High-throughput proteotyping of bacterial isolates by double barrel chromatography-tandem mass spectrometry based on microplate paramagnetic beads and phylopeptidomics

Karim Hayoun, Jean-Charles Gaillard, Olivier Pible, Béatrice Alpha-Bazin, J. Armengaud

High-throughput proteotyping of bacterial isolates by double barrel chromatography-tandem mass spectrometry based on microplate paramagnetic beads and phylopeptidomics

Karim Hayoun, Jean-Charles Gaillard, Olivier Pible, Béatrice Alpha-Bazin[#], Jean Armengaud[*#]

Laboratoire Innovations technologiques pour la Détection et le Diagnostic (Li2D), Service de Pharmacologie et Immunoanalyse (SPI), CEA, INRA, F-30207 Bagnols sur Cèze, France.

[#]These co-authors should be considered as co-last authors.

**Author to whom correspondence should be addressed: Jean Armengaud, CEA-Marcoule, DRF/Joliot/DMTS/SPI/Li2D, "Innovative Technologies for Detection and Diagnostics" Laboratory, BP 17171, F-30200 Bagnols-sur-Cèze, France; jean.armengaud@cea.fr; Tel: +33 4 66 79 62 77; Fax: +33 4 66 79 64 60.*

Running title: High-throughput proteotyping

Word count: 4,860 *Character count: 27,415*

Keywords: proteotyping, tandem mass spectrometry, isolates, microorganisms, taxonomy, sample preparation

Abstract

Tandem mass spectrometry-based proteotyping of microorganisms presents several advantages over whole-cell MALDI-TOF mass spectrometry: because a larger number of signals are recorded with better accuracy and precision, the approach allows for the identification of microorganisms at more resolved taxonomic levels, and can easily manage complex samples. Additionally, the use of SP3 paramagnetic beads for cell lysis and protein cleanup simplifies sample preparation for proteotyping. Based on these features, we have developed and tested a 96-well plate platform for high-throughput proteotyping of a large variety of bacteria. We evaluated the performance of the platform in terms of bacterial load and found no cross-contamination between wells. Likewise, phylopeptidomics analysis revealed no alteration in the relative quantifications of microorganisms. Finally, we applied this new format for rapid proteotyping of a large set of dental isolates using double-barrel chromatography coupled to tandem mass spectrometry, which maximizes the number of spectra per unit of time. The procedure allowed us to establish whether these isolates were pure strains or mixtures of strains and to identify the microorganisms at the most resolved taxonomic level.

Significance

The rapid and accurate identification of microorganisms is a clinical priority in medical diagnostics; however, specimens containing mixtures of microorganisms are difficult to analyze and the procedure is time-consuming. Tandem mass spectrometry proteotyping allows the fast identification of complex mixtures of microorganisms, known or unknown, and can also establish the biomass ratio of each component. We describe here a new workflow for preparing microbial samples in a 96-well-plate format for tandem mass

spectrometry proteotyping and document its advantages and limitations. We demonstrate that this new format coupled to a highly efficient double-barrel LC-MS/MS system allows proteotyping of 96 isolates in 55h.

Highlights

- We developed a 96-well-based SP3 platform for high-throughput tandem mass spectrometry proteotyping.
- Quantitative phylopeptidomics reveals that the 96-well platform yields an identical relative abundance of microorganisms to conventional in-tube methods.
- The 96-well platform can accept a broad range of sample material: from 2.8×10^5 to 1.7×10^9 cells.
- Double-barrel LC-MS/MS system allows proteotyping 96 samples in only 55 h.

Introduction

Proteotyping is a typing method for taxonomic identification of organisms according to their specific protein profile acquired by mass spectrometry [1]. Proteotyping can be performed by whole cell matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, an established technique for the identification of microorganisms based on the molecular mass profile of their low molecular weight and basic proteins. The technique is designed to query the mass spectrum acquired directly on the sample against a database containing profiles of thousands of previously recorded microorganisms [2]. The approach

enables the rapid and confident identification of, for example, bacterial pathogens for clinical diagnostics at low cost [3], and can be used for discriminating subspecies as illustrated for *Francisella tularensis* [4] or *Campylobacter coli* [5], and for identifying antibiotic resistance in specific cases [6]. Yeast and fungi can be also identified with the same principle [7]. The MALDI-TOF approach is not, however, universal, as the spectra of most environmental microorganisms have not yet been recorded and, more importantly, only pure microorganisms can be identified [2] due to the low number of mass features recorded. One of the ways to address these challenges is to extend microorganism proteotyping by measuring a larger number of signals by tandem mass spectrometry [1, 8]. Proteotyping by tandem mass spectrometry relies primarily on shotgun proteomics, wherein proteins present in the sample are proteolyzed into small peptides with trypsin; the generated peptides are then resolved by reverse phase chromatography and their sequence established by tandem mass spectrometry. The taxonomic information associated with the peptide sequences is subsequently analyzed to identify the taxa present in the sample based on taxon-specific peptide sequences. This approach has been exemplified with several bacteria [9], and is sufficiently powerful to differentiate phylogenetically closely related bacteria [10] and even cellular forms of the same bacterium (e.g., vegetative cells and spores) [11]. Another advantage of tandem mass spectrometry-based proteotyping is its applicability to mixtures of microorganisms in complex samples, such as blood cultures [12] or feces [13], and in the latter example no cultivation step is required. It can also be used to assess the biomass contributions of microorganisms in a complex sample by relying on the global inter-taxa fractions of shared peptides, as described in the "phylopeptidomics" principle [14], and has utility for monitoring antibiotic resistance biomarkers [15]. Using the shotgun approach, tandem mass spectrometry proteotyping can be applied without any *a priori* information, and is thus more appealing for identification than targeted mass spectrometry. For all of these reasons, tandem mass

spectrometry-based proteotyping is broadly applicable for clinical diagnostics, and also for in-depth characterization of environmental microbial communities [16].

The efficiency of tandem mass spectrometry proteotyping depends on the amount of generated peptides and, accordingly, sample preparation including protein extraction, purification and proteolysis, is a critical step in the process. In-gel [17], in-solution [18] and filter-aided sample preparation [19, 20] have been the most common approaches used in shotgun proteomics over the last decade. Hughes and co-workers [21, 22] have recently proposed an alternative sample preparation approach based on paramagnetic beads, termed single-pot solid-phase-enhanced sample preparation (SP3), wherein proteins are fixed onto magnetic beads and the protein/bead complexes can be easily retained using a magnet for washing and cleanup. Once bound to the bead surface, proteins are digested directly, and the resulting peptides are released ready for LC-MS/MS analysis. We previously showed that SP3 improved proteotyping performance over in-gel and S-Trap sample preparation with regards to peptide and protein identification [23]. Indeed, in the SP3 protocol, reduction and alkylation of proteins may be omitted and a short 15-minute proteolysis is sufficient, thus shortening sample preparation for tandem mass spectrometry proteotyping.

Sample preparation performed in tubes is time-consuming and labor-intensive when numerous samples have to be analyzed, and is thus not suited to high-throughput clinical diagnostics. Accordingly, the adaption of sample preparation from tube format to multi-well plate format is an attractive concept, and has been described for classical proteomic sample preparation requiring labeling [24], enrichment [25], or purification using FASP [26, 27], S-Trap [28], and more recently SP3 [29-31]. Here, we explored the possibility of performing 96-well plate sample preparation for tandem mass spectrometry proteotyping of microbial samples and compared the proteotyping outcomes against a single-tube format. We assessed magnet performance, established the range of bacterial cell load, and tested for cross-

contamination and the relative quantities of mixed organisms. Finally, we applied this new format coupled to a highly efficient double-barrel LC-MS/MS system for proteotyping a large set of dental isolates.

Materials and methods

Microbial culture, mixture preparation and sampling

Bacteria were cultured overnight under aerobic conditions at 30°C and 140 rpm agitation. *Bacillus subtilis* ATCC6633 (American Type Culture Collection), *Escherichia coli* BL21(DE3) (pET Expression System 30 kit; Novagen) and *Acinetobacter baumanii* CIP70.10 (Collection of Institut Pasteur, Paris, France) were grown in brain heart infusion broth (bioMérieux), lysogeny broth (BD Bacto) and tryptic soy broth (bioMérieux), respectively. *Saccharomyces cerevisiae* was obtained directly by dissolving 82 mg of baker's yeast (Lesaffre, France) in 25 mL of PBS (Gibco). The Mix3* asymmetric mixture of microorganisms (5.8×10^8 cells of *B. subtilis*, 3.5×10^7 cells of *E. coli* and 1.0×10^6 cells of *S. cerevisiae*) was prepared from a culture of *B. subtilis* (1.1×10^9 cells.mL^{-1}), *E. coli* (1.0×10^9 cells.mL^{-1}), and *S. cerevisiae* (3.0×10^7 cells.mL^{-1}) at an OD_{600nm} equal to 1.0. After mixing, cells were collected by centrifugation at 8,000 × g for 5 min and stored at -20°C until use. Dental microorganisms were collected from three healthy volunteers using a toothpick. The material from the three toothpicks was solubilized in 1.2 mL of PBS and, after thorough mixing, 10 µL of this solution was diluted into 1 mL of PBS. A volume of 100 µL was sprayed on lysogeny broth (1× and 0.1×), brain heart infusion broth (1× and 0.1×) and Columbia medium supplemented with 5% sheep blood (both from bioMérieux) agar plates and incubated overnight at 30°C.

