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Enzymatic protein digests do not assist in *E. coli* discrimination at the strain level using mass spectrometry

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ABSTRACT

Different procedures for matrix assisted laser desorption ionization time of flight mass spectrometry-based *E. coli* classification at the strain level using the enzymatic digestion of proteins from the cell lysate have been studied. The effects of ultrasonic energy as well as the effects of protein reduction and protein alkylation in the sample treatment and in the subsequent classification were assessed. The final optimal method for classification was then compared with an intact cell-based approach in a different set of samples. Our results show that *E. coli* classification at the strain level is possible as 12 different strains were correctly classified using intact cell analysis. Overall, the confidence level in classification was higher when the analysis was performed with the intact cell approach.

Keywords: Bacterial; *E. Coli*; Fingerprinting; MALDI; Classification.

1. Introduction

Bacterial classification, referred as the assignment of an isolate to a species formally described [1], has been generally accomplished based on conventional techniques that rely on biochemical reactions and phenotypic characteristics, such as Gram staining and colony morphology [2]. The complete identification with these methods is usually time-consuming and, in some cases, correct identification is not possible [3]. Thereby, molecular biology methods, mostly based on nucleic acid tests such as polymerase chain reaction (PCR), have been used as complementary or alternative approaches to overcome these disadvantages due to their rapidity and sensitivity [4]. However, these techniques require a high level of technical expertise and remain expensive [5].

In recent years, besides its wide field of application in conventional proteomics, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been increasingly applied with success for microbial identification, including bacteria, fungi and viruses [6-12]. A unique mass spectral fingerprint of the microorganism is acquired and used for identification at the species and strain-level. The rapidity, accuracy and cost-effectiveness make it a promising tool to replace current methods in clinical microbial diagnostics within the next few years [1, 13].

Nevertheless, the increment in published work led to an increase in the number of sample processing methods that differ in particular steps [14]. Nowadays, among all treatment procedures published regarding MALDI-TOF MS microbial profiling, two main approaches can be discerned. The direct analysis of intact cells, without any disruption process, has been referred as an easy way to achieve microbial identification [15]. On the other hand, cell disruption and protein extraction analysis or whole cell lysate analysis represent an

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alternative method for microorganism identification [14]. Whereas few biomarkers are required for identification at the species-level, much more are needed for strain differentiation [16]. Protein fraction analysis enhances the discriminatory power of the technique by increasing the number of reproducible peaks detected that are useful in intra-species classification. For instance, Bizzini et al. improved the identification yield of strains of different species of bacteria and yeasts, from 70,3% to 93,2% after a protein extraction step [17].

In this work, the usefulness of digesting the whole bacterial lysate as well as digesting the whole bacterial protein content for bacterial discrimination at the strain level through MALDI-TOF MS has been assessed. The effectiveness of ultrasonic assisted enzymatic digestion was compared with the conventional overnight digestion, in order to reduce the identification time to a few minutes. Finally, the optimized method was applied to a distinct set of 12 *E. coli* isolates in order to compare it with an intact cell procedure for classification.

2. Material and methods

2.1. *Apparatus*

A Branson Sonifier® SLPe 150W equipped with a 3 mm microtip from Branson Ultrasonics (Danbury, USA) and a sonicator model SONOPULS HD 2200 with a Cup Horn BB6 accessory, from Bandelin (Berlin, Germany), were used for cell lysis and for accelerating protein reduction, alkylation and digestion steps. A mini-centrifuge CM-50 from ELMI (Riga, Latvia) and a refrigerated centrifuge MPW-65R from MPW Med. Instruments (Warsaw, Poland) were used throughout the experiments when necessary. Deionized ultrapure water was obtained from a Simplicity™ system from Millipore (Milan, Italy). Mass spectrometry analyses were performed with a MALDI-TOF/TOF UltraFlex model from Bruker Daltonics (Bremen, Germany).

2.2. *Bacterial samples*

Escherichia coli T1 Mach (Invitrogen) (A), NovaBlue (Merck-Millipore) (B), DH5α (C) and BL21 (D) were used for method development. For method validation, a set of 12 previously characterized *E. coli* isolates recovered from wild animals was used (E-P): GV5A (E) [18], L196 (F) [19], WA57 (G) [20], L65 (H) [19], W4B[a] (I) [20], W151A (J) [20], W184 (K) [20], L63[†] (L) [19], L18 (M) [21], L40 (N) [21], L115 (O) [21] and L185 (P) [22].

