



## Comparative proteomics of *Brucella melitensis* is a useful toolbox for developing prophylactic interventions in a One-Health context

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### ABSTRACT

Brucellosis caused by *Brucella melitensis* is a zoonosis frequently reported in the Mediterranean and Middle-East regions and responsible for important economic losses and reduced animal welfare. To date, current strategies applied to control or eradicate the disease relies on diagnostic tests that suffer from limited specificity in non-vaccinated animals; while prophylactic measures, when applied, use a live attenuated bacterial strain characterized by residual virulence on adult pregnant animals and difficulties in distinguishing vaccinated from infected animals. To overcome these issues, studies are desired to elucidate the bacterial biology and the pathogenetic mechanisms of both the vaccinal strain and the pathogenic strains. Proteomics has a potential in tackling issues of One-Health concern; here, we employed label-free shotgun proteomics to investigate the protein repertoire of the vaccinal strain *B. melitensis* Rev.1 and compare it with the proteome of the *Brucella melitensis* 16 M, a reference strain representative of *B. melitensis* field strains. Comparative proteomics profiling underlines common and diverging traits between the two strains. Common features suggest the potential biochemical routes responsible for the residual virulence of the vaccinal strain, whilst the diverging traits are suggestive biochemical signatures to be further investigated to provide an optimized diagnostic capable of discriminating the vaccinated from infected animals. The data presented in this study are openly available in PRIDE data repository at <https://www.ebi.ac.uk/pride/>, reference number PXD022472.

### 1. Introduction

Brucellosis, also known as Malta fever, is among the most important zoonoses in the Mediterranean and Middle-East regions [1]. Brucellosis is of great relevance in the One-Health context, acknowledged its impact in the human and environmental sector, besides the pivotal role of the environment in feeding the pathogen transmission circle between the human and animal hosts [2,3]. The World Organization for Animal Health (OIE) has included brucellosis in the list of notifiable terrestrial and aquatic animal diseases due to its epidemiological features and the high risk to human and animal health (<https://www.oie.int/>).

Brucellosis in sheep and cattle is primarily caused by *Brucella melitensis*; *B. melitensis* infections have also been observed in camels and cattle, especially in those areas with extensive breeding of small ruminants [4]. Humans encounter *B. melitensis* via consumption of contaminated food (mainly unpasteurized dairy product), processing of meat from infected animals, contact with infected biologic material (reproductive secretions, aborted fetuses, wool from infected animals), including the accidental spray of infected biological fluids and the inhalation of contaminated dust particles [5].

Measures to control Brucellosis rely on a massive animal vaccination program along with the screening and culling of the infected animals.

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The currently adopted vaccine to protect sheep and goat against *B. melitensis* infection consists of a subcutaneous or conjunctival administration of the live attenuated *B. melitensis* Rev.1 strain. This confers a good immunity to animals, but its massive usage is discouraged since vaccination alone is not able to eradicate the disease and the residual virulence of the *B. melitensis* Rev.1 may induce abortion in pregnant animals or be released during lactation with harmful effects to humans [6,7]. Moreover, the O-polysaccharide (OPS) moiety administered with the vaccinal dose is also the target molecule of the serological diagnostic tests, hindering the differentiation of infected from vaccinated animals (DIVA) [7–9]. Besides, the antigens adopted as the target for the serological-based diagnosis are cross-reactive with other Gram-negative species leading to a reduced sensibility of the current diagnostic tests [10,11]. To overcome these issues, several studies are being performed to better understand the bacterial biology and the pathogenic mechanisms of both the vaccine strain and other commonly circulating *Brucella* strains [12–16]. Proteomics represents a valuable toolbox for addressing complex issues on the verge of the One-Health approach [17]. In the present study, a label-free-shotgun proteomic approach is employed to explore the protein profile of the vaccinal strain *B. melitensis* Rev.1 and then compare it with the proteome of the reference strain *Brucella melitensis* 16 M, representative of field strains *B. melitensis*. The identified protein repertoire enables the thorough investigation of the biology of the selected bacteria and the comparative evaluation of proteomes on a bacterial strain basis. A discussion on the biological role of the differentially expressed proteins and their potential implication as candidates for the DIVA diagnosis and/or the pathogenic mechanism is also provided.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*Brucella melitensis* 16 M and Rev.1 strains were provided by the OIE Reference Laboratory for Brucellosis at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM). Both *Brucella* strains were cultured as pure culture in the IZSAM's lab by an internal validated protocol [18]. Briefly, 16 M and Rev.1 strains were grown on tryptic soy agar plates (TSA, Biolife) for 72 h at 37 °C in an atmosphere with 5–10% CO<sub>2</sub>. Bacterial colonies were collected into sterile ice-cold 0.01 M phosphate-buffered saline (PBS), pH 7.0. Bacterial suspensions from four independent cultures were pooled for each strain and a final concentration of  $5 \times 10^6$  CFU/ml was obtained. Bacteria were pelleted by centrifugation at 12,000 xg for 5 min at 4 °C and washed three times with sterile ice-cold 0.01 M PBS. Bacterial cells were finally heat-inactivated at 98 °C for 15 min, cooled on ice and stored at –80 °C.