Cell lysis

Protein extraction was performed according to Hayoun et al. 2019. Briefly, 60 µL of 1× lithium dodecyl sulfate solution (Thermo Scientific) supplemented with 5% β-mercaptoethanol (v/v) was added per 1 mg of cell pellet. The sample was then incubated for 5 min at 99°C in a thermomixer (Eppendorf) and sonicated for 5 min in an ultrasonic water bath (VWR ultrasonic cleaner USC 300 T) working at 45 kHz and 200 W. Microorganisms were disrupted by bead beating in 2-mL screw-cap microtubes (Sarstedt) containing an equal amount (66.7 mg) of 0.1 mm silica beads, 0.1 mm glass beads and 0.5 mm glass beads as previously described [23]. Disruption was performed using a Precellys Evolution instrument (Bertin Technologies) operated at 10,000 rpm for 10 cycles of 30 s, with 30 s of pause between each cycle. Beads were removed by centrifugation at 16,000 × g for 1 min and the supernatant was transferred to a new microcentrifuge tube and then incubated at 99°C for 5 min.

SP3 proteolysis

A total of 500 µg of hydrophilic (ref. n°24152105050250) and hydrophobic (ref. n°44152105050250) SP3 commercial solutions (Sigma-Aldrich) at 10 mg/mL were mixed and suspended in 100 µL of Milli-Q water. Beads were retained using a nickel-plated neodymium magnet (N42 grade, Supermagnete, Webcraft, GmbH; reference Q-40-20-10-N), rinsed twice with 200 µL of Milli-Q water and then resuspended in 100 µL of Milli-Q water to give a 10 µg/µL stock solution, which was stored at 4°C until use as recommended [21]. For SP3 proteolysis, 40 µg of beads (4 µL) were added to 20 µL of cell lysate, followed by

acidification with 12 µL formic acid and activation of beads with 204 µL CH_3CN (85% final concentration). Proteins were then trapped using either MagnaBind (Thermo Scientific) or Smart2 MBS (Tecan) neodymium magnetic racks, or hand-held N42 magnet (Supermagnete). After removal of supernatant, proteins were washed twice with 200 µL of 70% ethanol and once with 180 µL CH_3CN. These steps for purifying proteins were performed at room temperature. A volume of 10 µL of digestion buffer comprising 1 µg/µL of Trypsin Gold (Promega) in 50 mM NH_4HCO_3 and supplemented with 0.01% of ProteaseMAX surfactant (Promega) was added to the beads. Purified proteins were digested for 15 min at 50°C, beads were then trapped, and the recovered digests were acidified with 1 µL of trifluoroacetic acid (TFA, 0.5% final concentration).

Liquid chromatography and tandem mass spectrometry

Peptides were identified either with a Q-Exactive HF (Thermo Scientific) or an LTQ-Orbitrap XL (Thermo Scientific) tandem mass spectrometer coupled to an ultimate 3000 nano LC system (Thermo Scientific), operated as described [32, 33]. Peptides (30 or 300 ng) were desalted on a reverse-phase PepMap 100 C18 µ-precolumn (5 mm, 100 Å, 300 mm i.d. × 5 mm, Thermo Scientific) before peptide separation on a nanoscale PepMap 100 C18 nanoLC column (3 mm, 100 Å, 75 mm i.d. × 50 cm, Thermo Scientific) at a flow rate of 0.3 $\mu L.min^{-1}$ using a 60 min gradient (2.5% B from 0 to 3 min, 2.5–25% B from 3 to 53 min and 25–40% B from 53 to 63 min) of mobile phase A (0.1% HCOOH/100% H_2O) and phase B (0.1% HCOOH/80% CH_3CN). The Q-Exactive HF mass spectrometer was operated in data-dependent acquisition mode with a Top20 strategy corresponding to the selection of the 20 most abundant precursor ions for serial fragmentation. Full-scan mass spectra were acquired from 350 to 1,800 *m/z*. Only peptides with 2 or 3 positive charges were selected for

fragmentation with a dynamic exclusion time of 10 sec and an isolation window of 1.6 *m/z*. The LTQ Orbitrap XL was operated with the same parameters but with a Top5 strategy corresponding to the selection of the 5 most abundant precursor ions, an isolation windows of 1 *m/z,* and a linear 60 min gradient (5% B from 0 to 3 min and 5–50% B from 3 to 63 min).

High-throughput proteotyping of microorganisms

Cross-contamination tests and microorganism screening were performed using a double-barrel LC-MS/MS system, based on the same principle as that described by Hosp et al. [34]. This parallel UHPLC system operating with two analytical columns reduces the idling time of the mass spectrometer during loading of the peptides to the reverse-phase column. Two pairs of PepMap 100 C18 µ-precolumns and PepMap 100 C18 nanoLC columns were mounted in tandem on the same chromatography platform coupled to the LTQ-Orbitrap XL tandem mass spectrometer, allowing elution of peptides from one column in parallel to washing and reconditioning of the other. In this case, peptides (30 ng) were desalted and resolved with a 27-min gradient consisting of 5–40% B from 3 to 30 min, after a 3-min equilibration at 5% B.

Data interpretation

For the Mix3* digests, MS/MS spectra were assigned to peptide sequences using a database comprising the polypeptide sequences from the annotated genomes of *B. subtilis* subsp. spizizenii ATCC6633, *E. coli* BL21-Gold (DE3) pLysS AG and *S. cerevisiae* S288C strains. The database contains 43,774 polypeptide sequences and 15,766,031 amino acid residues. The interpretation was performed using Mascot Daemon software version 2.6.1 (Matrix Science) with the following parameters: 5 ppm peptide tolerance, 0.02 Da MS/MS fragment tolerance, 2+ or 3+ peptide charges, a maximum of two missed cleavages, carbamidomethylation of cysteine as fixed modification, oxidation of methionine as variable

modification and trypsin as proteolytic enzyme. Microorganism proteotyping was performed by assigning MS/MS spectra against the NCBInr database (downloaded on 01/03/2018) comprising 108,307,546 protein sequences, using Mascot with the same parameters as above except that only one missed cleavage was allowed, and a p-value below 0.05 in homology threshold mode was set for peptide validation. The phylopeptidomics approach for identifying the microorganisms and quantifying their respective biomass contributions was performed as described [14]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017720.

MALDI-TOF mass spectrometry proteotyping

Material (<5 mg) from recent bacterial colonies (< 48h incubation) was transferred into a 1.5 mL tube and solubilized using 300 µL of Milli-Q water, and then with 900 µL of absolute ethanol. Cells were pelleted by centrifugation at 16,000 × g for 2 min. Proteins were extracted by resuspending the cellular pellets in 50 µL of 70 % formic acid, followed by addition of 50 µL of CH_3CN, and vortexing. Samples were centrifuged as described here-above. For each sample, one µL of protein supernatant was spotted onto a MTP384 ground steel MALDI plate (Bruker Daltonics), dried, overlaid with 1.5 µL of α-cyno-4-hydroxy-cinnamic acid matrix (saturated α-HCCA matrix was prepared in 50 % acetonitrile / 2.5 % TFA), and dried. This was done twice for analytical duplicates. MS spectra were recorded using an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 200-Hz Smartbeam laser. Spectra were recorded in the linear, positive mode from 2,000 to 20,000 *m/z*, cumulating 500-1,000 shots from different positions of the target spot. The Ion source 1, Ion source 2, Lens and the extraction delay time were set at 20.07 kV, 18.60 kV,

7.50 kV, and 200 ns, respectively. Each spectrum was the merged signal from 500-1,000 shots from different positions of the target spot. Calibration was performed externally using the Bruker Bacterial Test Standard (*Escherichia coli* protein extract including the additional proteins RNase A and myoglobin). Spectra were recorded with the flexAnalysis software (v3.4, Bruker Daltonics) and interpreted with the Biotyper software (v3.1, Bruker Daltonics) and the biotyper spectral database v7 which contains spectra profiles for 7,311 reference microorganisms. The proteotyping confidence is based on a score analysis as recommended by the manufacturer: i) a score > 2.3 was considered highly probable species identification, ii) a score between 2.0 and 2.3 was considered a secure genus identification and probable species identification, iii) a score between 1.7 and 1.999 was considered as a probable genus identification, iv) a score < 1.7 was considered as not a reliable identification.

