *Arcobacter butzleri*, *Helicobacter pullorum*, *Campylobacter jejuni* and *Campylobacter coli* – several other members of these genera were included in the reference library to determine the species specificity of the designed MALDI Biotyper reference database library. Strains that made up the reference database library were grown on Columbia agar, and yielded reproducible and unique mass spectra profiles, which were compared with the Bruker Biotyper database, version 2. The database was used to identify 144 clinical isolates using whole spectral profiles. Furthermore, reproducibility of MALDI-TOF MS results was evaluated with respect to age and/or storage of bacteria and different growth media. It was found that correct identification could be obtained even if the bacteria were stored at room temperature or at 4 °C up to 9 days before being tested. In addition, bacteria were correctly identified when grown on Campylosel agar; however, they were not when grown on modified charcoal cefoperazone deoxycholate agar. These results indicate that MALDI-TOF MS fingerprinting is a fast and reliable method for the identification of *Arcobacter* and *Helicobacter* species, and their distinction from phenotypically similar *Campylobacter* species, with applications in clinical diagnostics.

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INTRODUCTION

Identification of species belonging to the *Arcobacter*, *Helicobacter* and *Campylobacter* genera has become increasingly important, since many of them are recognized as human and/or animal pathogens. *Arcobacter butzleri* was found to be the fourth most frequently isolated *Campylobacter*-like organism in human clinical samples, before *Campylobacter lari*, but after *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus,* in Belgium and in France (Prouzet-Mauleon *et al.*, 2006; Vandenberg *et al.*, 2004). On several occasions, *Helicobacter pullorum* has been isolated from poultry (Zanoni *et al.*, 2007; Ceelen *et al.*, 2007; Atabay *et al.*, 1998; Neubauer & Hess, 2006b). A number of research groups have associated *H. pullorum* with gastroenteritis, diarrhoea, and liver and gall bladder disease in human patients (Young *et al.*, 2000; Stanley *et al.*, 1994; Castera *et al.*, 2006; Rocha *et al.*, 2005). For *H. pullorum* there is a lack of phenotypic identification methods, and as a result, this bacterium is commonly misidentified as thermophilic *Campylobacter* (Atabay *et al.*,

Abbreviations: ATCC, American Type Culture Collection; MALDI, matrix-associated laser desorption/ionization; NCTC, National Collection of Type Cultures; TOF, time of flight.

A table of identification data is available as supplementary material with the online version of this paper.

1998; Kuijper *et al.*, 2003). *C. jejuni* is the leading cause of bacterial gastroenteritis in developed countries (EFSA, 2005). In humans the majority (97 %) of food-borne diseases can be attributed to animals farmed for meat, especially poultry (Wilson *et al.*, 2008).

Various molecular DNA-based methods for the identification of *Arcobacter*, *Helicobacter* and *Campylobacter* species have been developed. These methods typically require the use of several species-specific PCR primers, hybridization probes or multiple restriction enzymes, and are usually not designed to differentiate all known species simultaneously (Bohr *et al.*, 2002; Jauk *et al.*, 2003; Wilson *et al.*, 2008; Neubauer & Hess, 2006a). Bacterial identification by matrix-associated laser desorption/ionization–time of flight (MALDI-TOF) MS is based on generating complex fingerprints of biomarker molecules by measuring the exact mass/charge ratio of peptides and proteins (Claydon *et al.*, 1996; Suh & Limbach, 2004). A number of species from the *Campylobacter* genus (Mandrell *et al.*, 2005; Kolinska *et al.*, 2008) have been characterized by MALDI-TOF MS. In addition, *Helicobacter pylori* and *Helicobacter mustelae* were analysed by MALDI-TOF MS (Winkler *et al.*, 1999), but not *H. pullorum* and *Helicobacter pametensis*. So far, no study applying MALDI-TOF MS based on the same sample preparation and technology, and using a whole spectral profile to differentiate potentially confounding *Arcobacter* and *Helicobacter* species together with phenotypically similar *Campylobacter* species has been reported.

