A shift in the virulence potential of 
*Corynebacterium pseudotuberculosis* biovar 
*ovis* after passage in a murine host 
demonstrated through comparative 
proteomics

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**Abstract**

**Background:** *Corynebacterium pseudotuberculosis* biovar *ovis*, a facultative intracellular pathogen, is the etiologic agent of caseous lymphadenitis in small ruminants. During the infection process, *C. pseudotuberculosis* changes its gene expression to resist different types of stresses and to evade the immune system of the host. However, factors contributing to the infectious process of this pathogen are still poorly documented. To better understand the *C. pseudotuberculosis* infection process and to identify potential factors which could be involved in its virulence, experimental infection was carried out in a murine model using the strain 1002*_ovis* and followed by a comparative proteomic analysis of the strain before and after passage.

**Results:** The experimental infection assays revealed that strain 1002*_ovis* exhibits low virulence potential. However, the strain recovered from the spleen of infected mice and used in a new infection challenge showed a dramatic change in its virulence potential. Label-free proteomic analysis of the culture supernatants of strain 1002*_ovis* before and after passage in mice revealed that 118 proteins were differentially expressed. The proteome exclusive to the recovered strain contained important virulence factors such as CP40 proteinase and phospholipase D exotoxin, the major virulence factor of *C. pseudotuberculosis*. Also, the proteome from recovered condition revealed different classes of proteins involved in detoxification processes, pathogenesis and export pathways, indicating the presence of distinct mechanisms that could contribute in the infectious process of this pathogen.

**Conclusions:** This study shows that *C. pseudotuberculosis* modifies its proteomic profile in the laboratory versus infection conditions and adapts to the host context during the infection process. The screening proteomic performed us enable identify known virulence factors, as well as potential proteins that could be related to virulence this pathogen. These results enhance our understanding of the factors that might influence in the virulence of *C. pseudotuberculosis*.

**Keywords:** *Corynebacterium pseudotuberculosis*, Bacterial label-free proteomic, Caseous lymphadenitis, Bacterial virulence, Serial passage, Extracellular proteins
**Background**

*Corynebacterium pseudotuberculosis* biovar *ovis* is a Gram-positive facultative intracellular pathogen. It is the etiologic agent of Caseous Lymphadenitis (CLA) in small ruminants, a disease characterized by abscess formation in lymph nodes and internal organs [1]. Cases of human infection caused by *C. pseudotuberculosis* have been reported and are associated with occupational exposure [1]. CLA is globally distributed and causes significant economic losses in goats, and sheep herds [2]. The pathogenic process of *C. pseudotuberculosis* in the host comprises two phases: (i) initial colonization and replication in lymph nodes that drain the site of infection, which is associated with pyogranuloma formation, and (ii) a secondary cycle of replication and dissemination via the lymphatic or circulatory systems. This dissemination is promoted by the action of phospholipase D (PLD) exotoxin, the major virulence factor of *C. pseudotuberculosis*, which allows this pathogen to contaminate visceral organs and lymph nodes, where it ultimately induces lesion formation [3–5].

Exported proteins reportedly favor the infection process in pathogenic bacteria; this class of proteins is involved in adhesion and invasion of host cells, nutrient acquisition, toxicity, and in the evasion of the host immune system [6]. Different strategies like the transposon mutagenesis have been adopted to identify *C. pseudotuberculosis* biovar *ovis* exported proteins [7]. Additionally, comparative proteomics has been applied to characterize the extracellular proteome of *C. pseudotuberculosis* biovar *ovis*, as well as, the extracellular immunoproteome (strains C231_ovi and 1002_ovi) [8–11]. In these studies, some proteins of the strain 1002_ovi, suspected to be virulence factors, were not detected suggesting this strain presents a low virulence. The surface proteome of *C. pseudotuberculosis* biovar *ovis* was also characterized using bacterial strains isolated from the lymph nodes of naturally infected sheep. This proteomic analysis allowed the identification of proteins that could favor the survival of this pathogen during the chronic phase of CLA [12].

The experimental passage of bacterial pathogens through in vitro or in an in vivo model is a strategy that has been applied to evaluate the virulence potential of several pathogens. By generating a confrontation between the pathogen and the dynamic network of host factors, including the immune system components, it helps to identify bacterial factors involved in virulence [12–19]. In this study, the strain 1002_ovi was experimentally inoculated in mice [20, 21] to identify factors which could contribute to virulence in *C. pseudotuberculosis* biovar *ovis*. Comparative proteomics of the culture supernatant from this strain collected before and after the experimental passage in mice was carried out to identify factors that might contribute to virulence of 1002_ovi.

**Methods**

**Bacterial strains and growth conditions**

The *C. pseudotuberculosis* biovar *ovis* strain 1002 (1002_ovi) was isolated from a goat in Brazil; this strain was cultivated under standard conditions in brain–heart infusion broth (BHI-HiMedia Laboratories Pvt. Ltd., India) at 37 °C. When necessary, 1.5% of agar was added to the medium for a solid culture. For extracellular proteomic analyses, 1002_ovi was grown in a chemically defined medium (CDM) [(NaH2PO4·7H2O (12.93 g/L), KH2PO4 (2.55 g/L), NH4Cl (1 g/L), MgSO4·7H2O (0.20 g/L), CaCl2 (0.02 g/L) and 0.05% (v/v) Tween 80], 4% (v/v) MEM Vitamins Solution (Invitrogen, Gaithersburg, MD, USA), 1% (v/v) MEM Amino Acids Solution (Invitrogen), 1% (v/v) MEM Non-Essential Amino Acids Solution (Invitrogen), and 1.2% (w/v) glucose at 37 °C [22].