### 2.2. Protein extraction and sample preparation

The pooled aliquot of each *Brucella* strain ( $5 \times 10^6$  cells each) was resuspended in 100 mM Tris/HCl pH 7.9 containing 6 M urea and lysed through multiple steps of centrifugation and sonication with a sonication probe at 50% power with 30"cycle. Extracted proteins were quantified using a Bradford-based Protein assay (Bio-Rad Laboratories, Segrate, MI).

### 2.3. Label-free proteomics analysis

Protein digestion has been performed according to the Filter-Aided Sample Preparation (FASP) protocol [19] as described in detail by Piras et al. [20].

Label-free proteomic analysis was performed on an ACQUITY MClass System (Waters Corporation) directly coupled to a high-definition Synapt G2-Si mass spectrometer (Waters Corp). according to Greco et al. [21].

Differential protein abundance was assessed by High-Definition

expression configuration mode (HDMS<sup>E</sup>) as described by Marini et al. [22]. Each sample was run in four technical replicates.

Progenesis QI for Proteomics v4.0.6403.35451 (Waters Ltd., Newcastle upon Tyne) software was used for protein quantification [23]. Database search was performed by the ion accounting method against the UniProtKB database (*Brucella melitensis* biotype 1, UniProtKB UP000000419). Differentially expressed proteins were established according to the following criteria: proteins identified in at least three out of four runs of the same sample; fold change of regulation  $> \pm 30\%$ ; *p*-value  $< 0.05$ , according to the analysis of variance (ANOVA).

The data presented in this study are made publicly available in the PRIDE data repository (<https://www.ebi.ac.uk/pride/>), reference number PXD022472.

### 2.4. Bioinformatic data analysis

Classification of the identified proteins into functional categories and/or abundance clusters was made under statistical supervision. The NeVOmics python-R based tool [24] was used to identify significantly enriched GO terms and KEGG pathways (FDR 1%) in the proteins of the two experimental groups (*i.e.* *Brucella melitensis* 16 M and *Brucella melitensis* Rev.1). To yield a better detailing of the functional comparison between the two bacterial strains, differentially expressed (DE) proteins of Rev. 1 and 16 M samples were further distinguished as low DE (IDE) and high DE (hDE) depending on the protein abundance ratio (1,3–2 and greater than 2-fold-changes, respectively). Subgroups were subjected to functional classification and comparative analysis independently. Regardless of the protein abundance, GO and KEGG enrichment has been obtained using as target organisms the *Brucella melitensis* group with the database versions updated respectively to the release 2020-08-11 for GO and 95.0+/09–15 for KEGG.

## 3. Results

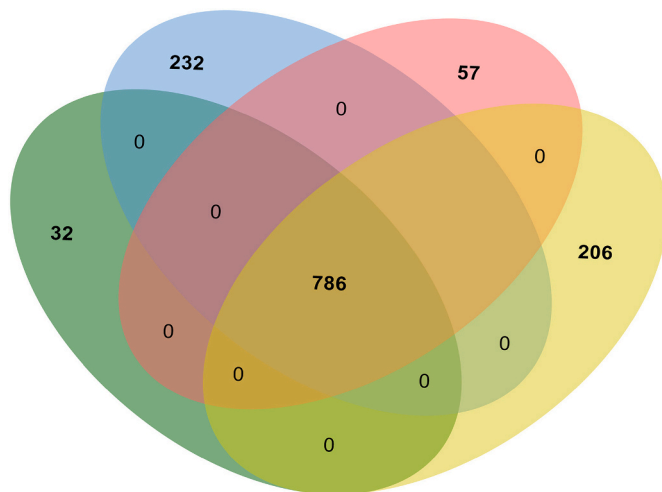
### 3.1. Protein identification in the Rev.1 and 16 M *Brucella* strains