2.3. *Standards and reagents*

Luria-Broth (Sigma-Aldrich, Steinheim, Germany) and Brain Heart Infusion (Oxoid) liquid mediums were used for *E. coli* growth. Phosphate saline buffer (PBS, pH = 7.4), sodium deoxycholate (NaDOC), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), acetonitrile, DL-dithiothreitol (DTT), iodoacetamide (IAA) and trypsin from porcine (proteomics grade) were also from Sigma-Aldrich. Ammonium bicarbonate (AmBic), formic acid, and α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Fluka (Buchs, Switzerland). Peptide and protein standards for mass calibration were purchased from Bruker Daltonics (Germany).

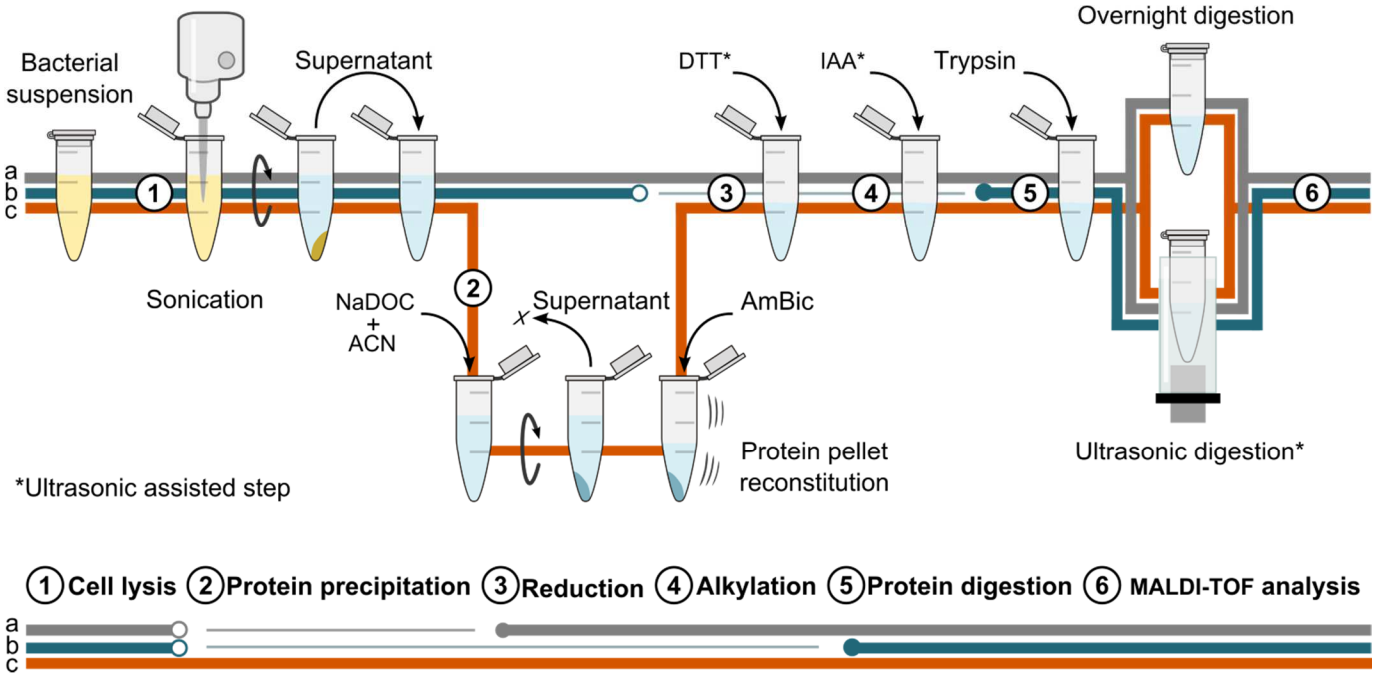

Figure 1. Overview of the different sample treatments applied in protein digestion. **(a)** Direct digestion of proteins from supernatant with the reduction and alkylation steps. **(b)** Direct digestion of proteins from supernatant without the reduction and alkylation steps. (c) Protein digestion after protein precipitation, resuspension and reduction and alkylation steps.