**Experimental infection of strain 1002_ovi in a murine model (in vivo assay)**

The standardization of the parameters for infection was performed according to Moraes et al. [20] and Ribeiro et al. [21]. Female BALB/c mice between six and eight weeks old were used in all experiments. They were provided by the Animal Care Facility of the Biological Sciences Institute from the Federal University of Minas Gerais and were handled by the guidelines of the UFMG Ethics Committee on Animal Testing (Permit Number: CETEA 103/2011). For the bacterial passage assay using the murine model, two groups of three mice each was infected via intraperitoneal injection with 10⁶ colony forming units (CFU) of strain 1002_ovi. Thirty-six hours after infection, all animals were sacrificed. Their spleens were aseptically removed to recover the bacterial strain, as described below: the spleen removed from each animal was then, individually macerated in sterile saline solution (0.9% NaCl₂), seeded onto BHI agar plates and incubated for 48 h at 37 °C. Subsequently, one recovered bacterial colony was cultured in BHI broth. The recovered bacteria were then referred to as Recovered (Rc). For the bacterial virulence assay, we used the freshly recovered bacteria and bacteria that did not contact the murine host as a control, which is referred to as Control (Ct). Groups of five mice were infected with Rc and Ct, via intraperitoneal injection of a suspension containing 10⁶ CFU or 10⁵ CFU. The animals’ survival rates were calculated and represented in GraphPad Prism v.5.0 (GraphPad Software, San Diego, CA, USA) using the Kaplan-Meier survival function. The results of 1002_ovi CFU count in the organs were calculated using the two-way ANOVA test.

**Preparation of proteins from culture filtrates for proteome analysis**

For proteomic analysis, the Ct and Rc (three independently recovered colonies) that was obtained from infected
mice spleens as described above were grown in CDM at OD_{600} = 0.8. The cultures were then centrifuged for 20 min at 2700 x g. The supernatants were then filtered using 0.22-μm filters, 30% (w/v) ammonium sulfate was added to the samples, and the pH of the mixtures was adjusted to 4.0. Next, 20 mL N-butanol was added to each sample. The samples were centrifuged for 10 min at 1350 xg and 4 °C. The interfacial precipitate was collected and resuspended in 1 mL of 20 mM Tris–HCl pH 7.2 [23]. Finally the concentration protein was determined by Bradford method [24].

2D-PAGE electrophoresis and Mass Spectrometry

The 2-DE procedure and in-gel protein digestion were performed as described previously [9, 10]. Approximately 300 μg of the protein extract from each condition was dissolved in rehydration buffer (Urea 7 M, thiourea 2 M, CHAPS 2%, Tris–HCl 40 mM, bromophenol blue 0.002%, DTT 75 mM, IPG Buffer 1%). Samples were applied to 18 cm pH 3–10 NL strips (GE Healthcare, Pittsburgh, USA). Isoelectric focusing (IEF) was performed using the apparatus IPGphor 2 (GE Healthcare) under the following voltages: 100 V 1 h, 500 V 2 h, 1000 V 2 h, 10,000 V 3 h, 10,000 V 6 h, 500 V 4 h. The IPG strips were placed on 12% acrylamide/bis acrylamide gels in an Ettan DaltSix II system (GE Healthcare). The gels were stained with Coomassie Blue G-250 staining solution, and 2-DE gels were scanned using an Image Scanner (GE Healthcare). The Image Master 2D Platinum 7 (GE Healthcare) software was used to analyze the generated images and all spots were matched and analyzed by gel-to-gel comparison. The quantification of the spots was calculated according percentage volume (% Vol) and spots with reproducible changes in abundance were considered to be differentially expressed. Protein spots were excised from the gels, and in-gel digestion was carried out using trypsin enzyme (Promega, Sequencing Grade Modified Trypsin, Madison, WI, USA). The peptides were then desalted and concentrated using ZIP TIP C18 tips (Eppendorf).

The samples were subsequently analyzed for MS and MS/MS modes, using an MALDI-TOF/TOF mass spectrometer Autoflex IIIITM (Bruker Daltonics, Billerica USA). The equipment was controlled in a positive/reflector way using the Flex-ControITM software (Bruker Daltonics). External calibration was performed using peptide standards samples (angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39, somatostatin 28, bradykinin Fragment 1–7, Renin Substrate tetra decapeptide porcine) (Bruker Daltonics). The peptides were added to the alpha-cyano-4-hydroxycinnamic acid matrix, applied on an AnchorChipTM 600 plate (Bruker Daltonics) and analyzed by Autoflex III. The search parameters were as follows: enzyme; trypsin; fixed modification, carbamidomethylation (Cys); variable modifications, oxidation (Met); mass values, monoisotopic; maximum missed cleavages, 1; and peptide mass tolerance of 0.005% Da (50 ppm). The results obtained by MS/MS were used to identify proteins utilizing the MASCOT_ (http://www.matrixscience.com) program and compared with the genomic data of the Actinobacteria class deposited in the NCBI nr database.

2D nanoUPLC-HDMSE data acquisition and Data Processing

The protein extracts from three biological replicates of each condition were concentrated using spin columns with a 10 kDa threshold (Millipore, Billerica, MA, USA) to perform the label-free proteomic analysis. The protein was denatured (0.1% RapiGEST SF at 60 °C for 15 min) (Waters, Milford, CA, USA), reduced (10 mM DTT), alkylated (10 mM iodoacetamide) and enzymatically digested with trypsin (Promega). The digestion process was stopped by adding 10 μL of 5% TFA (Fluka, Buchs, Germany), and glycogen phosphorylase (Sigma, Aldrich, P00489) was added to the digested samples after digest at 20 nmol.uL⁻¹ as an internal standard for normalization. Each replicate was injected using a two-dimensional reversed phase (2D RPxRP) nanoUPLC-MS (Nano Ultra Performance Liquid Chromatography Mass Spectrometry) approach with 171 multiplexed high definition mass spectrometry (HDMSE) label-free quantitation [25]. Qualitative and quantitative experiments were performed using both a 1 h reversed phase gradient from 7% to 40% (v/v) acetonitrile (0.1% v/v formic acid) at 500 nL.min⁻¹ and a nanoACQUITY UPLC 2D RPxRP Technology system [26]. A nanoACQUITY 174 UPLC HSS T3 1.8 μm, 75 μm x 15 cm column (pH 3) was used with an RP XBridge BEH130 C18 5 μm 300 μm x 50 mm nanoflow column (pH 10). Typical on-column sample loads were 250 ng of the total protein digests for each of the 5 fractions (250 ng/fraction/load). All analyses were performed using nano electrospray ionization in the positive ion mode nanoESI (+) and a NanoLockSpray (Waters, Manchester, UK) ionization source. The mass spectrometer was calibrated using an MS/MS spectrum of [Glu1]-Fibrinopeptide B human (Glu-Fib) solution (100 fmol.uL⁻¹) delivered through the NanoLockSpray source reference sprayer. Multiplexed data-independent (DIA) scanning with additional specificity and selectivity for non-linear ‘T-wave’ ion mobility (HDMSE) experiments were performed using a Synapt G2-S HDMS mass spectrometer (Waters, Manchester, UK).