Bioinformatics data analysis identified a total of 1313 proteins, corresponding to approximately 41% of the whole proteome of *Brucella melitensis* (UniProtKb UP000000419, 3179 entries). The protein dataset is "stratified" according to the relative abundance of the protein entries, calculated on the inter-sample (*i.e.* Rev.1 vs 16 M) basis. Specifically, proteins featured by a relative abundance within a tolerance window of  $\pm 30\%$  (*i.e.* relative fold change  $\leq 1.3$ ) are considered as equally expressed between the bacterial strains and labelled as "equally abundant" (EA); whereas, entries scoring an abundance above, or below, 30% of the abundance of their counterpart identified in the other samples group are considered as differentially expressed (DE). According to their fold change, the latter is further distinguished into two sub-groups namely low DE (IDE, fold change across bacterial strains ranging between 1.3 and 2) and high DE (hDE, fold change  $> 2$ ) respectively. A total of 786 proteins were considered as EA proteins and 527 proteins are classified as DE. Concerning the Rev.1 strain, 264 proteins are identified as DE, including 32 hDE proteins. The 16 M strain recorded 263 DE entries, 57 of which are hDE (Fig. 1). The full list of the identified protein dataset is provided as Supplementary material S1.

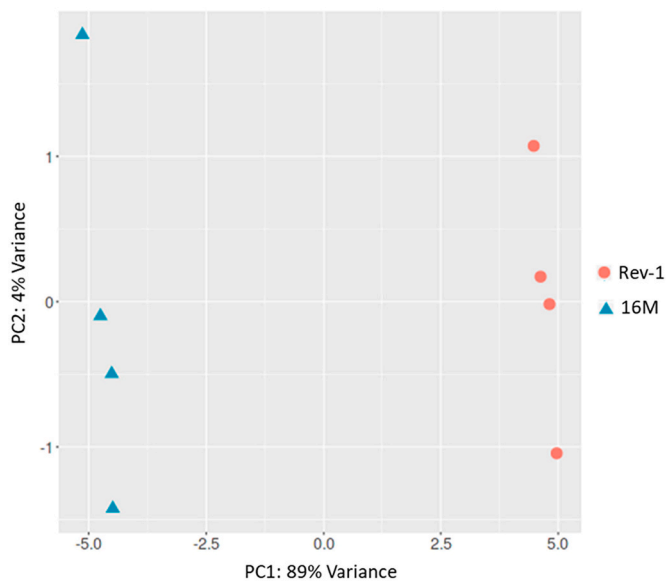
Depiction of the identified protein dataset in a PCA plot displays a clear clustering of the Rev.1 and 16 M protein dataset along the PC1 axis, which explains 89% of the total variability among the samples (Fig. 2). Also, the technical replicates of Rev.1 and 16 M samples representatives tend to cluster close together (4% variance depicted over the PC2), supporting the robustness of the obtained data.

### 3.2. Functional categorization of the EA protein repertoire

The EA protein repertoires of Rev.1 and 16 M *Brucella* strains are investigated from a functional perspective through NeVOmics. The