In this study, we established a reference database of selected *Arcobacter*, *Helicobacter* and *Campylobacter*. The second objective was to use the reference database to identify 144 clinical isolates and compare the results to molecular methods. The third objective was to evaluate the reproducibility using different growth media and age of bacteria that are relevant in a diagnostic lab.

METHODS

Bacterial strains. For establishing the database library, reference strains were obtained from the National Collection of Type Cultures (NCTC) and from the American Type Culture Collection (ATCC). Preliminary work was done first on *C. jejuni* NCTC 12744, *A. butzleri* NCTC 12481 and *H. pullorum* ATCC 51801 to optimize the method. For standardizing the culture method all bacteria were grown on Columbia (COS) agar containing 5 % sheep blood (bioMérieux), at 42 °C for 48 h under microaerobic conditions (GENbox microaer; bioMérieux). In addition, bacteria were grown on Campylosel (CAM) agar (bioMérieux) and on modified CCD (charcoal cefoperazone deoxycholate) agar [blood-free agar base with 32 mg cefoperazone l^{-1} and 10 mg amphotericin ml^{-1} (Oxoid)] for reproducibility testing. *Escherichia coli* strain DH5α (Invitrogen) was grown on COS agar at 37 °C for 24 h.

Reference strains used in this study to generate the database library are listed in Table 1, which includes their origin, species and other information. The reference library was then used to identify 144 clinical isolates that were obtained from humans, the environment and from different farm animals, but mostly poultry (Table 2).

PCR-RFLP analyses. To identify the clinical isolates by molecular methods, the protocol of Jauk *et al.* (2003) was followed. Briefly, the isolates were examined by PCR-RFLP. PCR based on the 16S rRNA gene of the genera *Arcobacter*, *Helicobacter* and *Campylobacter* amplified a 1216 bp fragment. The amplicons were digested with the restriction enzymes *Rsa*I and *Eco*RV. Additional differentiation was obtained using PCR assay based on the hippuricase-encoding gene (Marshall *et al.*, 1999).

Sample preparation for MALDI-TOF MS analysis. A single colony was removed from the agar plates using an inoculating loop and the material was placed in a vial containing 300 µl MilliQ purified water to suspend the bacteria. To inactivate the bacteria, 900 µl absolute ethanol was added to the vial. After centrifugation for 2 min at 20 000 ***g*** the supernatant was removed. Afterwards, a second centrifugation step was done to remove the ethanol completely. For cell-wall disruption, 50 µl formic acid (70 %) was added to the pellet and thoroughly mixed. Subsequently, 50 µl acetonitrile were added for protein extraction. After a centrifugation for 3 min at 20 000 ***g***, 1 µl supernatant containing the bacterial extract was transferred to a

Table 1. Reference strains used to establish the reference database for MALDI-TOF MS based species identification

Designation	Genus	Species	Subspecies	Origin
NCTC 12145	*Campylobacter*	*jejuni*	*jejuni*	Human
NCTC 12744	*Campylobacter*	*jejuni*	*jejuni*	Contaminated milk
ATCC 700819	*Campylobacter*	*jejuni*	*jejuni*	Human faeces
NCTC 12143	*Campylobacter*	*coli*		No information given
NCTC 12144	*Campylobacter*	*lari*		Child with mild diarrhoea
NCTC 11458	*Campylobacter*	*lari*		Child with mild diarrhoea
ATCC 35217	*Campylobacter*	*hyointestinalis*		Swine with proliferative enteritis
NCTC 10842	*Campylobacter*	*fetus*	*fetus*	Brain of sheep fetus
NCTC 12481	*Arcobacter*	*butzleri*		Human faeces
ATCC 49616	*Arcobacter*	*butzleri*		Human faeces
ATCC 49942	*Arcobacter*	*butzleri*		No information given
ATCC 49615	*Arcobacter*	*cryaerophilus*		Human blood
ATCC 51400	*Arcobacter*	*skirrowii*		Cow (abomasitis)
ATCC 51801	*Helicobacter*	*pullorum*		Asymptomatic broiler chicken
ATCC 51802	*Helicobacter*	*pullorum*		Human faeces
ATCC 51478	*Helicobacter*	*pametensis*		Tern
DH5α	*Escherichia*	*coli*		