2.4. *Bacterial growth and protein extraction*

E. coli strains A, B, C and D were cultured in Luria-Broth (LB) medium and incubated overnight at 37 ºC, 190 rpm in an orbital shaker. After incubation, cells were centrifuged at 8000 rpm for 5 min, decanted and washed twice with PBS-buffer. Cell disruption was performed with an ultrasonic probe (3 mm probe tip diameter; 35 % ultrasonic amplitude) during 6 minutes with intercalated cycles of ultrasonication (10 s) and rest periods without sonication (10 s). This procedure was performed in an ice-bath to prevent sample overheating. Cell debris was removed by centrifugation at 13000 rpm at 4 ºC for 10 min. The supernatant was collected to new vials and the pellet was discarded. Three procedures were then applied: direct enzymatic digestion of supernatant with (i) and without (ii) reduction and alkylation steps and enzymatic digestion of supernatant's protein content after precipitation (iii). The procedures are described in detail below and depicted in Fig.1

2.5. *Enzymatic digestion of supernatant*

Two aliquots of 50 μL of supernatant were made for each method tested. Direct digestion of proteins was performed with (Fig. 1a) and without (Fig. 1b) reduction and alkylation steps. The protein content of each aliquot was processed as follows. For protein reduction, 2 μL of DTT (10 mM final concentration in sample mixture) were added and the sample was ultrasonicated with the sonoreactor, SR, SONOPULS (50 % amplitude) for 2 min. Then, 2 μL of IAA (50 mM final concentration in sample mixture) were added and protein alkylation was enhanced with the SR (50 % amplitude) for 2 min. Finally, 44 μL of trypsin (0,01 μg/μL final concentration in sample mixture) were added to the sample and protein digestion was performed during 5 min with the SR (50 % amplitude), or during overnight (ca. 12 h) at 37ºC. Protein digestion was stopped with the addition of 2 μL of formic acid (1 % v/v final concentration in sample mixture).

2.6. *Enzymatic digestion of supernatant's protein content*

Two aliquots of 500 μL of supernatant were taken for each method tested and proteins were precipitated. For protein precipitation samples were made up to 0.02 % with NaDOC (2% m/v). Samples were incubated during 30 min. Then, TCA was added until solution was made up to 10 % v/v in this reagent, and the samples were incubated at 4 ºC for 1 h. After centrifugation at 13000 rpm, 4 ºC, during 15 min, the supernatant was discarded and the pellet was washed with 400 μL of cold acetone (-20 ºC), incubated during 15 min at -20 ºC and centrifuged at 13000 rpm, 4 ºC, for 15 min. The supernatant was discarded and the pellet was air dried to remove acetone residues. Finally, 50 μL of AmBic 25 mM were added to dissolve the proteins from the pellet. Proteins were reduced, alkylated and digested as described above (Fig. 1c).

2.7. *Sample treatment for whole bacterial analysis*

Twelve isolates of *E. coli* were used. The isolates were taken from wild animals and they were collected during a biological survey done within a program of the UTAD University. Bacteria were cultured in BHI (brain heart infusion) medium. After incubation, cells were centrifuged at 8000 rpm for 5 min, decanted and washed twice with MilliQ water. The resulting pellet was then resuspended in 250 μL of 0.1 % TFA and vortexed for 60 s. Three biological replicates for each isolate were done (Fig. SM-1).

2.8. *MALDI-TOF-MS analysis*

2.8.1. *Peptide digests*

The peptide digests were mixed (1:1) with α-CHCA matrix at a concentration of 10 mg/mL in acetonitrile/water (50 % v/v) and TFA (0.1%). Then, each sample was applied onto the MALDI sample plate (Bruker Daltonics parts no. 209519) in five different spots (1 μL per spot), air dried and analyzed without any further purification steps, in a Ultraflex II MALDI-TOF/TOF instrument from Bruker Daltonics equipped with a 200 Hz Smartbeam laser system. Data was acquired using the FlexControl software (version 3.3.108.0). Measurements were performed in the reflectron positive-ion mode. A total of 1500 spectra were acquired at each spot position with a laser frequency of 100 Hz. Close external calibration was performed with the $[M + H]^+$ monoisotopic peaks of bradykinin 1-7 (757.3992), angiotensin II (1046.5418), angiotensin I (1296.6848), substance P (1347.7345), bombesin (1619.8223), renin substrate (1758.9326), ACTH clip 1-17 (2093.0862), ACTH 18-39 (2465.1983), and somatostatin 28 (3147.4710). The peak lists were generated from the mass spectra using the peak detection algorithm SNAP (sophisticated numerical annotation procedure).