Results

Performance of Smart2 MBS and MagnaBind for 96-well plate proteotyping

SP3 magnetic-bead proteolysis of proteins requires low volumes of reagents and the resulting digests are ready for direct LC-MS/MS analysis [23]. It is important, however, to ensure that the final digests do not contain any magnetic beads that could disturb peptide injection or liquid chromatography, particularly for the 96-well plate configuration. We evaluated the performance of two commercial neodymium magnetic racks in terms of bead trapping: the Smart2 MBS (Tecan) and the MagnaBind (Thermo Scientific). The former rack has 108 cylindrical magnets arranged above and below each well, with direct contact between the bottom of each well and the magnet. The latter has 24 large magnets, placed in-between four wells. In this case, the magnets are covered by a plastic plate of 0.5 mm thickness, which

increases slightly the distance between the magnets and the beads as compared with the Smart2 MBS magnetic rack. The magnetic field for both devices is not given by the suppliers. The operational performances of the two magnetic racks were evaluated by processing the *E. coli* lysate. **Figure 1** shows how beads are retained at the bottom of each well and whether some beads could be present in the final peptide samples. As shown in **Figure 1 (panel A)**, the two magnetic racks have a different distribution of beads during magnetic retention. The Smart2 MBS magnet traps the SP3 beads in a small spot at the very bottom of the well, whereas the MagnaBind rack beads are concentrated on the wall of the tube but not at the bottom. With regards to the MagnaBind magnet, we observed a partial resuspension of some beads when removing the liquid during the different washing steps, which tended to diminish the amount of trapped material. Also, some beads were recovered in the final peptide sample. The attraction of the beads on the wall of the tube rather than at the bottom also made a difference when removing the fluid. In the former case, the fluid may scrub the beads upon aspiration, while this may be attenuated when the beads are at the bottom of the well. The presence of beads in the final digests was inspected by optical microscopy. **Figure 1B** shows the absence of magnetic beads in digests obtained using the Smart2 MBS rack, whereas some beads were observed in the digests using with the MagnaBind rack. Therefore, it is advisable that the use of MagnaBind magnet rack for proteolysis should be followed by a supplementary clean-up step before LC-MS/MS analysis of peptide samples, as beads may damage the chromatography system. This additional cleanup consists of introducing each digest into a new plate and, after magnet retention, re-aspirating the peptide samples. We measured the average peptide digest volume obtained with the two 96-well plate protein digestion formats. Both the Smart2 MBS and MagnaBind racks showed some sample losses, as only 8.02 µL (±4%) and 7.26 µL (±8%) were recovered, respectively, from the initial 10 µL enzymatic solution added. This volume loss can be explained by bead rehydration and

evaporation during the enzymatic digestion step. Based on these results, the Smart2 MBS magnet rack is recommended if sensitivity and simplicity are required. Accordingly, the next steps of proteotyping validation described were performed with the Smart2 MBS magnet rack.

A 96-well SP3 approach for high-throughput analysis performs equally well as in-tube format

We compared the proteotyping efficiency of the SP3 sample preparation in a 96-well format with an in-tube SP3 digestion using the Mix3* asymmetric mixture of microorganisms containing 94.2% of *B. subtilis*, 5.7% of *E. coli* and 0.2% of *S. cerevisiae* cells. A total of 37,137 (±4%) MS/MS spectra were recorded for the in-tube SP3 with a starting material of 0.34 mg of cellular pellet (wet weight). Most of these spectra (20,567, ±4%) could be attributed to peptide sequences, leading to the identification of 13,311 (±4%) peptides and 1,873 (±1%) proteins validated with at least two different peptide sequences. The peptide-spectrum matches (PSMs) found for each sample and the corresponding proteins are listed in **Supplementary Tables S1** and **S2**, respectively. The same proteolysis protocol performed in the 96-well plate format with the Smart2 MBS magnet had no significant impact on digestion efficiency and reproducibility, as it yielded 19,275 (±6%) PSMs, 12,436 (±4%) peptides and 1,715 (±2%) proteins (see **Supplementary Tables S3** and **S4** for the list of PSMs and proteins, respectively). Likewise, the same ratios of missed tryptic cleavage were found between the in-tube and 96-well platforms, with 79% of peptides without miss-cleavage, 19% with a single miss-cleavage and 2% with two miss-cleavages whatever the method. The detected peptides and identified proteins from the two conditions had similar physicochemical properties. These two conditions share most of their peptidome, with only 16.2 % and 8.7 % of unique peptides specific for in-tube and 96-wells proteolysis, respectively. **Figure 2** shows

a Venn diagram comparing the number of proteins validated with at least 2 peptides in both digestion conditions. When considering all the proteins identified in the 3 replicates, only 12.2 % and 4.2 % were specific to in-tube and 96-wells SP3, respectively. A total of 183 proteins among the 250 exclusively identified in the in-tube condition are also identified but with only a unique peptide in the 96-wells SP3 sample. Similarly, 70 of the 87 specific proteins presents in the 96-wells dataset are also found in the in-tube results with a unique peptide. As expected, experimental and analytical variability observed at the peptide level influenced the validation of a few number of proteins. However, these results demonstrate that such variability does not significantly change the confidence of the proteotyping analysis. Also, their proteotyping values were equivalent, as the number of peptides assigned to each microorganisms were comparable between the 96-well format (**Table S3**) and the in-tube format (**Table S1**): 665 and 791 for *S. cerevisiae*, 3671 and 4157 for *E. coli*, and 11542 and 12348 for *B. subtilis*.

Relative quantities of microorganisms in the Mix3* are conserved

A modified preparation protocol can result in alterations in accuracy or precision in the relative quantification of mixtures of organisms. This can be problematic in studies aiming to monitor simple or complex mixtures of microorganisms in different conditions or over time, for example, clinical, biomimetic, biotechnological or bioremediation microbiota setups. We thus questioned whether the relative quantification of microorganisms was comparable between in-tube- and in-plate-based SP3 protocols. The Mix3* sample is an excellent substrate for estimating alterations in relative quantity as the 3 different microorganisms are strongly heterogeneous in terms of cell wall and membrane structure: gram-positive bacteria, gram-negative bacteria and yeast. The relative quantities of the three organisms were assessed

by phylopeptidomics [14] in triplicate and are reported in **Table 1.** Results showed that the biomass ratio for the three organisms was comparable between in-tube and in-plate SP3 protocols and corresponded relatively well to the protein quantities that can be extracted from the cells.

Range of use of the SP3 96-well plate format for proteotyping

In routine diagnostics, proteotyping of microorganisms can be performed on samples containing various biomass quantities. Therefore, we investigated the application range of the SP3 96-well plate proteotyping method by testing different quantities of *E. coli* cellular lysate. The lysate from 1.7×10^9 *E. coli* cells corresponding to 300 µg of protein was diluted to give an equivalent of 5.7×10^4 cells (0.01 µg of protein) for the lowest level. **Figure 3** shows the bead retention achieved by the Smart2 MBS magnetic rack after the digestion step. We noted that the protein concentration has a direct effect on bead attraction efficiency. The presence of a large amount of protein enhances bead aggregation (for example, compare well A6 to well A3 in **Figure 3**). By contrast, when the protein quantity was excessive (above 100 µg), a loss of proteins during the washing steps was observed. The resulting peptides for each sample were analyzed by LC-MS/MS and the species present in the sample was identified by MS/MS proteotyping. The number of PSMs assigned to *E. coli* and the number of taxon-specific peptides are listed in **Supplementary Table S5**. As shown in **Figure 3**, an adequate number of species-specific peptides were obtained in all the quantities tested, except for the quantity of 10 ng of protein, equivalent to 5.7×10^4 cells. We also noted low values for species-specific peptides at 2.8×10^5 and 1.7×10^9 *E. coli* cells, with only two species-specific peptides in most replicates. In the latter case, the presence of an excess of proteins disturbed the retention of beads during the process and completion of digestion, as illustrated by the

strong decrease of MS/MS spectra (35%) and PSMs (56%). The SP3 96-well plate format is thus adaptable for proteotyping microorganisms in a broad range of material quantity from 2.8 $\times 10^5$ to 1.7 $\times 10^9$ cells.