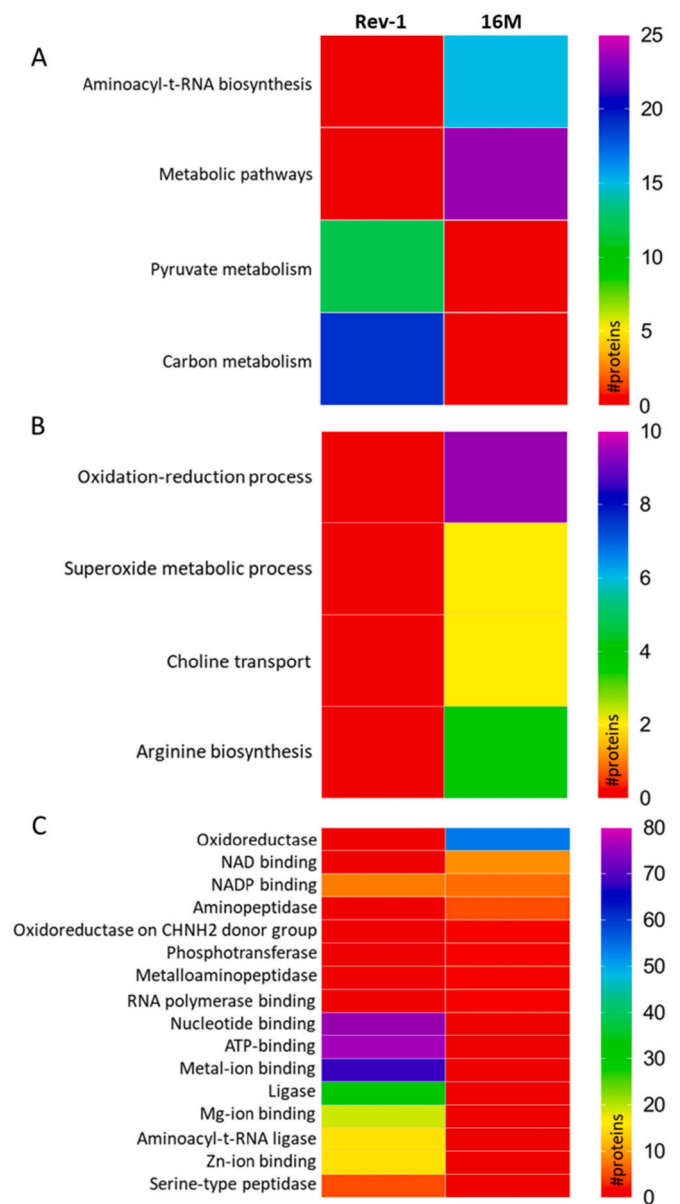


**Fig. 1.** Protein distribution across samples groups. The Venn diagram displays the protein dataset sorted according to their relative abundance and the *Brucella* strains they belong to. Green ensemble refers to Rev.1 protein dataset with a fold-change >2; blue refers to Rev.1 proteins with a fold-change between 1.3 and 2; pink concern the 16 M protein dataset with a fold-change >2 and yellow ensemble refers to 16 M proteins with a fold-change between 1.3 and 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Dataset distribution over a PCA plot on a bacterial strain basis. Identified proteins are scattered onto a PCA plot on a bacterial strain basis.

abundance of each functional category, meant as the sum of abundances of the protein belonging to a specific category, is then employed for the functional comparison of the two bacterial strains on the EA protein repertoire basis. The “sole” statistically enriched terms (*i.e.* over-represented) between the two sample groups are considered. The EA proteins of the Rev.1 strain are primarily involved in “metabolic pathways” category of the KEGG database and a minor, but statistically significant, portion of the Rev-1 EA protein list belongs to the “Aminoacyl-t-RNA biosynthesis” biochemical pathway (Fig. 3). On the other hand, EA proteins of the 16 M strain are attributed to “pyruvate metabolism” and “carbon metabolism” biochemical pathways. Functional classification of the EA proteins based on the biological processes annotation fails to identify statistically significant biological processes



**Fig. 3.** Functional classification of the EA proteins of the *Brucella* strains. Panel A depicts the functional classification, according to the KEGG database, of the EA proteins that are significantly overrepresented. Biological processes and molecular functions that are significantly over-represented are displayed in panel B and C, respectively.

relative to the Rev.1 samples. On the contrary, the majority of the 16 M EA proteins is involved in the oxidation-reduction process, and a minor portion of the functionally classified EA proteins are active in the arginine biosynthetic process, superoxide metabolic process and choline transport biological processes (Fig. 3).

Functional classification of the EA proteins at the level of molecular functions depicts a great number of the Rev.1 proteins implicated in binding substrates such as nucleotides, ATP and metal ions. Other functions, including ligase activity and Ser-type peptidase activities, are expressed by Rev.1 bacteria. The 16 M strain, instead, shows a lower diversity and different asset of the molecular functions when compared with the Rev.1 counterpart. Here, most of the molecular functions identified concern the oxidoreductase and stress-response activities; whilst a reduced number of proteins is involved in the NAD and NADP binding and aminopeptidase activity. A minor number of 16 M EA proteins is active in RNA polymerase binding, metalloaminopeptidase

activity, protein-N(PI)-phosphohistidine-sugar phosphotransferase activity and the oxidoreductase activity specific to the CH-NH<sub>2</sub> group of donors (Fig. 3).