2.8.2. *Intact bacterial analysis*

A volume of 10 μL of the bacterial suspension was mixed with 10 μL of a sinapinic acid matrix solution (20 mg/mL in 50 % acetonitrile/ 2 % TFA. Then, 1 μL of each sample was applied in quintuplicate onto a MALDI target plate (MTP 384 target plate ground steel T F). The same Ultraflex II MALDI-TOF/TOF instrument described above was used. Measurements were performed in the linear positive-ion mode from 2000 to 20000 m/z. Close external calibration was performed with the protein calibration standards I. Peak lists were generated with the FlexAnalysis software (version 3.3) using the peak detection algorithm Centroid.

3. Results and discussion

Currently, the identification of bacteria by MALDI-TOF-MS relies mostly on the analysis of intact bacteria due to the

simplicity of the procedures and the rapidity of the analysis. Yet, these procedures may not be suitable for the analysis of pathogenic bacteria due to the risk of contamination and exposure of the operator to the infectious agent. Alternatives relying on the analysis of inactivated bacterial lysates and enzymatic digests of proteins from the bacterial lysate have been recently developed [14]. New approaches to such alternatives are presented below.

3.1. *The effect of ultrasonic energy in the digestion of whole bacterial lysates.*

Ultrasound has been reported as a useful and reliable tool to accelerate not only protein digestion with trypsin, but also the entire protocol for protein identification by MS including protein reduction with DTT and alkylation with IAA [23 -26]. The mechanism responsible for the enhancement of protein enzymatic digestion with ultrasound is not fully understood yet, but it is thought to rely on cavitation [27]. The cavitation phenomena occurs when a ultrasound wave travels through a liquid media generating micro bubbles of gas, known as cavitation bubbles, which grow during successive rarefaction and compression cycles. Eventually, these bubbles reach an unstable size and undergo violent collapse generating localized spots of high pressure and high temperature, which can be used as micro reactors to enhance chemical and physical reactions. In this work, ultrasonic energy from a sonoreactor apparatus, SR, was used to accelerate the digestion of proteins from crude bacterial lysates and proteins obtained after precipitation of the bacterial lysate, as well as to enhance protein reduction and alkylation steps. The SR works as a small ultrasonic bath. However, the ultrasound intensity delivered is much higher than the one obtained from a normal ultrasonic bath. This it allows to speed up protein digestion with great efficiency [28]. Furthermore, ultrasonication with the SR is performed in closed vials. This prevents sample contamination and allows hazardous samples to be safely handled.

In the first part of this work, the ultrasonic (5 min) and overnight (12 h) enzymatic digestion of crude bacterial lysates were compared. As a general role, the mass spectra obtained after ultrasonication had lower intensity than the spectra obtained after overnight digestion (Fig. SM-2). Table SM-1 presents the number of mass peaks with relative intensity higher than 5 % or 10 % obtained after protein enzymatic digestion with the overnight (12 h) procedure or with the ultrasonic (5 min) procedure. As this table shows, the number of peaks with a relative intensity higher than 5 % obtained with the overnight procedure varied from 128 to 564, which represents a difference of more than 400 peaks between spectra of D and B strains, respectively. Regarding the ultrasound-assisted digestion, the number of mass peaks with a relative intensity higher than 5 % varied from 279 to 478 peaks. The same pattern was observed when only the mass peaks with a relative intensity higher than 10 % were considered. As a trend, the number of significant mass peaks