Table 2. Species identification results for the clinical isolates using MALDI-TOF MS in comparison to PCR-RFLP

Isolation and identification	No. of isolates					
	A. butzleri	*H. pullorum*	*C. coli*	*C. jejuni*	*C. hyointestinalis*	*C. fetus*
Origin						
Human	3	8	–	–	–	–
Broiler chicken	–	1	19	62	–	–
Swine	–	–	34	1	–	–
Layer chicken	–	–	3	3	–	–
Bovine	–	–	1	2	2	–
Cat	–	–	–	–	–	1
Duck	–	–	–	1	–	–
Environment	–	–	–	3	–	–
Method						
MALDI-TOF MS*	3/3	9/9	57/57	72/72	2/2	1/1
PCR-RFLP†	3/3	9/9	57/57	72/72	0/2	0/1

*MALDI-TOF MS represents an identification at species level log(score) value of $\geqslant 2.0$.
†PCR-RFLP analyses were carried out according to the protocol of Jauk *et al.* (2003).

sample position of a ground/polished steel MALDI target plate and allowed to dry at room temperature. Each sample was spotted six times onto the MALDI target plate to test technical replication. Then, the sample was overlaid with 2 µl matrix (α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluoroacetic acid) and dried again. All steps were performed at room temperature.

MALDI-TOF MS parameters. Mass spectra were collected using Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker) in linear mode, i.e. using a mass range of 2000 to 20 000 Da (parameter setting: IS1 20.0 kV, IS2 18.7 kV, lens 6.25 kV, detector gain 1634 V). Five hundred single spectra (10 × 50 laser shots) were summarized with a 50 Hz nitrogen laser for each sample. The instrument was externally calibrated with *E. coli* strain DH5α ribosomal proteins, e.g. RL36 4364.3 *m/z*, RS32 5095.8 *m/z*, RS34 5380.4 *m/z*, RS33meth. 6254.4 *m/z*, RL29 7273.5 *m/z* and RS19 10299.1 *m/z*.

Data visualization and analysis. Each individual spectrum was scrutinized by eye using the flexAnalysis software 3.0 (Bruker Daltonik) and atypical spectra were excluded from further analysis (e.g. flat line spectra, spectra containing high matrix background signal). A reference database library was established for MALDI-TOF MS-based species identification following the manufacturer's recommendations for Ultraflex measurement and the MALDI Biotyper 1.1 software package (Bruker Daltonik). In brief, for each database entry, at least 30 individually measured mass spectra fingerprints were imported into the MALDI Biotyper 1.1 software. Eight independent measurements (i.e. bacteria were grown at eight different times over the course of 3 months and subsequently measured) were obtained at six different spots each. After smoothing, baseline correction and peak-picking, the resulting peak lists (up to 70 peak masses) were used by the program to calculate and to store a main spectrum containing the information about mean peak masses, mean peak intensities and peak frequency.

Species identification of clinical isolates by MALDI-TOF MS. The MALDI-TOF MS reference database was used to identify and differentiate 144 clinical isolates. For correct identification of species, a generated peak list was matched against the established reference library using the integrated pattern-matching algorithm of MALDI Biotyper 1.1 software. Briefly, the software calculates log(score) values, that is, a log(score) between 1.7 and 2.0 represents genus identification. A log(score) value of $\geqslant 2.0$ represents an identification at species level. Anything less than a 1.7 log(score) was rated as not identifiable by the software.

MALDI-TOF MS reproducibility test. To test the reproducibility of MALDI-TOF MS-based species identification, 15 field strains were selected randomly and the reproducibility of their spectra under different conditions was tested. At first, bacteria were grown on COS agar for 48 h at 42 °C. Subsequently they were stored for 2, 4, 6 and 9 days at room temperature (20 °C), and at 4 °C, to see if the age and/or the storage conditions of the bacteria have an influence on the results. Secondly, these selected strains were grown for 48 h at 42 °C on CAM and mCCD agars, which are regularly used to isolate such bacteria.