### 3.3. Comparative evaluation of the DE proteins of Rev.1 and 16 M strains

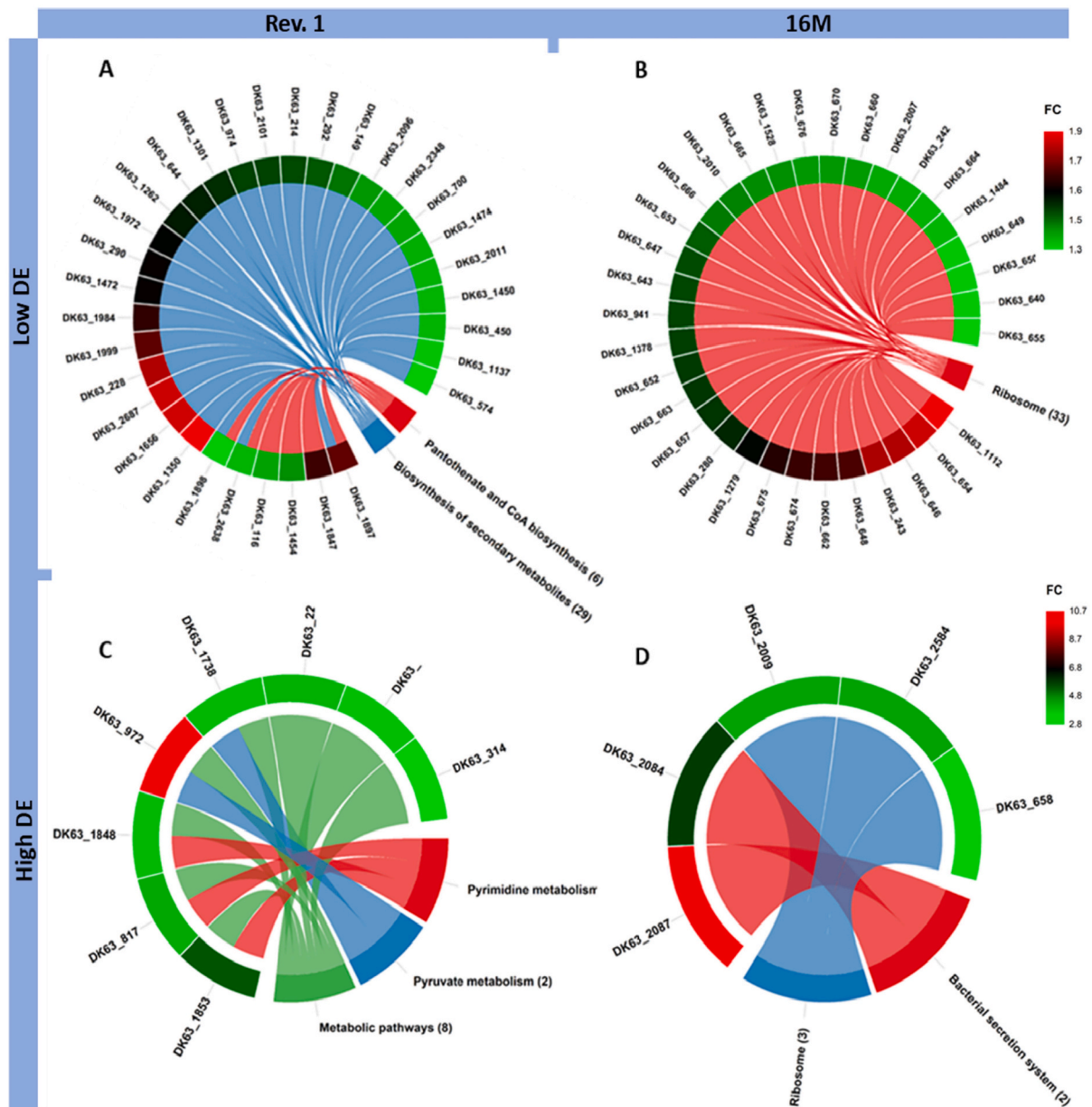
To provide a “discriminatory” featuring between the vaccinal and the field strain, DE proteins are distinguished as lDE (fold change between 1.3 and 2) and hDE (fold change >2).

lDE proteins of the Rev.1 samples are principally involved in the “biosynthesis of secondary metabolites” and “Pantothenate and CoA biosynthesis” KEGG pathways. On the other hand, the lDE proteins of the 16 M strain are classified in the “Ribosome” KEGG functional class (Fig. 4, panel A and B).

Classification of the hDE proteins into KEGG functional classes reveals different biological functions of the Rev.1 and the 16 M strains

despite the categorization of the proteins falls within “broad” functional classes. hDE proteins of Rev.1 are classified as “metabolic pathways” and “pyruvate metabolism” KEGG classes. hDE proteins of the 16 M samples are instead classified in the “Ribosome” and the “Bacteria secretion system” categories of the database (Fig. 4, panel C and D).