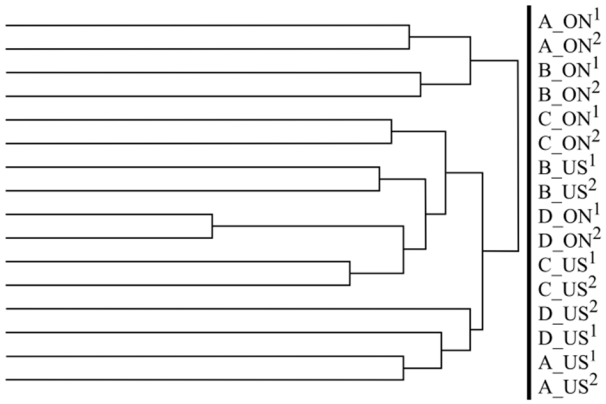

Figure 2. Hierarchical clustering of peak lists obtained by MALDI-TOF MS from overnight (ON) and ultrasound (US) assisted enzymatic digestion of crude bacterial lysates of E. coli strains A, B, C and D. Two replicates were performed in each procedure. Cluster analysis was accomplished with BACTERIAL IDENTIFICATION software (Euclidean distance, minimum variance 0.1%).

is higher in spectra from overnight digested samples than in spectra from samples digested with ultrasound., with the exception of strain D. In this case, one of the main reasons for the discrepancy in the size of the list of peptides can be related with ion suppression effect, as can be seen by the mass spectrum obtained after the overnight digestion of proteins from strain D_ON (Fig. SM-2). This spectrum clearly shows a high intense mass peak at 1480 m/z. The major effect is the decrease of the relative intensities of the remaining mass peaks, which in turn are not selected for analysis, since they do not meet the relative intensity threshold. This is very important because the higher the number of peaks selected for analysis the higher the possibility to find differences between strains. The mass spectra obtained were analysed by hierarchical clustering to study the classification achieved by each sample treatment. As shown in Fig. 2, most replicates of the different samples are grouped in pairs, according to the strain and to the type of sample treatment used. This shows that both, the overnight and ultrasonic sample treatments; render reproducible mass spectra, which can be used to distinguish bacterial samples at the strain level. Furthermore, it is clear from this result that the sample treatment is the variable that most influences the classification of the strains, as they are grouped as a function of the sample treatment used. This result is of great importance because it reveals that sets of data obtained from two different sample treatments should not be used to classify bacteria.

3.2. *Influence of protein reduction and alkylation in the sample treatment for bacterial classification.*

Protein reduction with DTT followed by alkylation with IAA is used in proteomics to break disulphide bonds between cysteine residues and to prevent their formation, respectively. These reactions introduce a chemical modification which destroys the native structure of the protein and

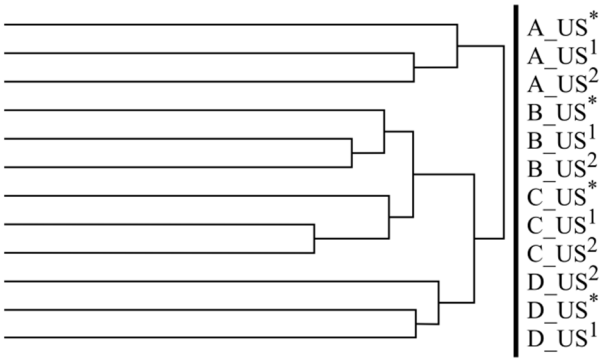

Figure 3. Hierarchical clustering of peak lists obtained by MALDI-TOF MS from ultrasound assisted enzymatic digestion of crude bacterial lysates of E. coli strains A, B, C and D, with (US) and without (US*) previous reduction and alkylation treatments. Two replicates were performed in the US procedure. Cluster analysis was accomplished with BACTERIAL IDENTIFICATION software (Euclidean distance, minimum variance 0.1%).