No cross-contamination occurs for proteotyping in a 96-well plate format

The 96-well plate SP3 format is an attractive solution for high-throughput proteotyping. However, cross-contamination of unwanted material between wells, due to the addition/removal of reagents or mixing steps, could be disadvantageous as compared with an in-tube protocol where the use of separate tubes limits inter-sample contamination. The possibility of cross-contamination was tested by parallel microplate digestion of two model bacteria, *A. baumanii* and *E. coli*, which are phylogenetically distant and thus easily distinguishable. In this case, one species was introduced into a well and the other species was then introduced in all surrounding wells (**Figure 4**). The reciprocal experiment was also set-up and both experiments were performed twice. After plate processing, the resulting 36 digests were subjected to nanoLC-MS/MS and proteotyping identification. The detailed proteotyping data are detailed in **Supplementary Table S6**. The 36 samples were accurately identified at the species level by 1,026 (±3%) PSMs and 10 (±13%) assigned specific peptides for *A. baumanii*, and 1,273 (±2%) PSMs and 2 (±20%) assigned specific peptides for *E. coli*. The presence of *E. coli* in *A. baumanii* samples, or the reverse, was not detected through PSMs or specific peptides. These results demonstrate that sample homogenization, liquid disposal, reagent removal and incubation steps during the protocol do not have any impact on the purity of samples. Thus, the method can be easily applied to a microplate platform without any risk of cross-contamination.

Application of the 96-well plate format for screening isolates by tandem mass-spectrometry proteotyping

To assess the real-life performance of the 96-well plate SP3 format for proteotyping clinically-relevant microorganisms, we sampled dental plaque and plated microbial isolates on agar. A total of 48 isolates were randomly selected and treated in duplicate with the 96-well plate SP3 protocol to produce peptides. The 96 peptide pools were then analyzed by double-barrel chromatography-tandem mass spectrometry to establish whether the samples corresponded to a pure isolate or to a mixture of microorganisms, and to identify them at the most relevant taxonomical level. The proteotyping results with the confident taxonomical level reached for the 96 samples are shown in **Figure 5** and the corresponding number of MS/MS spectra, PSMs and specific peptides are listed in **Supplementary Table S7**. Considering all the samples, a mean of 3,037 (±15%) MS/MS spectra were recorded for each sample and 1,184 (±22%) peptide sequences were identified. Notably, the same identification result was obtained for each duplicate, demonstrating the good reproducibility of the sample preparation method for a large number of samples. As expected, different taxonomical levels could be ascertained depending on the sample. The 48 isolates were assigned at the species level, with a significant number of species-specific peptides or assigned PSMs. As shown in **Figure 5**, the identified microorganisms were quite diverse, with *Stenotrophomonas maltophilia* (8 isolates), *Pseudomonas aeruginosa* (8 isolates), *Staphylococcus aureus* (5 isolates), *Serratia marcescens* (4 isolates) and *Lactococcus lactis* (3 isolates) being the most represented. Several isolates belonging to various species of *Rothia* (4 isolates), *Neisseria* (6 isolates) and *Staphylococcus* (8 isolates) were also identified. For example, *Rothia dentocariosa*, *Rothia aeria*, and *Rothia* sp. HMSC067H10 (*Rothia dentocariosa*-like) were classified. Among the 48 isolates, five corresponded to mixtures of two microorganisms: *L. lactis* and *Staphylococcus warneri* (sample 2), *S. marcescens* and *Cronobacter dublinensis*

(sample 16), *S. marcescens* and *S. maltophilia* (sample 17), *Massilia* sp. Leaf139 (*Massilia timonae*-like) and *Curtobacterium flaccumfaciens* (sample 25) and *P. aeruginosa* and *Microbacterium* sp. Ag1 (sample 30). The tandem mass spectrometry identification thus highlighted the presence of mixtures of bacteria and could precisely identify the most abundant species. *S. marcescens* was the predominant bacterium of sample 16 analyzed in positions G4 and H4 with the presence of a low amount of *C. dublinensis* (8% of the total assigned PSMs), and of sample 17 in positions A5 and B5 with some *S. maltophilia* (21% of the total signal). Sample 30 corresponded mainly to *P. aeruginosa* with a small contamination of *Microbacterium* sp. Ag1 (3% of the assigned PSMs). Overall, the optimized approach combining the 96-well plate SP3 preparation, tandem mass spectrometry and phylopeptidomics led to the characterization of 96 samples in 55 hours. Twenty-four of the described samples were analyzed by MALDI-TOF mass spectrometry, after protein extraction in order to maximize the identification (**Supplementary Table S8**). Only 3 samples were identified with highly probable species score, 7 with a probable species score, 9 with a probable genus score, and 5 could not be identified. The MALDI-TOF results of 10 samples are concordant with MS/MS proteotyping, among which only two bacteria with a highly probable species identification and four with secure genus identification and probable species identification.

Discussion

SP3 paramagnetic beads offer fast purification and proteolysis of proteins [21, 22]. In the present study, we assessed their application for microorganism identification by tandem mass spectrometry proteotyping after optimization, and we found them to be more efficient and quicker than other sample preparation methods [23]. The 96-well plate format allows simultaneous treatment of many samples, resulting in significant time saving. The results

indicate a low variability of in-tube and 96-wells SP3 proteolysis, with 83.6 % of shared proteins. An important part of the specific proteins are detected in both conditions but not validated with at least 2 peptides. This rather low experimental variability can be explained by small differences between in-tube and 96-wells procedures in terms of contact area liquid volume recovered after digestion. Furthermore, this format opens the door for fully automatized sample preparation, which is important in the framework of clinical diagnostics of highly virulent pathogens that require minimal handling by operators. We show here that the SP3 96-well plate format is adaptable for proteotyping microorganisms over a broad range of cellular material, from 10^5 to 10^9 cells. Such quantities are easily obtainable by picking a colony from an agar plate or by centrifuging a few milliliters of liquid culture. We did, however, note some difficulties in protein purification for larger quantities of material. Thus, the operator should not overload the wells. A recent publication by Muller et al. [31] presents an implementation of SP3 using a liquid handling Bravo system (Agilent Technologies) for a fast and automated 96-well format sample preparation for performing proteomics. In terms of protein concentration, our method is conform to the reported observations with capabilities to work with less than 100 ng of proteins. The *E. coli* cells used in our study are much smaller than HeLa cells. The volume of the former is roughly 1000 smaller than the later. Therefore, for the same input material, a larger number of cells is required: approximately 10^5 *E. coli* cells are equivalent in terms of proteins to 100 HeLa cells. In addition to the interesting conclusions presented by Muller et al. [31], our results demonstrates that excess sample may have an impact on the procedure and that the conformation of magnets on the rack may lead to a decrease of protein recovery. As demonstrated here, tandem mass proteotyping with sample preparation performed in 96-well plates does not seem to carry a risk of cross-contamination of samples under standard conditions. Nevertheless, for quality control, we would recommend using specific wells on the 96-well plate for systematic controls with

known strains and calibrated bacterial loads. This would allow the assessment of i) correct sample processing and ii) the absence of cross-contamination in each plate. The easy handling and low steps of the 96-well SP3 protocol presented in our publication is adapted for automated system, as already described in the literature. The Smart2 MBS magnetic rack selected for this efficient beads retention is part of a workstation (Tecan) and can be used for automation without any need of protocol adaptation.

Whole-cell MALDI-TOF mass spectrometry has been successfully implemented in clinical laboratories because sample preparation is simple and fast, it does not require heavy cell loads, and there is limited operator-contact with pathogens. The tandem mass proteotyping pipeline presented here, and more specifically the sample preparation in the 96-well format, holds the same qualities. While whole-cell MALDI-TOF mass spectrometry has quickly become a standard approach for identifying human pathogens because of its simplicity to implement and low cost [35, 36], some limitations have been highlighted [1]. In particular, several microorganisms are not well identified at the species level; for example, *Streptococcus pneumoniae* and *Streptococcus mitis* cannot be easily discriminated with this methodology [37]. Tandem mass spectrometry proteotyping is based on many more experimental features than whole-cell MALDI-TOF mass spectrometry: thousands of peptide masses recorded with high accuracy (error below 5 ppm) *versus* less than one hundred protein molecular weights with low accuracy (error above 400 ppm), respectively. Thus, the former approach is more discriminant than the latter as confirmed with the comparison carried out in the present study. The MS/MS spectra were recorded using a mass tolerant precursor of 5 ppm, a mass fragment ion of 0,02 Da and an isolation window of 1.6 m/z. These values recommended by Thermo Scientific for measuring MS/MS spectra should be used for interpreting MS/MS proteotyping results. Because of the extra-large database used, appropriate control of the FDR is key for avoiding false-positive identification of

microorganisms. Noteworthy, the additional signals recorded by MS/MS could increase the sensitivity of strategies related to high throughput dereplication of isolates [38] and could improve discrimination of strains at taxonomic ranks below the species level [39]. Therefore, tandem mass spectrometry proteotyping could be used for systematically characterizing clinical isolates as a performant first-line epidemiological tool. The 96-well plate format developed here is perfectly adapted for such an objective.