Functional classification of the proteins according to the biological processes depicts lDE proteins of the Rev.1 bacteria strongly involved in anabolism and biosynthetic processes of a variety of biological compounds as well as the expression of gene regulators and RNA processing process. On the other hand, the 16 M counterpart depicts a minor functional diversity and a high concern of this bacteria in “translation”, “protein folding”, “protein repair” and “response to oxidative stress” processes. Statistically over-represented hDE proteins of the 16 M samples are functionally categorized into pathogenesis and cellular iron ion homeostasis (Fig. 5); whereas no hDE protein of the Rev.1 samples scored a statistically significant over-representation.



**Fig. 4.** Functional classification of the Differentially Expressed (DE) proteins of the *Brucella melitensis* Rev.1 (panel A, C) and 16 M (panel B, D) strains. The figure depicts Chord graphs summarizing the functional classification of the KEGG classes that are significantly over-represented between the sample groups. Panel A and B are relative to the low DE proteins of the Rev.1 and 16 M strains, respectively. Panel C and D concern the high DE proteins of the vaccinal and field strain, respectively. Kegg accession entries displayed in the Chord graphs are translated into UniProtKB accession numbers and provided as Supplementary material S2.

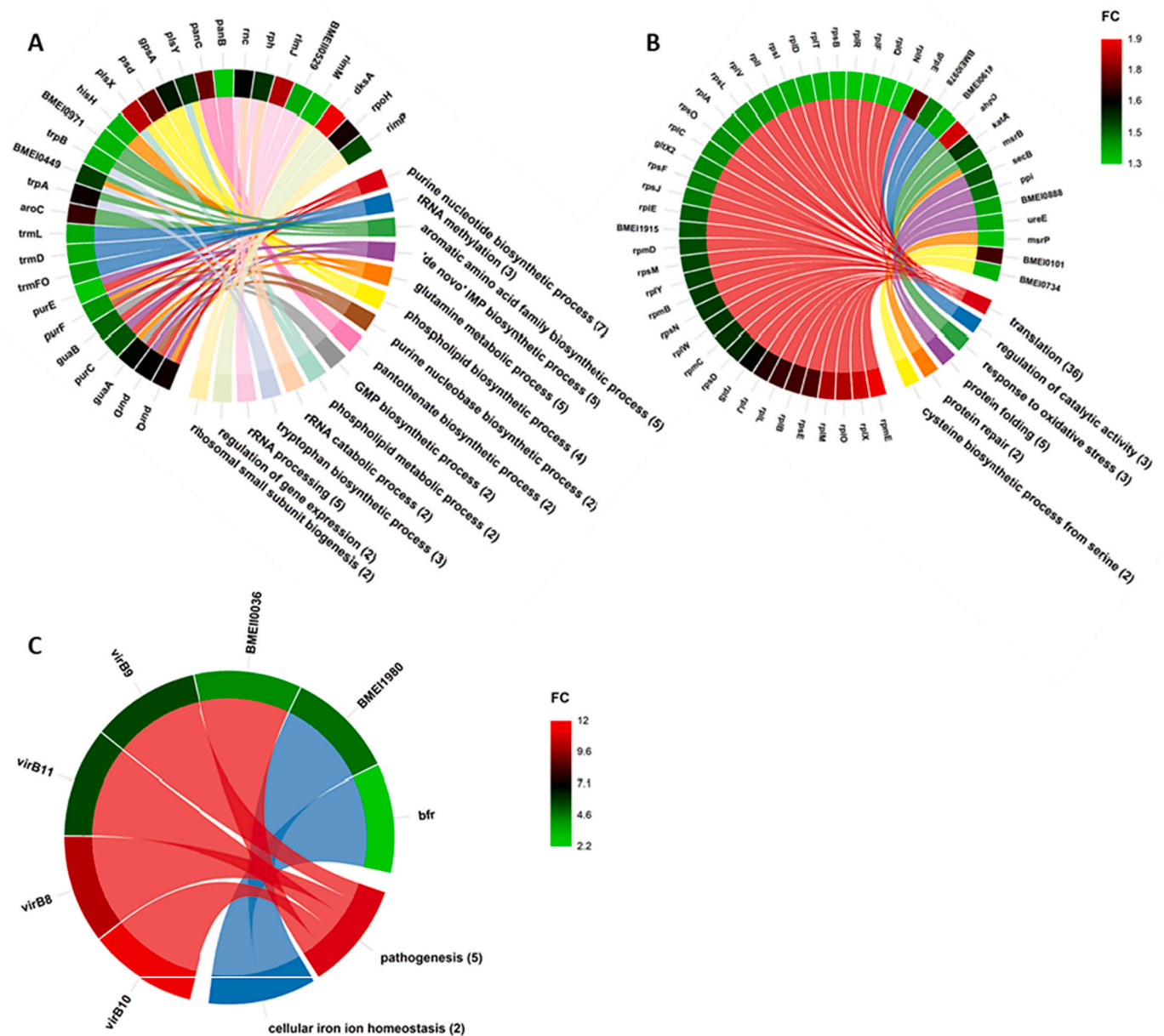


Fig. 5. Functional classification into biological processes of the Differentially Expressed (DE) proteins of the *Brucella melitensis* Rev.1 and 16 M strains. Chord graphs depict the biological processes that are statistically over-represented in the low DE (lDE) of the Rev.1 and 16 M strains (panel A and B respectively). Biological processes categorization of the high DE (hDE) proteins of the 16 M samples are depicted in the panel C. No statistically significant enriched terms have been highlighted in the protein dataset of the vaccinal strain.

A deeper functional detailing of the DE proteins depicted a higher functional diversity of the 16 M samples as compared to the Rev.1 counterpart. Here, lDE proteins of the Rev.1 samples highlight concern of this strain in the DNA-dependent ATPase, aspartate-tRNA ligase and gluconate-2 dehydrogenase activity. The field strain, instead, reported a higher functional diversity ranging from broadening the array of substrate molecules and the activity on protein biosynthesis and the production of ribosome constituents. hDE proteins of the 16 M samples confirm the involvement of the field strain in the production of the structural components of the ribosome as well as its involvement in pathogenesis as supported by the higher level of proteins concerned in the ferric ion binding (Supplementary material S3).

4. Discussion and conclusion

Nowadays, brucellosis is among the most significant bacterial

zoonosis. *Brucella* spp. infections are endemic in several areas of the globe and considered as a neglected reemerging disease in many, formerly, Brucella-free countries. Tackling the spreading of *Brucella* spp. requires multidisciplinary approaches as the One-Health, acknowledged the economy and public health concerns of the infections. Controlling *Brucella* spp. dissemination spans from the effective monitoring and treatment of the infections in the human being population to the control of the animal infection routes, including the limitation of pathogen shedding in the environment, aimed at interrupting the pathogen infective/transmission cycle [3,25].