RESULTS AND DISCUSSION

Detection and characterization of infectious micro-organisms in a reasonably fast and reliable manner from biological and environmental samples has a high priority. The genera *Arcobacter*, *Helicobacter* and *Campylobacter* belong to the rRNA superfamily VI. The differentiation between members of these three genera is challenging, particularly when employing biochemical tests as the sole criterion due to the inconsistency of the phenotypic profiles observed among strains (On, 1996). To overcome the problems related to classical phenotypic species identification methods, this study evaluated the capability of MALDI-TOF MS to differentiate and identify these species. For comparison, PCR-RFLP, a classical genotypic method, was chosen to characterize clinical strains.

The MALDI-TOF MS reference database was established with 16 well-characterized culture collection strains from different sources representing 10 different species of *Arcobacter*, *Helicobacter* and *Campylobacter*. These species yielded reproducible and unique mass spectral profiles,

which were compared with Bruker Biotyper database, version 2. However, the Bruker Biotyper database version 2 did not contain spectra from *H. pametensis*, as a result this bacterium did not match with any other spectra in the database. Therefore, the *H. pametensis* mass spectral profile is unique. All reference strains used represented the majority of clinically relevant species from the three genera. In addition, *E. coli* DH5α spectra were included in the reference database library.

The most common *Arcobacter* species were analysed, namely *A. butzleri*, *Arcobacter skirrowii* and *Arcobacter cryaerophilus* (Fig. 1). Interestingly, mass signal patterns of *A. cryaerophilus* and *A. skirrowii* as well as *A. butzleri* and *A. skirrowii* shared a number of common mass peaks that were 100 % frequent (in 135 spectra). Mass signal patterns of *A. cryaerophilus* were completely different to *A. butzleri*. However, the spectra of this *A. cryaerophilus* were found to have a number of small peak-shifts compared to the *A. cryaerophilus* type-strain in the Bruker Biotyper database, version 2. As a result, our reference *A. cryaerophilus* strain mass spectra profile did not reliably match the Bruker *A. cryaerophilus* type strain. This confirms the need to have a number of different strains of the same species coming from different sources in the reference database for correct identification.

Similarly, the two *Helicobacter* species, *H. pullorum* and *H. pametensis* (Fig. 2), had very different mass signal patterns and could be easily discriminated. Finally, five species from the genus *Campylobacter* widely found as human and/or animal pathogens were analysed, *C. jejuni*, *C. coli*, *C. lari*, *Campylobacter hyointestinalis* and *C. fetus*. Representative fingerprint mass spectra clearly demonstrate that all five species produced unique molecular profiles and could be easily differentiated (Fig. 3). Within the species fingerprints, it was observed that for thermophilic *Campylobacter* (i.e. *C. jejuni*, *C. coli* and *C. lari*) the mass signal patterns were very different to the non-thermophilic *Campylobacter* (i.e. *C. fetus* and *C. hyointestinalis*). Therefore, the 10 species could be easily discriminated by means of a direct comparison of the whole mass spectra fingerprints.

Different mass spectrometric methods for analysing whole bacteria cells for intact proteins, including the identification of one or few specific protein biomarker ions, have been reported. Mandrell *et al.* (2005) focused on *Campylobacter* 'species-identifying' biomarker ions (SIBS) using MALDI-TOF MS and reported that there were SIBS types associated with the source. In addition, biomarker ions in the 9 to 14 kDa range were reported to be diagnostic for *Campylobacter* species. However, one of the biomarkers [7035 Da see Fig. 3 (indicated by an asterisk)] published by Mandrell *et al.* (2005) was found to be 100 % frequent (in 211 spectra) for thermophilic *Campylobacter* but not observed in non-thermophilic *Campylobacter* in this study. This biomarker was extracted and identified to be a 50S ribosomal L29 protein by Fagerquist *et al.* (2006). In addition, specific *C. jejuni* biomarker 10 276 Da and specific *C. coli* biomarkers 10 032 and 12 855 Da proposed by Mandrell *et al.* (2005) were not frequently detected in this study. Therefore, species identification using one or few biomarker ions characteristic for a given species may