The lack of reference spectra for most environmental bacteria and uncultivable microorganisms is another drawback of whole-cell MALDI-TOF mass spectrometry [1]. As shown here for dental isolates, tandem mass spectrometry proteotyping is applicable to any microorganism and, based on the generalist database, can be used to ascertain the phylogenetic lineage of the isolate. Whole-cell MALDI-TOF mass spectrometry is not reliable for characterizing samples containing multiple microorganisms [10]. Here, we confirmed that tandem mass spectrometry proteotyping can be applied to samples containing several microorganisms; indeed, complex mixtures can be characterized in terms of taxa present, but also in terms of biomass contributions [14, 40].

In conclusion, the application of an SP3 approach in a 96-well plate format maintains the performance of our previously developed SP3 in-tube method with the added advantages of simpler and quicker processing of many more samples. The illustrative examples presented here demonstrate the potential of combining SP3 sample preparation and phylopeptidomics analysis for rapid and accurate screening of microorganisms, either as isolates or as mixed samples, including uncharacterized strains. We believe this new workflow will become a powerful strategy for high throughput proteotyping of microorganisms from clinical or environmental samples.

References

[1] L. Grenga, O. Pible, J. Armengaud, Pathogen proteotyping: a rapidly developing application of mass spectrometry to address clinical concerns, Clinical Mass Spectrometry 14 (2019) 9-17.

[2] J.P. Lavigne, P. Espinal, C. Dunyach-Remy, N. Messad, A. Pantel, A. Sotto, Mass spectrometry: a revolution in clinical microbiology?, Clin Chem Lab Med 51(2) (2013) 257-70.

[3] A. Karger, Current developments to use linear MALDI-TOF spectra for the identification and typing of bacteria and the characterization of other cells/organisms related to infectious diseases, Proteomics Clin Appl 10(9-10) (2016) 982-993.

[4] E. Durighello, L. Bellanger, E. Ezan, J. Armengaud, Proteogenomic biomarkers for identification of Francisella species and subspecies by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry, Anal Chem 86(19) (2014) 9394-8.

[5] M.F. Emele, S.S. Mozina, R. Lugert, W. Bohne, W.O. Masanta, T. Riedel, U. Gross, O. Bader, A.E. Zautner, Proteotyping as alternate typing method to differentiate Campylobacter coli clades, Sci Rep 9(1) (2019) 4244.

[6] C.G. Carvalhaes, A.C. Ramos, L.C.G. Oliveira, M.A. Juliano, A.C. Gales, Rapid detection of ceftazidime/avibactam resistance by MALDI-TOF MS, J Antimicrob Chemother 73(9) (2018) 2579-2582.

[7] R. Patel, A Moldy Application of MALDI: MALDI-ToF Mass Spectrometry for Fungal Identification, J Fungi (Basel) 5(1) (2019).

[8] R. Karlsson, L. Gonzales-Siles, F. Boulund, L. Svensson-Stadler, S. Skovbjerg, A. Karlsson, M. Davidson, S. Hulth, E. Kristiansson, E.R. Moore, Proteotyping: Proteomic characterization, classification and identification of microorganisms--A prospectus, Syst Appl Microbiol 38(4) (2015) 246-57.

[9] F. Boulund, R. Karlsson, L. Gonzales-Siles, A. Johnning, N. Karami, O. Al-Bayati, C. Ahren, E.R.B. Moore, E. Kristiansson, Typing and Characterization of Bacteria Using Bottom-up Tandem Mass Spectrometry Proteomics, Mol Cell Proteomics 16(6) (2017) 1052-1063.

[10] R. Karlsson, L. Gonzales-Siles, M. Gomila, A. Busquets, F. Salva-Serra, D. Jaen-Luchoro, H.E. Jakobsson, A. Karlsson, F. Boulund, E. Kristiansson, E.R.B. Moore, Proteotyping bacteria: Characterization, differentiation and identification of pneumococcus

and other species within the Mitis Group of the genus Streptococcus by tandem mass spectrometry proteomics, PLoS One 13(12) (2018) e0208804.
[11] C. Mappa, O. Pible, J. Armengaud, B. Alpha-Bazin, Assessing the ratio of Bacillus spores and vegetative cells by shotgun proteomics, Environ Sci Pollut Res Int (2018).
[12] E.M. Berendsen, E. Levin, R. Braakman, A. Prodan, H.C. van Leeuwen, A. Paauw, Untargeted accurate identification of highly pathogenic bacteria directly from blood culture flasks, Int J Med Microbiol 310(1) (2020) 151376.
[13] M. Abbondio, A. Palomba, A. Tanca, C. Fraumene, D. Pagnozzi, M. Serra, F. Marongiu, E. Laconi, S. Uzzau, Fecal Metaproteomic Analysis Reveals Unique Changes of the Gut Microbiome Functions After Consumption of Sourdough Carasau Bread, Front Microbiol 10 (2019) 1733.
[14] O. Pible, F. Allain, V. Jouffret, K. Culotta, G. Miotello, J. Armengaud, Estimating relative biomasses of organisms in microbiota using “phylopeptidomics”. Microbiome 8 (2020) 30.
[15] C.Y. Chen, C.G. Clark, S. Langner, D.A. Boyd, A. Bharat, S.J. McCorrister, A.G. McArthur, M.R. Graham, G.R. Westmacott, G. Van Domselaar, Detection of Antimicrobial Resistance Using Proteomics and the Comprehensive Antibiotic Resistance Database: a Case Study, Proteomics Clin Appl (2019) e1800182.
[16] J. Armengaud, Next-generation proteomics faces new challenges in environmental biotechnology, Curr Opin Biotechnol 38 (2016) 174-82.
[17] E.M. Hartmann, F. Allain, J.C. Gaillard, O. Pible, J. Armengaud, Taking the shortcut for high-throughput shotgun proteomic analysis of bacteria, Methods Mol Biol 1197 (2014) 275-85.
[18] K.R. Ludwig, M.M. Schroll, A.B. Hummon, Comparison of In-Solution, FASP, and S-Trap Based Digestion Methods for Bottom-Up Proteomic Studies, J Proteome Res 17(7) (2018) 2480-2490.
[19] J.R. Wisniewski, Filter Aided Sample Preparation - A tutorial, Anal Chim Acta 1090 (2019) 23-30.
[20] A. Zougman, P.J. Selby, R.E. Banks, Suspension trapping (STrap) sample preparation method for bottom-up proteomics analysis, Proteomics 14(9) (2014) 1006-0.
[21] C.S. Hughes, S. Foehr, D.A. Garfield, E.E. Furlong, L.M. Steinmetz, J. Krijgsveld, Ultrasensitive proteome analysis using paramagnetic bead technology, Mol Syst Biol 10 (2014) 757.