To date, in line with the One-Health concept, a steadily growing number of studies are being performed integrating the contribution of multiple disciplines (e.g. human and animal sciences, environmental microbiology, social sciences etc.) to provide a multifaced solution to complex health issues, as it is the case of brucellosis [3,26–28].

The current study employs a proteomics approach for the

comparative evaluation of the functional asset of two *Brucella* strains (i.e. the vaccinal strain, Rev.1 and the reference strain, 16 M). Proteomics is a valuable tool to tackle complex issues of One-Health concern [17]. The study relies on a state-of-art gel-free proteomics approach, enabling a more thorough survey of the *Brucella* protein repertoires than other gel-based approaches. This ensures a wider dynamic range of the analyzed proteins as well as the inclusion of both less abundant entries and large dimension proteins [29].

The elucidation of the functional peculiarities of both the vaccinal and the reference strain could provide essential knowledge for the improvement of the current diagnostic tests, e.g. enabling the discrimination between infected and immunized animals. Also, the thorough investigation of the functional asset of the *Brucella* strains might be beneficial for the development of new vaccine formulate(s); thus, contributing to more effective approaches in affected countries and supporting the One-Health perspective [30–33]. The qualitative analysis of the protein dataset of the two bacterial strains did not underline important differences in terms of a comparative evaluation. This observation is easily imputable to the taxonomical relatedness of the investigated bacterial strains, whose protein repertoire is reasonably similar in qualitative terms, leveraging the different protein abundances in driving the changes of phenotypic behaviour of these bacterial specimens. The proteome dataset of the two sample groups was re-organized as a function of the relative abundance of the identified proteins and an “equally abundant” (EA) and “differentially expressed” (DE) cluster were initially distinguished. The EA proteins are meant as the proteins with no (or negligible) difference of abundance between the investigated bacterial strains. The functional classification of the EA proteome portion of the two bacterial strains highlights a common involvement of both *Brucella* strains in the central metabolism and the protein biosynthesis and turnover. The EA proteins include entries featured by multiple physiological functions as is the case of the aminopeptidases and the serine-type peptidases. Many bacterial peptidases are reported as virulence factors acknowledged their ability to degrade proteinaceous structures that facilitate the pathogen colonization and/or protection of pathogens from the host’s immune system [34–37]. Moreover, peptidases are part of the bacterial secretion system providing a significant contribution to bacterial virulence [38]. Based on the above, production of peptidases by the Rev.1 samples is likely to be due to the residual virulence of the vaccinal strains and further detailing of these pathways would be beneficial for the development of safer vaccinal formulates. Residual virulence of the Rev.1 strain might also be supported by the identification of EA proteins with ligase activity. Indeed, proteins with ligase activity are proven to participate through various mechanisms to the pathogenicity and infections of diverse bacteria [39].