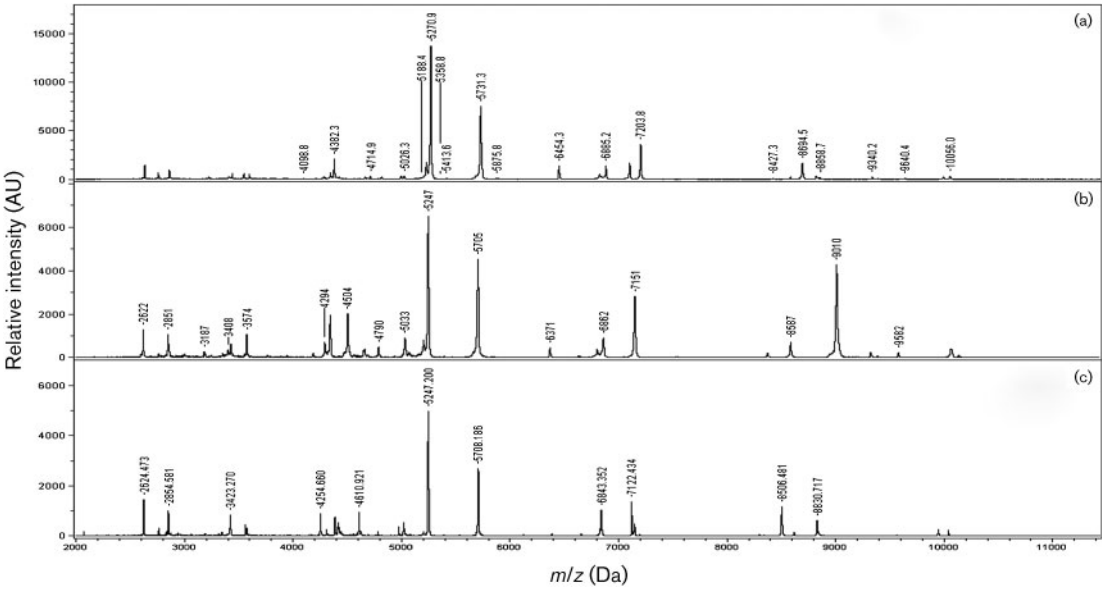

Fig. 1. MALDI-TOF MS profiles obtained from the analysis of *A. butzleri* (a), *A. skirrowii* (b) and *A. cryaerophilus* (c). The relative intensities of the ions [in arbitrary units (AU)] are shown on the *y*-axis, and the masses (in Da) of the ions are shown on the *x*-axis. The *m*/*z* value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular mass of the protein.