[22] C.S. Hughes, S. Moggridge, T. Muller, P.H. Sorensen, G.B. Morin, J. Krijgsveld, Single-pot, solid-phase-enhanced sample preparation for proteomics experiments, Nat Protoc 14(1) (2019) 68-85.
[23] K. Hayoun, D. Gouveia, L. Grenga, O. Pible, J. Armengaud, B. Alpha-Bazin, Evaluation of Sample Preparation Methods for Fast Proteotyping of Microorganisms by Tandem Mass Spectrometry, Front Microbiol 10 (2019) 1985.
[24] L. Dayon, A. Nunez Galindo, J. Corthesy, O. Cominetti, M. Kussmann, Comprehensive and Scalable Highly Automated MS-Based Proteomic Workflow for Clinical Biomarker Discovery in Human Plasma, J Proteome Res 13(8) (2014) 3837-3845.
[25] S.J. Humphrey, O. Karayel, D.E. James, M. Mann, High-throughput and high-sensitivity phosphoproteomics with the EasyPhos platform, Nat Protoc 13(9) (2018) 1897-1916.
[26] J. Potriquet, M. Laohaviroj, J.M. Bethony, J. Mulvenna, A modified FASP protocol for high-throughput preparation of protein samples for mass spectrometry, PLoS One 12(7) (2017) e0175967.
[27] Y. Yu, S. Bekele, R. Pieper, Quick 96FASP for high throughput quantitative proteome analysis, J Proteomics 166 (2017) 1-7.
[28] M. HaileMariam, R.V. Eguez, H. Singh, S. Bekele, G. Ameni, R. Pieper, Y. Yu, S-Trap, an Ultrafast Sample-Preparation Approach for Shotgun Proteomics, J Proteome Res 17(9) (2018) 2917-2924.
[29] L.F. Dagley, G. Infusini, R.H. Larsen, J.J. Sandow, A.I. Webb, Universal Solid-Phase Protein Preparation (USP(3)) for Bottom-up and Top-down Proteomics, J Proteome Res 18(7) (2019) 2915-2924.
[30] M.A. Gonzalez-Lozano, F. Koopmans, I. Paliukhovich, A.B. Smit, K.W. Li, A Fast and Economical Sample Preparation Protocol for Interaction Proteomics Analysis, Proteomics 19(9) (2019) e1900027.
[31] T. Müller, M. Kalxdorf, R. Longuespée, D.N. Kazdal, A. Stenzinger, J. Krijgsveld, Automated sample preparation with SP3 for low-input clinical proteomics, Mol Syst Biol 16(1) (2020) e9111.
[32] G. Klein, C. Mathe, M. Biola-Clier, S. Devineau, E. Drouineau, E. Hatem, L. Marichal, B. Alonso, J.C. Gaillard, G. Lagniel, J. Armengaud, M. Carriere, S. Chedin, Y. Boulard, S. Pin, J.P. Renault, J.C. Aude, J. Labarre, RNA-binding proteins are a major target of silica nanoparticles in cell extracts, Nanotoxicology 10(10) (2016) 1555-1564.
[33] J. Trapp, D. Gouveia, C. Almunia, O. Pible, D. Degli Esposti, J.-C. Gaillard, A. Chaumot, O. Geffard, J. Armengaud, Digging Deeper Into the Pyriproxyfen-Response of the

Amphipod Gammarus fossarum With a Next-Generation Ultra-High-Field Orbitrap Analyser: New Perspectives for Environmental Toxicoproteomics, Frontiers in Environmental Science 6(54) (2018).
[34] F. Hosp, R.A. Scheltema, H.C. Eberl, N.A. Kulak, E.C. Keilhauer, K. Mayr, M. Mann, A Double-Barrel Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) System to Quantify 96 Interactomes per Day, Mol Cell Proteomics 14(7) (2015) 2030-41.
[35] B. Rodriguez-Sanchez, E. Cercenado, A.T. Coste, G. Greub, Review of the impact of MALDI-TOF MS in public health and hospital hygiene, 2018, Euro Surveill 24(4) (2019).
[36] N. Singhal, M. Kumar, P.K. Kanaujia, J.S. Virdi, MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis, Front Microbiol 6 (2015) 791.
[37] R. Varghese, R. Jayaraman, B. Veeraraghavan, Current challenges in the accurate identification of Streptococcus pneumoniae and its serogroups/serotypes in the vaccine era, J Microbiol Methods 141 (2017) 48-54.
[38] C. Dumolin, M. Aerts, B. Verheyde, S. Schellaert, T. Vandamme, F. Van der Jeugt, E. De Canck, M. Cnockaert, A.D. Wieme, I. Cleenwerck, J. Peiren, P. Dawyndt, P. Vandamme, A. Carlier, Introducing SPeDE: High-Throughput Dereplication and Accurate Determination of Microbial Diversity from Matrix-Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry Data, mSystems 4(5) (2019).
[39] M. Sauget, B. Valot, X. Bertrand, D. Hocquet, Can MALDI-TOF Mass Spectrometry Reasonably Type Bacteria?, Trends Microbiol 25(6) (2017) 447-455.
[40] M. Kleiner, E. Thorson, C.E. Sharp, X. Dong, D. Liu, C. Li, M. Strous, Assessing species biomass contributions in microbial communities via metaproteomics, Nat Commun 8(1) (2017) 1558.

Acknowledgements

KH thanks the Région Occitanie for supporting part of his PhD fellowship. This work was funded in part by the French joint ministerial program of R&D against CBRNE threats and the ANR program “Phylopeptidomics” (grant number ANR-17-CE18-0023-01). The authors are indebted to Rosa Maria Gomila Ribas (Universitat de les Illes Balears, Serveis Cientificotècnics, Palma) for performing the MALDI-TOF measurements in a contractual

service mode and Sebastian Alberti (Universitat de les Illes Balears) for implementing an excellent technical platform.

Authors contribution statement

Karim Hayoun: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft **Jean-Charles Gaillard**: Methodology, Investigation **Olivier Pible**: Conceptualization, Formal analysis, Software, Writing-Review & Editing **Béatrice Alpha-Bazin**: Conceptualization, Validation, Investigation, Supervision, Writing-Review & Editing **Jean Armengaud**: Conceptualization, Validation, Resources, Data curation, Writing-Original Draft, Writing-Review & Editing, Supervision. All co-authors approved the final version.

Table 1. Assessment of *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevisiae* biomass ratio (Mix3*; n = 3)

Strain	Cell number ratio	Protein biomass ratio	Biomass ratio estimated by phylopeptidomics	
			In-tube SP3	96-well plate SP3
B. subtilis	94.2%	79% (±9%)	77% (±4%)	78% (±4%)
E. coli	5.7%	16% (±21%)	19% (±4%)	18% (±3%)
S. cerevisiae	0.2%	5% (±6%)	4% (±1%)	4% (±1%)

FIGURE LEGENDS

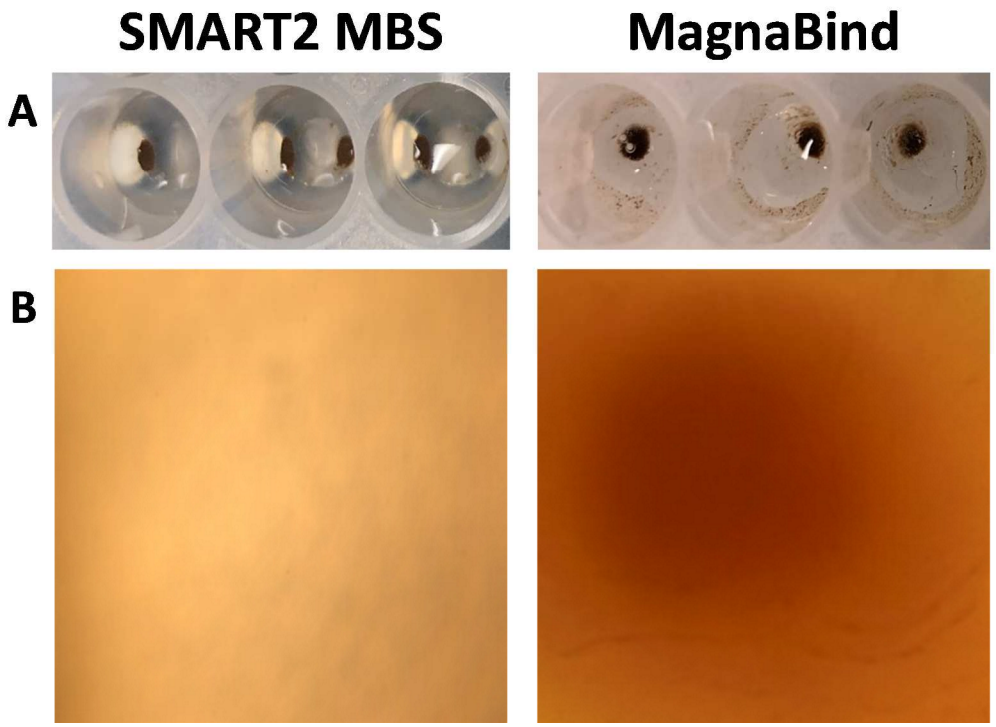
Figure 1. Comparison of SMART2 MBS (Tecan) and MagnaBind (Thermo Scientific) magnetic racks for processing SP3 proteolysis of *Escherichia coli* lysates (n = 3). (A) Photography of beads trapped with the magnet after the digestion step. **(B)** Microscopic visualization of the presence of magnetic beads in peptide digests at ×40 magnification (ZEISS Axiolab optical microscope).