Although featured by a large portion of EA proteins, DE proteins are likely to drive the peculiar behaviour of the two bacterial strains. Accordingly, a recent investigation reports that a minor portion of DE proteins enables the diverse biological functions existing among *Brucella melitensis* strains [40]. Transcriptomic investigation of *Brucella Rev. 1* and 16 M strains highlighted that more than 400 diverse genes, encoding for crucial processes such as metabolic, biosynthetic, and transport processes, are DE between the two sample groups [41]. In the current study, DE proteins of the vaccinal strain depict a stable metabolism as witnessed by the active concern of the DE protein repertoire in the biosynthetic process of secondary metabolites and, more broadly, the anabolism. Anabolism observed in the Rev.1 strain includes the massive expression of the *LeuA* gene encoding for 2-isopropylmalate synthase, a key protein in the biosynthetic process of leucine that, among the other functions, has been linked to virulence [42]. Also, *LeuA* is considered as one of the most promising targets for the development of alternative antimicrobials against intracellular pathogens [43], and its identification in the Rev.1 strain may explain the permanence of a residual virulence in the vaccinal strain as already pointed by the EA proteins. The identification of proteins involved in the secondary metabolism was

expected considering that this work was performed on cultured bacterial strains, and using heterogeneous substrates array may result in the production of novel compounds [44,45]. Comparatively, this represents the major functional duty of the Rev.1 strain and differentiates it from the reference strain whose functional featuring registered a far more abundant level of proteins involved in specific virulence processes. Over-representation of the Alkyl hydroperoxide reductase (AhpD), Catalase (KatA) methionine sulfoxide reductase (Msr) in the 16 M samples is likely associated with a protective role against the oxidative stress exerted by the immune cell effectors [46–48], and we speculate a crucial role in the escape of the bacteria from the host’s immune system and the completion of its infective process. AhpD overexpression has been linked to an increased propensity of biofilm formation in *Campylobacter jejuni* underlining, once again, the potential role of AhpD in virulence [49]. The correlates of virulence found in the 16 M are expected according to its natural behaviour and might be exploited as candidates to design differential diagnostics. Unfortunately, the DE proteins (both IDE and hDE) highlighted in the current study are not unique or exclusively imputable to *Brucella spp.*, thus likely unsuitable as targets for a definitive diagnosis of Brucellosis. Nevertheless, the changing abundance levels registered among the vaccinal and field strains might be implemented in novel companion diagnostic strategies aimed at more efficient discrimination of the infected and vaccinated animals (DIVA) out of the positively screened animals. In addition to their diagnostic potential, DE proteins are likely to have an important role in optimising prophylactic strategies. Despite the in-depth and comprehensive proteomic investigation that we have performed, an intrinsic limitation of our study could be linked to the relatively low level of protein ontology annotation of the publicly available databases. This could have affected the total number of protein identifications and, therefore, some functional characteristics of the two *Brucella* strains which are likely omitted from our results. Nevertheless, the recent sequencing of the *Brucella melitensis Rev.1* strain genome [50] might open new routes for a more comprehensive functional annotation, thus implementing it in the *Brucella* database, enabling a further deepening of the results achieved in the present survey. Specifically, studies aimed at a better elucidation of the functional asset of both the vaccinal and the reference strain are warmly required to clarify the bacterial biology and the amendment of the vaccinal formulates. This, along with the identification of suitable biomarkers for the differential diagnosis of *Brucella* infection, represents the key point for the design of effective diagnostic tests capable of discriminating vaccinated from infected subjects, with beneficial implication for both the environmental pathogen circulation and the veterinary and human health system.

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## Declaration of Competing Interest

None.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.onehlt.2021.100253>.

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