Following the identification of proteins, the quantitative data were packaged using dedicated algorithms [27] and searching against a database with default parameters to account for ions [28]. The databases used were reversed on-the-fly during the database queries and appended to the original database to assess the false positive rate during identification. For proper spectra...
processing and database searching conditions, the ProteinLynxGlobalServer v.2.5.2 (PLGS) with IdentityE and ExpressionE informatics v.2.5.2 (Waters, Manchester, UK) was used. UniProtKB (release 2013_01) with manually reviewed annotations was used, and the search conditions were based on taxonomy (Corynebacterium pseudotuberculosis). One missed cleavage by trypsin was allowed be up to 1 and various modifications as carbamidomethyl (C), Acetyl N terminal, phosphoryl (STY) and oxidation (M) were allowed [29]. The proteins collected were organized by the PLGS ExpressionE tool algorithm into a statistically significant list that corresponded to higher or lower regulation ratios between the different groups. For protein quantitation, we used the PLGS v2.5.2 software with the IdentifyE algorithm using the Hi3 methodology. The search threshold to accept each spectrum was the default value for a false discovery rate 4%. The quantitation values were averaged over all samples, and the standard deviations of \( p < 0.05 \), which were determined using the ExpressionE software, refer to the differences between biological replicates.

### Bioinformatic analysis

The proteins identified in 1002_\textit{ovis} under both conditions were analyzed using the following prediction tools: SecretomeP 2.0 server, to predict proteins exported from non-classical systems (positive prediction score greater than 0.5) [30] and PIPS software, to predict proteins in the pathogenicity islands [31]. Gene ontology (GO) functional annotations were generated using the Blast2GO tool [32].

### Results

The main objective of this study was to assay the virulence of 1002_\textit{ovis} in a murine model after passage through mice. We thus carried out an in vivo survival assay using BALB/c mice infected with bacteria that did not contact with murine model (Ct) and bacteria recovered (Rc) from mice spleens. In this assay using an infective dose (10\(^5\) CFU), caseous lesions were detected in different organs (liver, left kidney and right kidney) of all the animals infected only with Rc (data not shown). Altogether, these results showed that the serial passage process in a murine model increased the virulence potential of strain 1002_\textit{ovis}. In addition, these results confirmed the low virulence of this strain, which was previously suggested based on the composition of its extracellular proteome [8–10].

After passage in BALB/c mice, a dramatic change in the virulence potential of strain 1002_\textit{ovis} was observed. We thus hypothesized that this phenotypic change was visible at the proteome level since \textit{C. pseudotuberculosis} virulence relies on the production of a proteinaceous virulence factor. Thus, considering the importance of extracellular proteins for bacterial virulence, the proteomic analysis was conducted on the extracellular proteomes of 1002_\textit{ovis} recovered from infected mice spleens in comparison to the control condition, using two proteomics approaches: 2-DE and 2D nanoUPLC-HDMS\textsuperscript{E}. The electrophoretic resolution of the extracellular protein extract of Ct and Rc condition allowed the visualization of spots distributed over \( \text{pH} \) 3–10 (Fig. 2). A total of 14 spots were found to be differentially expressed between Ct and Rc condition, these spots were excised out of the gel, and identified by MS/MS (Table 1). In the LC/MS analysis, we used the label-free quantitative proteomic to evaluate the relative difference between the proteome of Rc and Ct condition. In this analysis, only proteins which presented \( p < 0.05 \) and differential expression (log2 ratios) equal or greater than a factor of 1.2 were considered, as described previously [33]. We detected a total of 118 expressed differentially proteins, between Ct and Rc condition (Fig. 3) (Table 2 and Additional file 1). Also, 48 proteins were assigned only to Ct (Additional file 2) and 32 proteins were exclusive to Rc (Table 3) The information about sequence coverage and a number of identified peptides for each protein sequence identified, as well as the information about the native peptide are available at Additional file 3: Table S3.

The proteins identified in both conditions were analyzed by SecretomeP [29] to assess whether these proteins could be exported by non-classical secretion systems. Among the expressed differentially proteins 31\% (37 proteins) were predicted as secreted through non-classical secretion systems. In turn, when analyzed the exclusive proteome of each condition 19\% (6 proteins) and 27\% (13 proteins) were considered to be exported by non-classical secretion systems for recovered and control condition, respectively. The PIPS tool was used to evaluate whether the genes that encode the proteins which were differentially expressed and identified in the exclusive proteome of the Rc condition are included in predicted pathogenicity islands. According these analysis 16 proteins was encoded by genes located on a predicted pathogenicity island; these
proteins are related to cellular metabolism, pathogenesis, transport pathway, stress response and unknown function (Additional file 4). To classify the proteins identified in functional groups, we used the Blast2Go tool [31]; according to this analysis, the proteins were grouped into 17 biological processes (Fig. 4). Among these proteins, we identified processes that are directly involved in bacterial virulence, such as protein transport, pathogenesis, cell adhesion and stress response (Table 2).

Important factors directly linked to *C. pseudotuberculosis* virulence, like the PLD phospholipase, as well as, the CP40 protease were detected only in the proteome of recovered 1002_ovis (Tables 1 and 3). Also, components of several secretion systems were also activated in the bacteria recovered. These include proteins related to hemin uptake, ATP-binding cassette (ABC) transporters and the Opp transporter, like OppA, OppC, and OppD. Proteins related to detoxification process were also specifically identified in the Rc supernatant: e.g. the glutaredoxin-like protein NrdH, which belongs to the NrdH-redoxins, a family of small protein disulfide oxidoreductases [34], mycothiol glutathione reductase present in Actinobacteria [35] and copper resistance protein CopC (Tables 2 and 3). In addition, we have identified 31 proteins in the recovered condition that also were detected in a strain of *C. pseudotuberculosis* isolated directly from ovine lymph nodes [12] (Tables 2 and 3). Proteins involved in the resistance to antimicrobial agents, such as penicillin-binding proteins, metallo-beta-lactamase, and penicillin-binding protein transpeptidase and proteases like Clp protease involved in the expression of cytotoxins in

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**Fig. 1** Survival of Balb/C mice infected with strain 1002_ovis. **a** The survival rate was measured to determine the virulence profile of strain 1002_ovis control and recovered in mice infected with 10^6 CFU of bacteria Ct = control condition, Rc recovered condition. **b** Survival rates of mice infected with 10^7 CFU of Ct and Rc. **c** CFU in the spleen of BALB/c mice infected with control and recovered condition for the first five days of infection. **d** CFU in the different organs (spleen, left kidney, right kidney and liver) of BALB/c mice infected with control and recovered condition after four weeks of infection. The mortality rates were measured daily. Results represent three independent experiments. *P* values of *<0.05* were considered to be statistically significant, and asterisks indicate statistically significant differences.