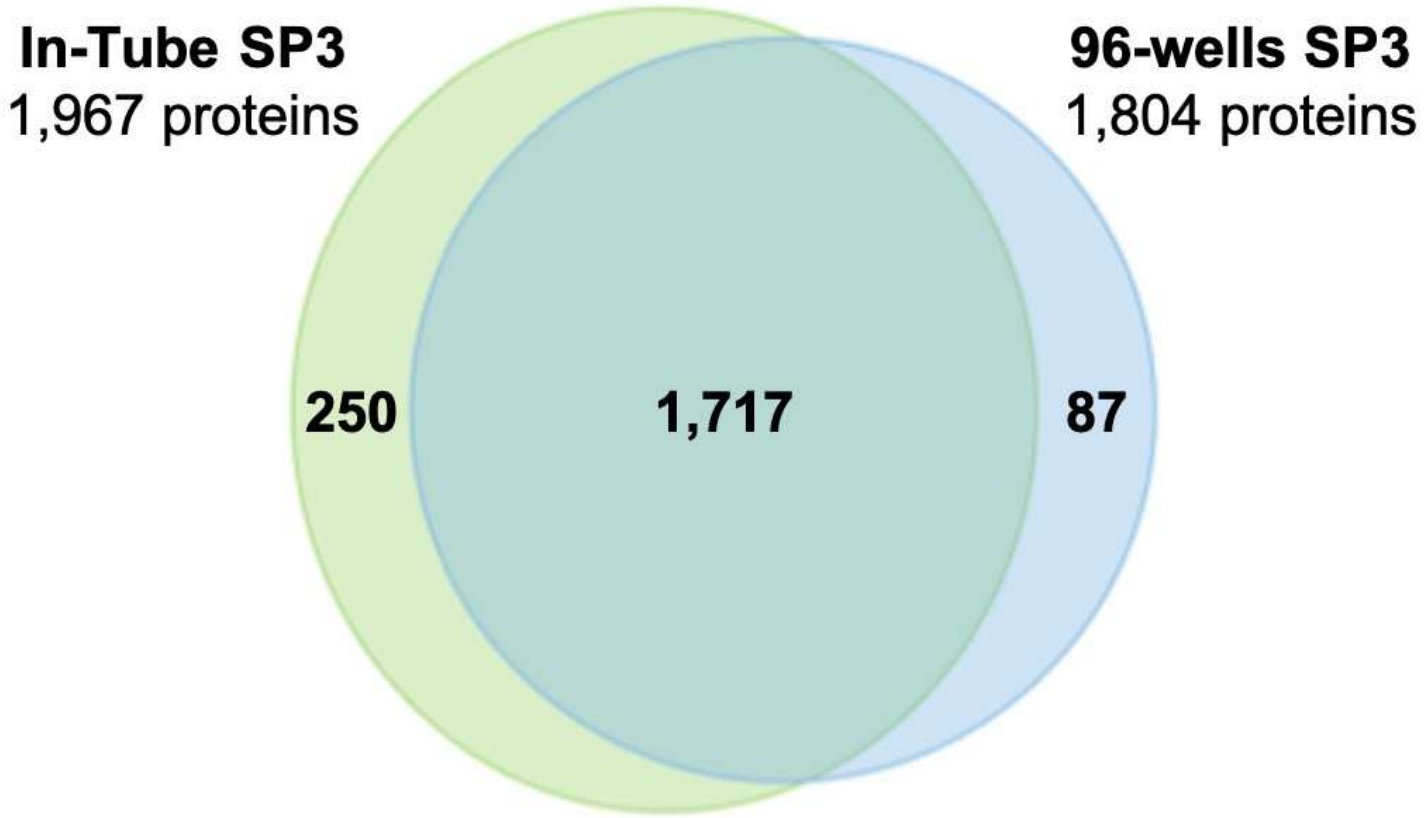
Figure 2. Venn diagram representing the number of Mix3* proteins identified in In-Tube SP3 and 96-wells SP3 proteolysis (n = 3). This diagram includes only proteins validated with at least 2 peptides.

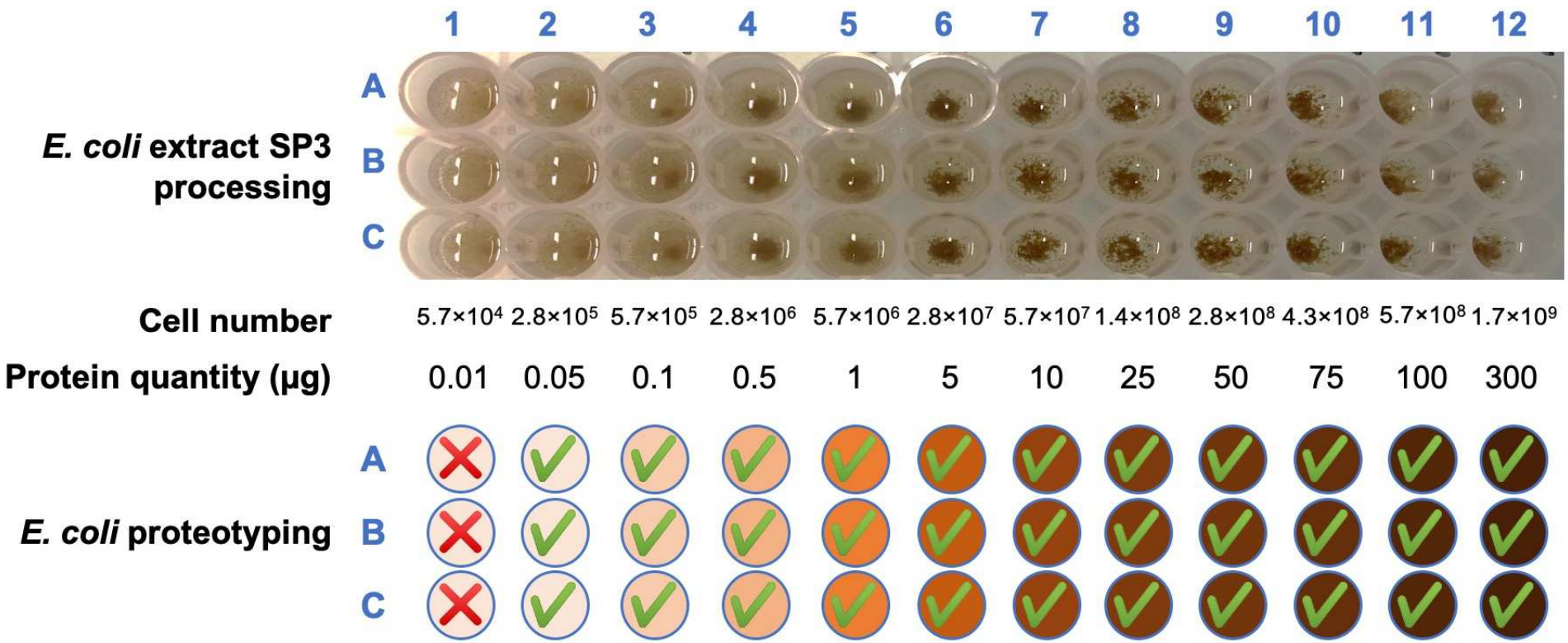
Figure 3. Microplate paramagnetic bead proteolysis and proteotyping results for various quantities of microorganisms (n = 3).

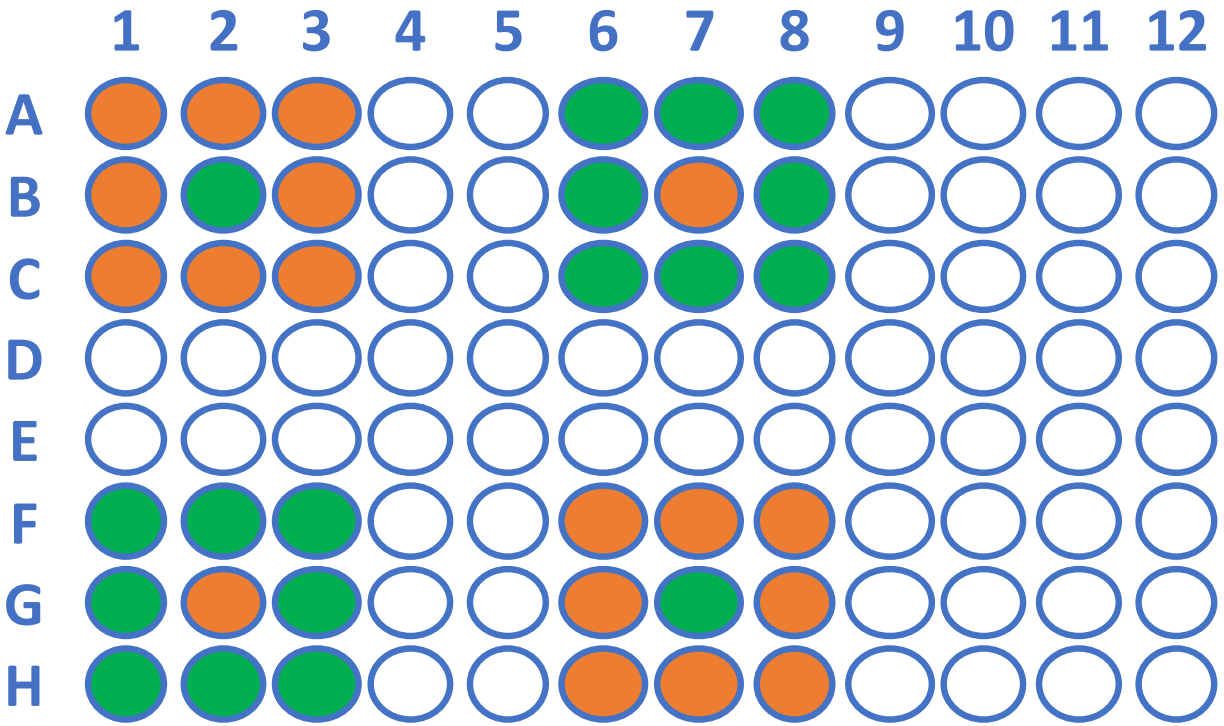
Figure 4. Schematic view of *Acinetobacter baumanii* (orange spots) and *Escherichia coli* (green spots) digests for validating the absence of cross-contamination.