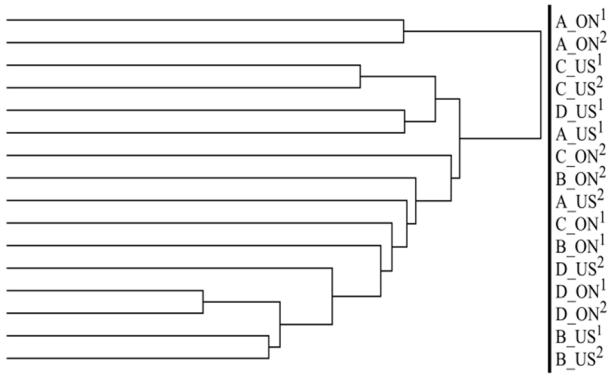

Figure 4. Hierarchical clustering of peak lists obtained by MALDI-TOF MS from overnight (ON) and ultrasound (US) assisted enzymatic digestion of precipitated proteins of E. coli strains A, B, C and D. Two replicates were performed in each procedure. Cluster analysis was accomplished with BACTERIAL IDENTIFICATION software (Euclidean distance, minimum variance 0.1 %).

helps enzymatic digestion by exposing cleavage sites that otherwise would remain hindered by the native structure. In the previous section, the analysis was done after protein reduction and alkylation. To assess the influence of protein reduction and alkylation on bacteria classification, direct enzymatic digestion assisted with ultrasound energy was performed on a sample set without reduction and alkylation steps. The number of peptides obtained with a relative intensity higher than 5% in the non-reduced and non-alkylated samples was 536, 403, 333 and 481 for strains A, B, C and D, respectively (Table SM-2). These values are of the same magnitude than the ones obtained in samples where protein reduction and alkylation was performed (Table SM-1). Fig. 3 presents the hierarchical clustering analysis of the spectra obtained for ultrasonic treated samples, both non-reduced and non-alkylated together with reduced and alkylated. As may be seen, 4 main groups were formed, each one corresponding to a different strain. These results suggest that performing protein reduction and alkylation on proteins from crude bacterial lysates before the enzymatic digestion has a minor effect in the classification obtained by MALDI-TOF-MS analysis.

3.3. *Evaluation of the discriminatory power of the protein content in the bacterial classification.*

When working with bacterial lysates, protein precipitation is routinely used to concentrate and separate the protein content from other components present in the lysate. Yet, one of the main disadvantages of this approach is the incomplete protein recovery from the precipitate [29]. In this work, proteins were precipitated from bacterial lysates with TCA/NaDOC. After precipitation, the pellet was resuspended in ammonium bicarbonate buffer (25 mM), then the proteins were reduced and alkylated, and finally digested with trypsin by the overnight (12 h) or the ultrasonic assisted (5 min) procedures. Results are shown in Fig. SM-3. As a general rule, the intensity of the spectra was lower than for the analysis of crude bacterial lysates. Even though the number of mass peaks obtained per spectra was similar to the ones obtained for the crude bacterial lysates, a higher variance between replicates was observed (Table SM-3). Fig. 4 presents the hierarchical clustering analysis of the spectra obtained after overnight and ultrasound assisted enzymatic digestion. Once again, the same strains are grouped in pairs according to the procedure used for protein digestion. For instance, replicates of the ultrasound assisted digestion of proteins from strain D were found closely related, but distant from spectra of the same strain obtained after overnight enzymatic digestion. This confirms that bacterial identification by MS seems highly dependent on the sample treatment used.

3.4. *Evaluation of the discriminatory power of the digested protein content analysis in a set of E.* coli *isolates*

According to the results presented in the sections above, the protein digestion of the crude bacterial lysate assisted with ultrasonic energy appears to be the most favourable method for strain discrimination. In order to evaluate the sensitivity and robustness of the method, 12 *E. coli* isolates recovered from wild animals previously classified according to the antimicrobial resistance gene profile were tested. Results presented in Fig. 5, show that nearly half of the replicated isolates were clustered grouped together after ultrasonic assisted enzymatic digestion. However, others were clustered distantlydistantly related, as shown for example in the replicates of isolate K. Therefore, it is possible to verify that this approach may have limitations. On the other hand, as may be observed in Fig. 6, the direct analysis of whole bacterial cells allows the classification of almost all strains. This suggest that this method is highly reproducible and also that the MALDI-TOF-based mass spectrometry can be used to