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**Fig. 2** Two-dimensional electrophoresis of the extracellular proteins 1002_ovis after following passage process. **a** Control condition. **b** Recovered condition. Red circle: spot proteins identified by MS/MS.
**Staphylococcus aureus** and **Listeria monocytogenes** [36, 37] were found induced in Rc supernatant.

**Discussion**

To investigate the protein factors that could influence the adaptive processes of *C. pseudotuberculosis* biovar *ovis* during the infection process, we combined a unique bacterial passage experiment in mice with proteomic analyses of 1002* _ovis* culture supernatants, collected before and after passage. In the first analysis, we observed that strain 1002* _ovis* (isolated from caprine) exhibited a low virulence potential, which is consistent with previous reports indicating the low virulence potential of this strain [38, 39]. Although a recent in silico analysis of the 1002* _ovis* genome predicted various genes involved in virulence [40], studies examining the exoproteome of this strain under laboratory growth conditions failed to detect many of these virulence proteins (e.g., PLD exotoxin or proteins involved in the pathway of cell invasion, detoxification) [8–10].

One explanation for this relies on the fact that after being first isolated, strains 1002* _ovis* have been maintained, in vitro, under laboratory conditions with extensive passages on the culture medium, which may alter the gene

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**Table 1** List of proteins identified in 1002* _ovis* control and recovered by 2D-PAGE-MS/MS

| Spot | Description | Accession | MW(kDa)/p.I | Peptides Number | Mascot Score | Molecular function |
|------|-------------|-----------|-------------|----------------|--------------|--------------------|
| 5, 6, 7 | Hypothetical protein | ADL20032 | 24.30/9.24 | 2 | 189 | Unknown function |
| 11, 29 | Trypsin-like serine protease | ADL20653 | 25.72/6.49 | 2 | 96 | Serine-type endopeptidase activity |
| 15 | Hypothetical protein | ADL21714 | 42.04/5.22 | 4 | 159 | Catalytic activity |
| 20, 34 | Corynomycolyl transferase | ADL21610 | 41.80/7.05 | 2 | 58 | Transferase activity |
| 16 | Cytochrome c oxidase sub II | ADL21302 | 40.33/6.03 | 2 | 96 | Cytochrome-c oxidase activity |
| 21 | Hypothetical protein | ADL21914 | 12.30/5.04 | 2 | 53 | Unknown function |
| 12 | Hypothetical protein | ADL19922 | 19.86/4.30 | 2 | 145 | Calcium ion binding |
| 8 | Hypothetical protein | ADL09626 | 24.30/9.24 | 3 | 228 | Unknown |
| 27 | Hypothetical protein | ADL20508 | 31.62/9.52 | 2 | 66 | Unknown |
| 22 | Phospholipase D | ADL19935 | 34.09/8.91 | 4 | 286 | Sphingomyelin phosphodiesterase D activity |
| 3 | Enolase | ADL20605 | 45.17/4.68 | 3 | 271 | Phosphopyruvate hydratase activity |
| 17 | Trehalose corynomycolyl transferase B | ADL21814 | 36.67/6.90 | 5 | 245 | Transferase activity, transferring acyl groups other than |
| 24 | Hypothetical protein | ADL21714 | 40.90/5.05 | 3 | 190 | Catalytic activity |

**Fig. 3** Volcano Plot show Log(2) Fold Change of the differentially expressed proteins detected by label-free proteomics between the recovered and control condition. Green: Up-regulated proteins; Grey: unchanged proteins; Red: Down-regulated proteins
Table 2: Proteins differentially produced among the recovered and control condition