Figure 5. Proteotyping results of isolates from dental plaque. The identified microorganisms are indicated on the corresponding wells from the 96-well plate and their taxonomical classes are distinguished by colors: *Gammaproteobacteria* (grey), *Betaproteobacteria* (orange), *Bacilli* (blue) and *Acinetobacteria* (green).

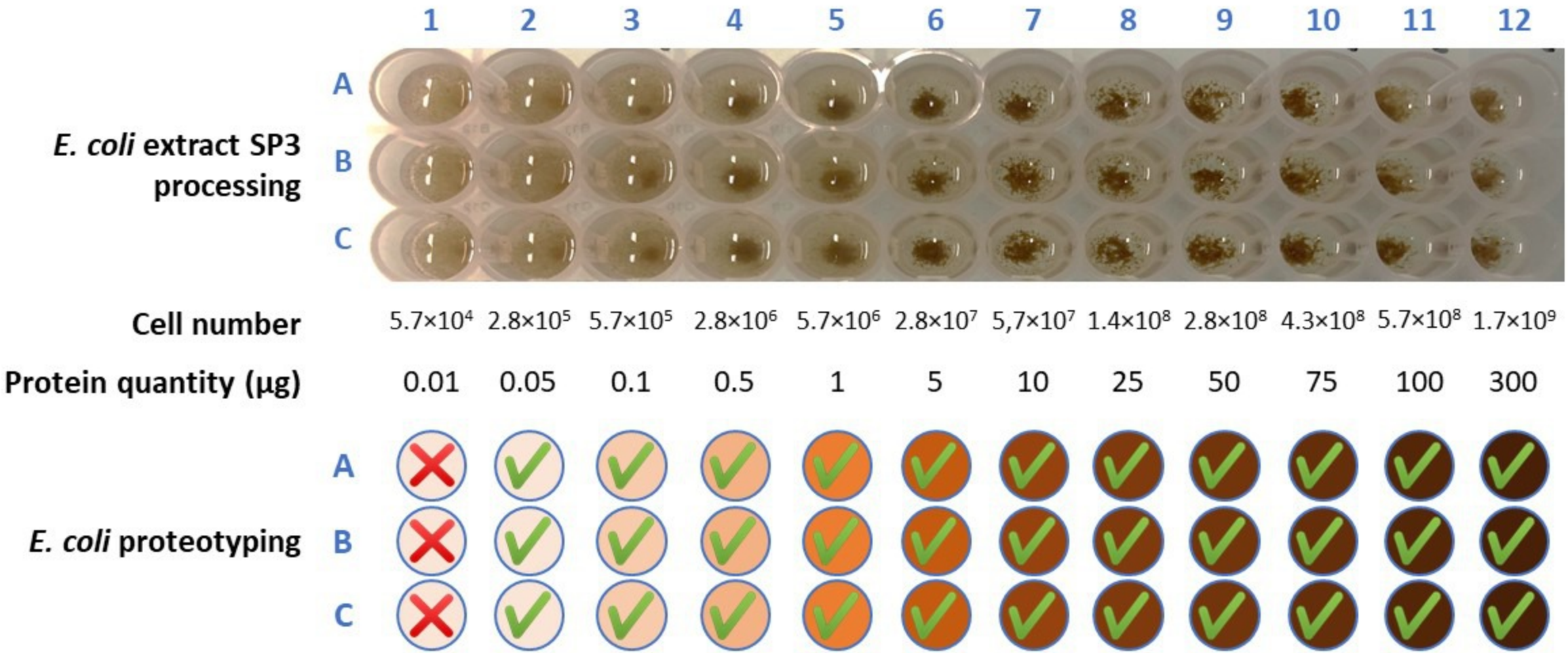

SMART2 MBS
MagnaBind
A
B


In-Tube SP3
1,967 proteins
96-wells SP3
1,804 proteins
250
1,717
87


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
E. coli extract SP3 processing
Cell number
5.7×10⁴ 2.8×10⁵ 5.7×10⁵ 2.8×10⁶ 5.7×10⁶ 2.8×10⁷ 5.7×10⁷ 1.4×10⁸ 2.8×10⁸ 4.3×10⁸ 5.7×10⁸ 1.7×10⁹
Protein quantity (µg)
0.01 0.05 0.1 0.5 1 5 10 25 50 75 100 300
A
B
C
E. coli proteotyping


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

	1	2	3	4	5	6	7	8	9	10	11	11
A	*L. lactis*	*P. aeruginosa*	*L. lactis*	*M.* sp. Ag1	*S. marcescens* *S. maltophilia*	*S. maltophilia*	*M. sp. Leaf139* *C. flaccumfaciens*	*S. salivarius*	*N. meningitidis*	*S. epidermidis*	*S. maltophilia*	*N. sicca*
B	*L. lactis*	*P. aeruginosa*	*L. lactis*	*M.* sp. Ag1	*S. marcescens* *S. maltophilia*	*S. maltophilia*	*M. sp. Leaf139* *C. flaccumfaciens*	*S. salivarius*	*N. meningitidis*	*S. epidermidis*	*S. maltophilia*	*N. sicca*
C	*L. Lactis* *S. warneri*	*S. marcescens*	*S. aureus*	*P. aeruginosa*	*P. aeruginosa*	*S. marcescens*	*S. maltophilia*	*P. aeruginosa* *M.* sp. Ag1	*G. haemolysans*	*S. maltophilia*	*P. fluorescens*	*R. dentocariosa*
D	*L. Lactis* *S. warneri*	*S. marcescens*	*S. aureus*	*P. aeruginosa*	*P. aeruginosa*	*S. marcescens*	*S. maltophilia*	*P. aeruginosa* *M.* sp. Ag1	*G. haemolysans*	*S. maltophilia*	*P. fluorescens*	*R. dentocariosa*
E	*K. rhizophila*	*S. aureus*	*P. aeruginosa*	*S. capitis*	*S. maltophila*	*S. aureus*	*R. aeria*	*P. fluorescens*	*R.* sp. HMSC067H10	*S. maltophilia*	*N.* sp. oral taxon 014	*P. aeruginosa*
F	*K. rhizophila*	*S. aureus*	*P. aeruginosa*	*S. capitis*	*S. maltophila*	*S. aureus*	*R. aeria*	*P. fluorescens*	*R.* sp. HMSC067H10	*S. maltophilia*	*N.* sp. oral taxon 014	*P. aeruginosa*
G	*S. aureus*	*P. aeruginosa*	*P. fluorescens*	*S. marcescens* *C. dublinensis*	*M.* sp. Ag1	*P. aeruginosa*	*R. dentocariosa*	*N. flavescens*	*N.* sp. HMSC15G01	*S. maltophilia*	*S. aureus*	*N.* sp. HMSC15G01
H	*S. aureus*	*P. aeruginosa*	*P. fluorescens*	*S. marcescens* *C. dublinensis*	*M.* sp. Ag1	*P. Aeruginosa*	*R. dentocariosa*	*N. flavescens*	*N.* sp. HMSC15G01	*S. maltophilia*	*S. aureus*	*N.* sp. HMSC15G01


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
E. coli extract SP3 processing
Cell number
5.7×10⁴ 2.8×10⁵ 5.7×10⁵ 2.8×10⁶ 5.7×10⁶ 2.8×10⁷ 5,7×10⁷ 1.4×10⁸ 2.8×10⁸ 4.3×10⁸ 5.7×10⁸ 1.7×10⁹
Protein quantity (µg)
0.01 0.05 0.1 0.5 1 5 10 25 50 75 100 300
A
B
C
E. coli proteotyping