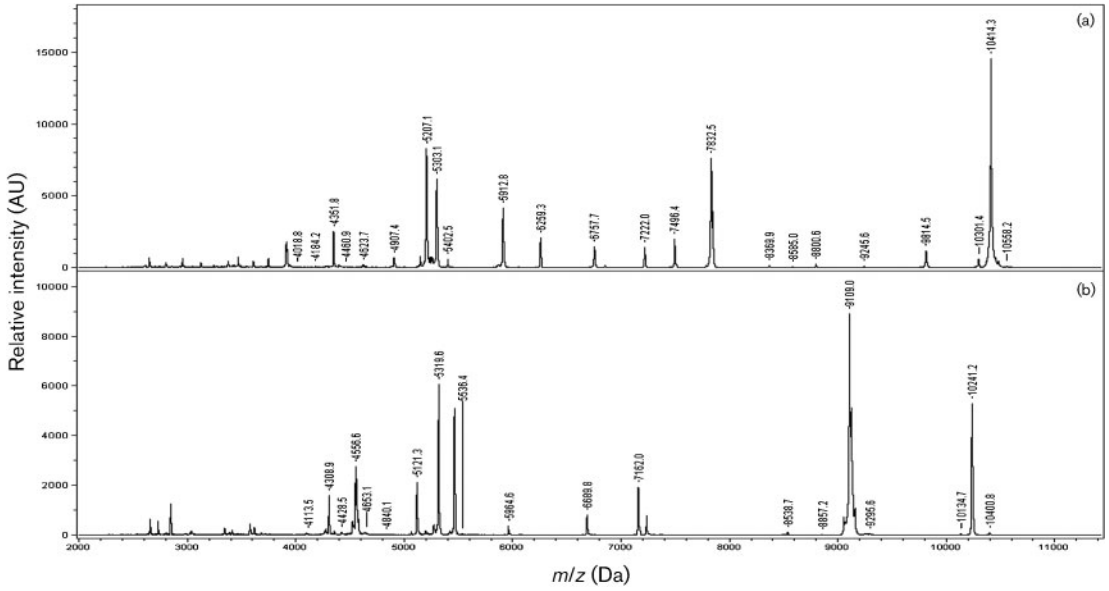

Fig. 2. MALDI-TOF MS profiles obtained from the analysis of *H. pullorum* (a) and *H. pametensis* (b). The relative intensities of the ions [in arbitrary units (AU)] are shown on the *y*-axis, and the masses (in Da) of the ions are shown on the *x*-axis. The *m*/*z* value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular mass of the protein.

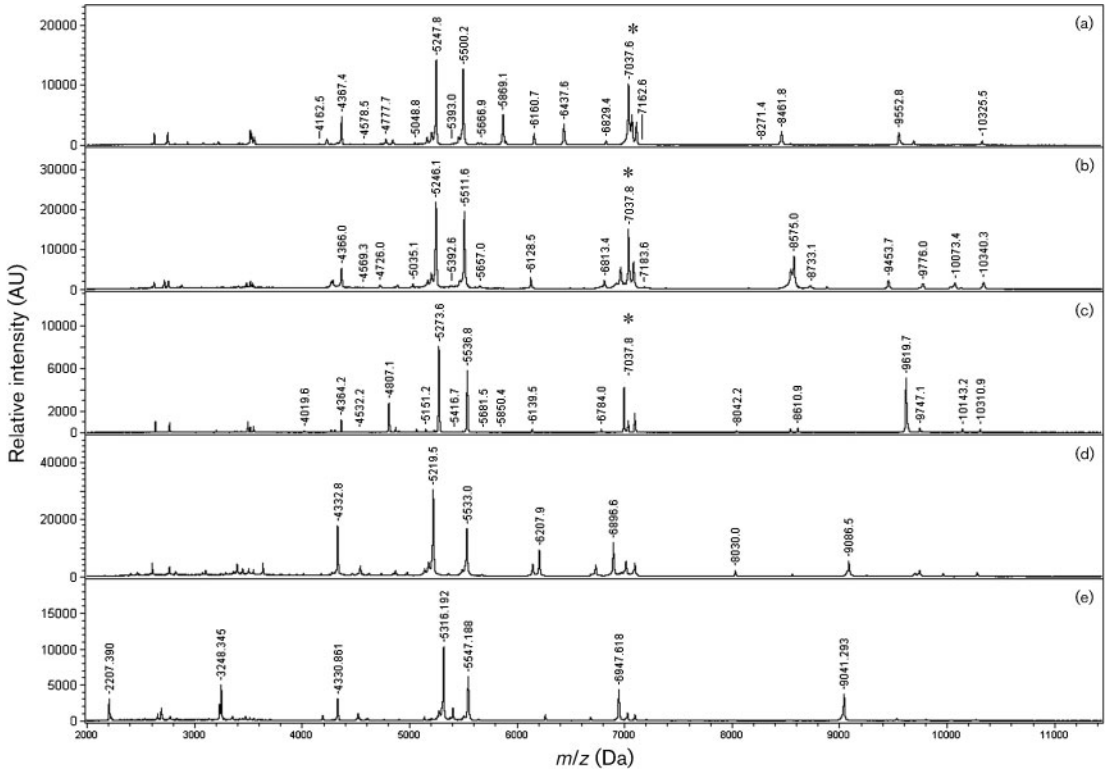

Fig. 3. MALDI-TOF MS profiles obtained from the analysis of *C. jejuni* (a), *C. coli* (b), *C. lari* (c), *C. fetus* (d) and *C. hyointestinalis* (e). The 50S ribosomal L29 protein 'biomarker' (indicated by an asterisk). The relative intensities of the ions [in arbitrary units (AU)] are shown on the *y*-axis, and the masses (in Da) of the ions are shown on the *x*-axis. The *m*/*z* value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular mass of the protein.