| Accession | Description                                                                 | Score   | Fold Change Log_{2} | SecretomeP |
|-----------|------------------------------------------------------------------------------|---------|---------------------|------------|
| D9QSH9_CORP1 | Periplasmic binding protein LacI                                                | 5601.78 | 3.26                | 0.612642   |
| D9Q6G4_CORP1 | Oligopeptide binding protein oppA                                              | 4120.1  | 3.00                | 0.892226   |
| D9Q4T5_CORP1 | ABC transporter domain containing ATP                                        | 1264.05 | 2.57                | 0.084974   |
| D9Q7K5_CORP1 | Oligopeptide binding protein oppA                                              | 33697.17| 2.11                | 0.873687   |
| D9Q5BB_CORP1 | Oligopeptide binding protein oppA                                              | 852.88  | 1.88                | 0.849217   |
| D9Q6C3_CORP1 | ABC type metal ion transport system permease                                  | 650.43  | 1.59                | 0.078043   |
| D9Q796_CORP1 | Glutamate binding protein GluB                                                | 6254.68 | −1.46               | 0.840325   |
| D9Q7W9_CORP1 | Iron(3+)-hydroxamate-binding protein fhuD                                    | 2774.62 | −1.62               | 0.824030   |
| D9Q71_CORP1  | Septum formation initiator protein                                            | 2071.46 | 1.38                | 0.551153   |
| D9Q5H7_CORP1 | Hypothetical protein                                                         | 115906.3| 1.51                | 0.840443   |
| D9Q71_CORP1  | GTP binding protein YchF                                                     | 3487.98 | 2.68                | 0.042575   |
| D9Q5F7_CORP1 | Chromosome partitioning protein ParB                                         | 2467.24 | 2.44                | 0.052395   |
| D9Q5G6_CORP1 | DNA polymerase III subunit beta                                               | 1907.74 | 1.80                | 0.071008   |
| D9Q5V6_CORP1 | Nucleoid associated protein                                                   | 68097.59| 1.59                | 0.070074   |
| D9Q6J8_CORP1 | DNA directed RNA polymerase subunit                                           | 29671.46| 1.38                | 0.094910   |
| D9Q748_CORP1 | tRNA rRNA methyltransferase                                                   | 2467.24 | 1.27                | 0.060356   |
| D9Q8L3_CORP1 | DNA directed RNA polymerase subunit omega                                     | 3784.13 | −1.21               | 0.700214   |
| D9Q6D1_CORP1 | DNA directed RNA polymerase subunit beta                                      | 2611.89 | −1.27               | 0.067182   |
| D9Q8AS_CORP1 | RNA polymerase-binding protein RbpA                                           | 10787.51| −1.75               | 0.103548   |
| D9Q584_CORP1 | 30S ribosomal protein S6                                                     | 20750.74| 4.82                | 0.047667   |
| D9Q6E4_CORP1 | Elongation factor G                                                         | 16882.71| 3.25                | 0.083231   |
| D9Q3I3_CORP1 | Peptidyl prolyl cis trans isomerase                                          | 61648.39| 2.91                | 0.142641   |
| D9Q835_CORP1 | Phenylalanine tRNA ligase beta subunit                                       | 1269.7  | 2.74                | 0.064869   |
| D9Q6L0_CORP1 | 50S ribosomal protein L13                                                    | 5689.37 | 2.64                | 0.101816   |
| D9Q6H2_CORP1 | 50S ribosomal protein L5β                                                    | 3269.32 | 2.12                | 0.076250   |
| D9Q918_CORP1 | Proline tRNA ligase b                                                       | 932.79  | 2.12                | 0.072151   |
| D9Q6C0_CORP1 | 50S ribosomal protein L10β                                                   | 27143.51| 1.86                | 0.031374   |
| D9Q6F6_CORP1 | 50S ribosomal protein L23β                                                   | 6947.79 | 1.85                | 0.060878   |
| D9Q6H1_CORP1 | 50S ribosomal protein L24                                                    | 27887.33| 1.75                | 0.078408   |
| F9Y2W9_CORP1 | Hypothetical protein                                                         | 3152.39 | 1.75                | 0.591013   |
| D9Q6H6_CORP1 | 30S ribosomal protein S8β                                                    | 4941.19 | 1.56                | 0.088407   |
| D9Q6F3_CORP1 | 30S ribosomal protein S10β                                                   | 25117.55| 1.54                | 0.048124   |
| D9Q6G2_CORP1 | 50S ribosomal protein L29                                                    | 2467.24 | 1.44                | 0.050948   |
| D9Q401_CORP1 | 50S ribosomal protein L27β                                                   | 2467.24 | 1.38                | 0.081399   |
| D9Q7E8_CORP1 | 50S ribosomal protein L25                                                    | 1358.05 | −1.28               | 0.037225   |
| D9Q6H8_CORP1 | 50S ribosomal protein L18                                                    | 8920.94 | −1.31               | 0.049024   |
| D9Q754_CORP1 | Homoserine dehydrogenase                                                    | 698.17  | −1.40               | 0.035138   |
Table 2  Proteins differentially produced among the recovered and control condition (Continued)

| Accession    | Description                                | Fold Change | p-Value |
|--------------|--------------------------------------------|-------------|---------|
| D9Q6B7_CORP1 | 50S ribosomal protein L1                   | 10218.08    | 0.633387|
| D9Q4T4_CORP1 | ATP dependent chaperone protein ClpB       | 1883,16     | 0.045308|
| D9Q8N9_CORP1 | Aspartate tRNA ligase                      | 1004,33     | 0.092415|
| D9Q752_CORP1 | Arginine tRNA ligase                       | 2679,11     | 0.051908|
| Pathogenesis |                                            |             |         |
| D9Q8M7_CORP1 | Metallopeptidase family M24                | 3213,83     | 0.050024|
| D9Q608_CORP1 | Penicillin binding protein transpeptidaseb | 1215,32     | 0.859830|
| D9Q827_CORP1 | Metallo beta lactamase superfamily proteinc| 629,38      | 0.144158|
| D9Q721_CORP1 | Hypothetical proteinc                      | 112025      | 0.260801|
| D9Q7K8_CORP1 | Trypsin like serine protease               | 35041,27    | 0.648370|
| D9Q416_CORP1 | ATP dependent Clp protease proteolyticb    | 2467,24     | 0.087255|
| D9Q639_CORP1 | Secreted hydrolaseb                       | 22798,13    | 0.072385|
| D9Q588_CORP1 | Penicillin binding proteinb                | 9951,61     | 0.916125|
| Energy metabolism |                                      |             |         |
| D9Q787_CORP1 | Glucose-6-phosphate isomerase              | 1025,89     | 0.058841|
| D9Q7G0_CORP1 | Enolaseb                                   | 53290,95    | 0.068928|
| D9Q651_CORP1 | Succinate dehydrogenase flavoprotein       | 797,48      | 0.159059|
| D9Q4P2_CORP1 | Acetate kinasedb                           | 10828,79    | 0.063340|
| D9Q8G5_CORP1 | Aconitase hydratasedb                     | 4250,81     | 0.217637|
| D9Q4Z7_CORP1 | Phosphenolpyruvate carboxykinase GTPb      | 8764,35     | 0.147167|
| D9Q7X0_CORP1 | 6 phosphofructokinase                      | 1806,65     | 0.052885|
| D9Q648_CORP1 | Dihydrolipoyl dehydrogenase               | 4110,08     | 0.047180|
| D9Q7T8_CORP1 | ATP synthase subunit alpha                 | 2467,24     | 0.070875|
| D9Q752_CORP1 | Citrate synthase                          | 6299,21     | 0.116042|
| D9Q89S_CORP1 | 6-Phosphogluconate dehydrogenase          | 4246,26     | 0.050906|
| Lipid metabolism |                                      |             |         |
| D9Q520_CORP1 | Glycerophosphoryl diester phosphodiesterc  | 2494,25     | 0.802154|
| D9Q718_CORP1 | Methylmalonyl CoA carboxyltransferase 1b   | 2467,24     | 0.049504|
| Amino acid metabolism |                       |             |         |
| D9Q5X8_CORP1 | Aspartokinaseb                             | 1944,81     | 0.043575|
| D9Q4C2_CORP1 | Succinyl CoA Coenzyme A transferase        | 10894,63    | 0.061344|
| D9Q3L8_CORP1 | Glutamine synthetase                      | 320,71      | 0.263700|
| D9Q8H7_CORP1 | Cysteine desulfurase                      | 1689,36     | 0.067087|
| Stress response |                                 |             |         |
| D9Q929_CORP1 | Mycothione glutathione reductase           | 490,36      | 0.085017|
| D9Q5T5_CORP1 | Glyoxalase Bloomycin resistance proteinc   | 8420,32     | 0.226764|
| D9Q424_CORP1 | DSBA oxidoreductase                       | 12179,8     | 0.061566|
| D9Q566_CORP1 | Universal stress protein Ab                | 2498,69     | 0.034684|
| D9Q4P4_CORP1 | Ferredoxin ferredoxin NADP reductaseb      | 1086,71     | 0.083585|
| D9Q824_CORP1 | Stress related proteinb                    | 2467,24     | 0.035291|
| D9Q692_CORP1 | Thiol disulfide isomerase thioredoxin      | 3721,88     | 0.438415|
| Metabolism of nucleotides and nucleic acids |                    |             |         |
| D9Q4Y6_CORP1 | Deoxyctydine triphosphate deaminase        | 887,26      | 0.216897|
| Accession | Description | Fold Change | p-value |
|-----------|-------------|-------------|---------|
| D9Q6J1_CORP1 | Adenylate kinase | 15629,86 | 2,21 | 0.059568 |
| D9Q8L4_CORP1 | Guanylate kinase | 2467,24 | 1,34 | 0.050095 |
| D9Q6T2_CORP1 | Ribokinase | 890,09 | −1,23 | 0.032324 |
| D9Q4E9_CORP1 | Adenylosuccinate lyase | 1441,99 | −1,54 | 0.035597 |
| D9Q6P0_CORP1 | D methionine binding lipoprotein metQ | 11519,67 | −1,93 | 0.817217 |