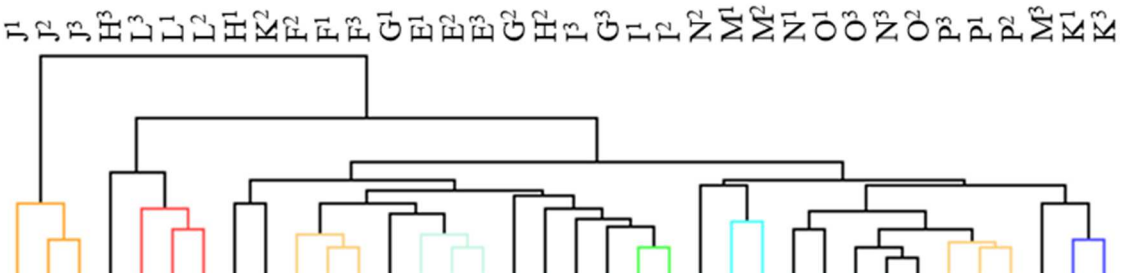

Figure 5. Hierarchical clustering of peak lists obtained by MALDI-TOF MS from ultrasound assisted enzymatic digestion of crude bacterial lysates of twelve (E-P) E. coli isolates characterized by the antimicrobial resistance gene profile. Three biological replicates were performed for each isolate.

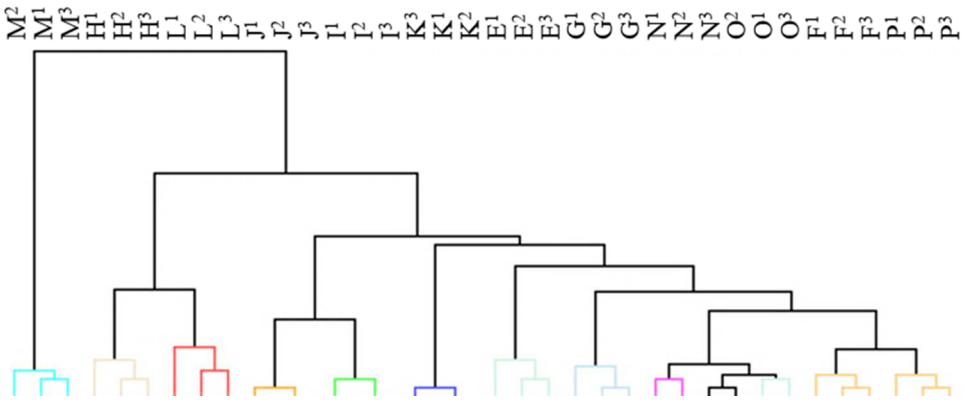

Figure 6. Hierarchical clustering of peak lists obtained by MALDI-TOF MS from whole cell analysis of twelve (E-P) E. coli isolates characterized by the antimicrobial resistance gene profile. Three biological replicates were performed for each isolate.

differentiate bacteria of the same species at the strain level. This approach, whole cell mass spectrometry (WC-MS), has been a widely adopted routine procedure for bacterial differentiation.

4. Conclusions

Different sample treatments for bacterial identification relying on the analysis of digested bacterial lysates have been compared. Crude bacterial lysates or proteins obtained after precipitation were enzymatically digested during overnight or with the aid of ultrasonic energy. In addition, the effect of protein reduction and alkylation on the classification was also studied. The results showed that reduction and alkylation have little effect in the classification of bacteria when ultrasonic energy was used to enhance enzymatic digestion of crude bacterial extracts. It was also verified that samples were grouped according to the sample treatment used rather than the corresponding bacterial strain. Even though, samples corresponding to the same strain and with the same sample treatment were grouped together in the majority of the studied cases. Nevertheless, the use of enzymatic digests failed in classifying 12 wild *E. coli* strains whilst such classification was achieved with the analysis of the intact bacteria. Therefore the use of digested samples is not recommended for classification of *E. coli* strains. Also bacterial classification through MALDI-based mass spectrometry is highly dependent on the sample treatment used and therefore classification studies must be always done using the same sample treatment. In fact this study seems to suggest that there is an urgent need to method standardization for MALDI-TOF sample preparation for microbial identification. It must be highlighted that this study was performed with gram negative species. Several known difficulties regarding mass spectrometry are inherent to the peptidoglycan layer of the gram positive bacterial cell wall, wherein a previous disruption step may be required to improve signal record. Therefore, extrapolation of these outcomes to other cases should be done carefully.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/131/0. Supplementary Material includes Figures SM_1, SM_2 and SM_3.

Acknowledgments

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