lead to incorrect results (Kolinska *et al.*, 2008). In another study, *C. jejuni*, *C. coli* and *C. fetus* were compared with *H. pylori* and *H. mustelae* by direct analysis of individual culture colonies in 50 % methanol using MALDI-TOF MS. A few specific biomarker ions in the 10 to 20 kDa range were reported to be the most discriminative of those observed (Winkler *et al.*, 1999). However, in this study mass signal patterns were only observed in the 2 to 11 kDa range for *Arcobacter*, *Helicobacter* and for *Campylobacter*.

For evaluation of the method, 144 clinical isolates were used in this study. In parallel to MALDI-TOF MS identification, all strains were analysed by PCR-RFLP. Identification results of the clinical isolates obtained by MALDI-TOF MS and PCR-RFLP are shown in Table 2. A list of log(score) data is available as Supplementary Table S1 with the online journal. All clinical isolates obtained from humans, the environment and from different farm animals, mostly poultry, gave sufficient spectra for species identification by MALDI-TOF MS. In total, MALDI-TOF MS identified all 144 clinical isolates at species level, i.e. log(score) $\geqslant$2.0 and no differences in results were found associated with the source of the isolate. PCR-RFLP could not differentiate between *C. fetus* and *C. hyointestinalis* in three clinical isolates (898, 1147 and 1307). In all these cases, a differentiation at the species level with MALDI-TOF MS was possible.

To investigate the stability of clinical isolate identification, 15 different strains were grown according to the standard procedure and subsequently stored at room temperature (20 °C) and at 4 °C, and tested after 2, 4, 6 and 9 days. In all cases, MS resulted in identical, correct identification results related to the reference database (data not shown). This finding supports the results of another investigation (Mellmann *et al.*, 2008), where non-fermenting bacteria were stored at room temperature up to 7 days. This can be a great advantage if samples are collected together for a single run or where samples need to be reinvestigated. It is noteworthy that bacteria grown for 48 h on COS or CAM agar gave the best results, e.g. good quality spectra, reliable log(score). However, bacteria grown longer than 72 h gave poor spectra profiles, lower intensity and unspecific peaks. Nevertheless, in most cases correct identification of the bacteria could be obtained. In another study MALDI-TOF MS was used for identification of *Listeria*, it was reported that extended periods of growth (4 days tested) did not affect spectra quality and results (Barbuddhe *et al.*, 2008). Obviously, this is species dependent. However, in a separate study *E. coli* was analysed using MALDI-TOF MS, where growth time did not affect the bacterial molecular profile significantly, hence, the incubation time span was up to 48 h (Mazzeo *et al.*, 2006).

To determine the influence of cultivation media on the quality of spectra, the 15 clinical strains were grown on different types of solid media used to isolate such bacteria. Species identification was possible at the species level if bacteria were grown on CAM agar, which was not used to create the reference database. Interestingly *Arcobacter*, *Helicobacter* and *Campylobacter* grown on mCCD agar could not be used for MALDI-TOF MS species identification as very poor spectra patterns were obtained, if any; consequently, no identification was possible (data not shown). Apparently, mCCD agar contaminants interfere with ionization of biomolecules of the bacteria. In fact, in some cases it was not possible to pick up bacteria biomass from mCCD agar because the bacteria were strongly attached on the surface of this agar. The importance of mCCD agar could not be overlooked as a drawback of MALDI-TOF MS as this agar is commonly used to isolate strains of *Campylobacteraceae*. This means that additional subculturing is necessary if the bacteria are going to be used for MALDI-TOF MS identification. To the best of our knowledge, no study reporting results on the use of mCCD agar for micro-organism identification by MALDI-TOF MS has been published.

Altogether, these data show that MALDI-TOF MS fingerprinting is a fast and reliable method for the identification of *Arcobacter* and *Helicobacter* species, and their distinction from phenotypically similar *Campylobacter* species with applications in clinical diagnostics. As a result, considering the speed with which reliable identification can be obtained, this technique is well suited for large-scale research and diagnostic analyses.

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