**Carbohydrate metabolism**

| Accession | Description | Fold Change | p-value |
|-----------|-------------|-------------|---------|
| D9Q8V2_CORP1 | UDP glucose 4 epimerase | 2001,76 | 3,13 | 0.094403 |
| D9Q6V6_CORP1 | Phosphomannomutase ManB | 1730,63 | 2,05 | 0.053146 |
| D9Q659_CORP1 | Formate acetyltransferase | 5456,95 | 1,54 | 0.539548 |
| D9Q423_CORP1 | Ribose-5-phosphate isomerase B | 2467,24 | 1,38 | 0.064467 |
| D9Q6V1_CORP1 | Mannose-1-phosphate guanylyltransferase | 1612,45 | −1,21 | 0.068085 |

**Nitrogen metabolism**

| Accession | Description | Fold Change | p-value |
|-----------|-------------|-------------|---------|
| D9Q4Q8_CORP1 | Cytochrome c nitrate reductase small | 1118,33 | 2,68 | 0.901856 |

**Unknown function**

| Accession | Description | Fold Change | p-value |
|-----------|-------------|-------------|---------|
| D9Q6T0_CORP1 | Hypothetical protein | 2277,6 | 3,62 | 0.050552 |
| D9Q4R2_CORP1 | Hypothetical protein | 442,07 | 3,35 | 0.866986 |
| D9Q6N1_CORP1 | Hypothetical protein | 561,84 | 3,02 | 0.062141 |
| D9Q8Q4_CORP1 | Hypothetical protein | 7271,5 | 2,96 | 0.974016 |
| D9Q832_CORP1 | Hypothetical protein | 1774,59 | 2,90 | 0.752478 |
| D9Q358_CORP1 | Hypothetical protein | 837,6 | 2,78 | 0.231421 |
| D9Q7M9_CORP1 | Hypothetical protein | 3246,28 | 2,60 | 0.147602 |
| D9Q76_CORP1 | Hypothetical protein | 3751,96 | 2,42 | 0.707595 |
| D9Q739_CORP1 | Hypothetical protein | 2845,77 | 2,28 | 0.836229 |
| D9Q4C5_CORP1 | Hypothetical protein | 1339,3 | 1,83 | 0.023133 |
| D9Q5C3_CORP1 | Hypothetical protein | 111234,6 | 1,49 | 0.946918 |
| D9Q700_CORP1 | Hypothetical protein | 2467,24 | 1,49 | 0.072810 |
| D9Q657_CORP1 | Hypothetical protein | 1172,66 | 1,41 | 0.830926 |
| D9Q6F2_CORP1 | Hypothetical protein | 2467,24 | 1,34 | 0.061860 |
| D9Q7X5_CORP1 | Hypothetical protein | 38716,45 | −1,21 | 0.825761 |
| D9Q4T9_CORP1 | Hypothetical protein | 553,76 | −1,28 | 0.934591 |
| D9Q6R6_CORP1 | Hypothetical protein | 1457,62 | −1,40 | 0.206908 |
| D9Q890_CORP1 | Hypothetical protein | 1948,52 | −1,51 | 0.847549 |
| D9Q6M6_CORP1 | Hypothetical protein | 1935,68 | −1,90 | 0.823541 |

**Others**

| Accession | Description | Fold Change | p-value |
|-----------|-------------|-------------|---------|
| D9Q6S_CORP1 | Maltotriose binding protein | 5210,9 | 5,22 | 0.864851 |
| D9Q4A3_CORP1 | DsbG protein | 3101,13 | 2,06 | 0.814366 |
| D9Q6N9_CORP1 | D methionine binding lipoprotein metQ | 2665,58 | 1,79 | 0.764416 |
| D9Q732_CORP1 | Carbonic anhydrase | 689,15 | 1,66 | 0.130559 |
| D9Q6W6_CORP1 | Lipoprotein LpqB | 1484,31 | 1,63 | 0.670057 |
| D9Q556_CORP1 | LSR2 like protein | 2714,21 | 1,49 | 0.096802 |
| D9Q5Q0_CORP1 | UPF0145 protein | 2467,24 | 1,37 | 0.025009 |
| D9Q7W0_CORP1 | Hypothetical protein | 1682,98 | 1,26 | 0.039678 |
| D9Q701_CORP1 | UPF0182 protein | 1682,98 | 1,26 | 0.869411 |
| D9Q8A3_CORP1 | Protein yceI | 16885,01 | 1,21 | 0.901679 |
### Table 2: Proteins differentially produced among the recovered and control condition (Continued)

| Accession     | Description                              | Score | Fold Change (Ratio) | p-value |
|---------------|------------------------------------------|-------|---------------------|---------|
| D9Q5X4_CORP1  | Serine aspartate repeat containing protein | 528.36| −1.82               | 0.892317|
| D9Q826_CORP1  | DoxX family protein                      | 697.26| −2.08               | 0.614317|
| D9Q7W3_CORP1  | Mycothiol acetyltransferase              | 947.33| −2.11               | 0.214833|
| D9Q407_CORP1  | Ornithine cyclodeaminase                 | 2566.18| −2.58              | 0.048247|

*Fold change - Ratio values to: 1002Rc:11002Ct_Log(2)Ratio ≥ 1.2 proteins with p < 0.05*
*Identified in an isolated of C. pseudotuberculosis from ovine lymph nodes [Rees et al. [12]]*
*Induced in 1002_ovis during to stress nitrosative [Pacheco et al. [57], Silva et al. [58]]*
*Predicted LPXTG cell wall-anchoring motif*

### Table 3: List of proteins identified in the exclusive proteome of recovered-condition

| Accession   | Description                              | Score | Biological process   | SecretomeP |
|-------------|------------------------------------------|-------|----------------------|------------|
| D9Q869_CORP1| Esterasea                              | 251.44| Others               | 0.862935   |
| D9Q575_CORP1| Cation transport protein                | 1961.29| Transport           | 0.062276   |
| D9Q5NS_CORP1| Uncharacterized iron regulated membraneb| 46.77 | Transport            | 0.855681   |
| D9Q3T9_CORP1| Pyridoxamine kinase                    | 216.2 | Cofactor metabolism  | 0.083313   |
| D9Q751_CORP1| Phosphoserine aminotransferase         | 639.64| Amino acid metabolism| 0.151778   |
| D9Q537_CORP1| LytR family transcriptional regulatora| 375.8 | Transcription        | 0.766483   |
| D9Q7F2_CORP1| Multicopper oxidase                    | 74.63 | Stress response      | 0.278840   |
| D9Q25_CORP1 | ABC transporter substrate binding lipoprotein | 283.38| Transport             | 0.452814   |
| D9Q6P2_CORP1| Manganese ABC transporter substrate bindinga| 236.6 | Transport             | 0.774461   |
| D9Q48_CORP1 | Phosphate ABC transporter phosphate bindinga| 125.4 | Transport             | 0.840195   |
| D9Q400_CORP1| D alanyl D alanine carboxypeptidase OS  | 426.74| Others               | 0.232261   |
| D9Q4T7_CORP1| Hypothetical protein                   | 157.52| Unknown function     | 0.349026   |
| D9Q5A_CORP1 | Hypothetical protein                   | 218.02| Unknown function     | 0.907333   |
| D9Q46_CORP1 | Hypothetical protein                   | 510.32| Unknown function     | 0.066368   |
| D9Q5B_CORP1 | Glucosamine-6-phosphate deaminaseb     | 524.55| Carbohydrate metabolism | 0.079507 |
| D9Q4J_CORP1 | Glutamate racemase                     | 343.98| Cell wall organization| 0.040278   |
| D9Q7N_CORP1 | O-methyltransferase                    | 619.11| DNA process          | 0.032455   |
| D9Q63_CORP1 | Gamma type carbonic anhydratase        | 577.75| Others               | 0.035357   |
| D9Q4X_CORP1 | Urease accessory protein UreD          | 333.12| Others               | 0.055896   |
| D9Q5O_CORP1 | Phospholipase D                      | 40.25 | Pathogenesis         | 0.409585   |
| D9Q5B CORP1 | Copper resistance protein CopC        | 4315.26| Stress response      | 0.964015   |
| D9Q493_CORP1| Glutaredoxin like protein mrDH        | 725.98| Stress response      | 0.033036   |
| D9Q66_CORP1 | ATP dependent RNA helicase rhE        | 1438.25| Transcription        | 0.060627   |
| D9Q4M_CORP1 | Cell wall channel                     | 4008.59| Transport             | 0.025882   |
| D9Q4V_CORP1 | CP40                                   | 558.79| Pathogenesis         | 0.926013   |
| D9Q49_CORP1 | Hypothetical protein                   | 1278.45| Unknown function     | 0.953803   |
| D9Q68_CORP1 | Hypothetical protein                   | 326.47| Unknown function     | 0.918886   |
| D9Q4S_CORP1 | Hypothetical protein                   | 2795.11| Unknown function     | 0.890081   |
| D9Q4N_CORP1 | Hypothetical protein                   | 708.75| Unknown function     | 0.857505   |
| D9Q5N_CORP1 | Hypothetical protein                   | 475.62| Unknown function     | 0.472378   |
| D9Q4L_CORP1 | Hypothetical protein                   | 5324.08| Unknown function     | 0.038893   |
| D9Q4T_CORP1 | Hypothetical protein                   | 732.37| Unknown function     | 0.037132   |

*Induced in 1002_ovis during to stress nitrosative [Pacheco et al. [57], Silva et al. [58]]*
*Identified in an isolated of C. pseudotuberculosis from ovine lymph nodes [Rees et al. [12]]*
expression profile of the strain, especially for effectors related to bacterial virulence. This phenomenon has also been reported in other pathogens such as *Mycobacterium bovis*, *Helicobacter pylori*, *S. aureus*, and *L. monocytogenes*. In vitro passages of these bacteria on culture medium altered both bacterial physiology and virulence profile [41–44]. However, we showed that the bacterial passage process in a murine model changed the virulence potential of strain 1002 _ovis_. Previous reports on experimental serial passages showed that pathogens such as *H. pylori*, *Escherichia coli*, *Xenorhabdus nematiphila*, *Arcobacter butzleri*, and *Salmonella enterica* also exhibited altered virulence profiles after in vivo passage in a host, which helped identifying factors that contribute to infectious process [14–19]. Thus, as observed in these pathogens, the recovered condition also showed increased capacity to persist into host, when compared with control condition. The altered physiology and virulence status observed in 1002 _ovis_ is supported by our proteomic analyses, where several proteins involved in processes favoring infection and host adaptation were differentially expressed after passage in mice.

Although our study focused on the *C. pseudotuberculosis* extracellular proteins, cytoplasmic proteins were also detected in the proteomic analyses. The presence of cytoplasmic proteins in the extracellular fraction is reported in several other proteomic studies [8–10, 12, 45]. It may be partially due to cell lysis and thus, be considered artifacts. However, cytoplasmic proteins in the culture supernatant may act as _moonlighting_ proteins and be exported via a non-classical secretion pathway [30, 46]. The _moonlighting_ proteins are described both Gram-positive and Gram-negative bacteria, and can be detected in different subcellular locations (cytoplasm, membrane, cell surface, and extracellular environment) and exhibit distinct functional behavior depending on the host cell type [46, 47]. Interestingly, some proteins, such as Chromosome partitioning protein ParB, Phosphoenolpyruvate carboxykinase GTP, Methylmalonyl CoA carboxyltransferase 12S subunit, Acetate kinase, and Enolase, induced in the Rc supernatants were identified only in the membrane shaving of *C. pseudotuberculosis* harvested directly from ovine lymph nodes [12].

The passage process in mice was also able to induce other proteins identified in Rc supernatants, and which contribute to the adhesion process. Proteins with an LPTXG domain, which characterizes the cell-wall anchored proteins, were identified and included monomers of membrane pilus. This latter class of proteins is described in pathogenic *Corynebacterium* species and may contribute especially in the process of cellular adhesion [48]. In *Campylobacter jejuni*, serial passages in mice induce the expression of invasiveness and increase the capacity of cell invasion [13]. Components of the Opp system were induced by the passage process, too. The Opp system facilitates the uptake of extracellular peptides, which are further used as carbon and nitrogen sources for bacterial nutrition [49]. Proteins that comprise the Opp system also were induced in a field isolated of *C. pseudotuberculosis* biovar _ovis_, when

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**Fig. 4** Biological processes differentially regulated in 1002 _ovis_ after passage in mice. Analysis of the differentially expressed proteins grouped into biological processes for strain 1002 _ovis_ after passage in mice.
compared with the strain C231_ovis a laboratory reference strain [12, 50]. In the pathogen Mycobacterium avium the OppA gene was highly expressed during the infection in a mouse model [51]. We have identified known secreted virulence factors as CP40 serine protease, which previously shown to be necessary for C. pseudotuberculosis virulence potential and to induce an immune response [52, 53].

An important factor that precedes the chronic stage of infection by C. pseudotuberculosis is the capacity of this pathogen to disseminate within the host, which consequently favors the establishment of the disease [3]. In C. pseudotuberculosis, this process is mediated by the action of PLD exotoxin, a major virulence factor of this pathogen [54, 55] that catalyzes the dissociation of sphingomyelin and increases vascular permeability, which contributes to the dissemination process of C. pseudotuberculosis in the host. Here, PLD was only detected in the proteome of the Rc condition. This result is noteworthy because, a previous proteomic study performed by our research group, PLD was not identified in the extracellular proteome of 1002_ovis [8–10]. McKean et al. [5] showed that pld expression is expressed by different environmental factors, thus during the infection and recuperation process 1002_ovis was exposed to different environmental and stimulus, which may have affected the pld expression. A study showed that a pld mutant strain is indeed unable to disseminate and yields reduced virulence [55]. Here, we observed the presence of caseous lesions in different organs only at the end of experimental infection, only in the group of mice infected with the Rc condition. Altogether, the observations suggest that the expression of PLD can be modified by the passage in the host and can thus change the virulence potential of 1002_ovis.

Another attribute of PLD is its capacity to alter the viability of macrophage cells during the infection [5]. However, before promoting macrophages lysis, C. pseudotuberculosis has to be able to resist the hostile environment inside macrophages mainly against reactive oxygen species (ROS) and reactive nitrogen species (RNS). Thus, the induction of proteins involved in detoxification processes in Rc could be contributed for its resistance against ROS and RNS. The inductions of proteins related to oxidative stress also were observed in Shigella flexneri, after recuperation process in an in vivo infection model. We detected the mycothione glutathione reductase, a component of the mycothiol system, which is present in Mycobacterium and Rhodococcus genera. This system is used as an alternative mechanism of disulphide reduction and contributes to the cytosolic redox homeostasis and the resistance to ROS [35]. Glutaredoxin-like protein, NrdH, which plays an important role in the resistance to ROS, and is present in C. glutamicum [34] and M. tuberculosis [56] was also detected.

On the other hand, some proteins like dihydroxybiphenyl dioxygenase, Metallo beta lactamase superfamily protein, Formamidopyrimidine DNA glycosylase, MerR family transcriptional regulator, which were induced by 1002_ovis during the exposition to nitric oxide [57, 58] were also found induced in this study in the recovered condition. These proteins are related to different processes of resistance to nitrosative stress, DNA repair, antibiotic resistance, and transcription, these results show a set of proteins involved in the adaptation process of 1002_ovis to nitric oxide, which could contribute to the pathogenic process of this pathogen. Another type of defense of the host immune system against bacterial infection is the utilization of copper [59]. Here, CopC, a protein related to copper resistance, was detected in recovered 1002_ovis. In M. tuberculosis, proteins involved in copper resistance are essential to virulence [60, 61]. Thus, the association of this factor related to an antioxidant system with PLD could promote an effective pathway of defense against the action of the innate immune system and consequently contributes to virulence process of C. pseudotuberculosis.

Conclusion
In conclusion, the virulence potential and proteomic profiles of strain 1002_ovis undergo dramatic changes after recovery from experimentally infected mice. The proteomic screening outlined, after the serial passage in murine model showed a set of proteins that were induced in the recovered condition. Into this group were detected known secreted virulence factors, as well as some proteins which could contribute in its virulence. Therefore, more study is necessary to show the true role of these proteins in the virulence of C. pseudotuberculosis. Altogether, our results demonstrate that in vitro passages alter the expression of C. pseudotuberculosis exoproteome leading to a reduced virulence and that a single passage in vivo, in a murine model, can induce significant changes in the C. pseudotuberculosis extracellular proteome, contributing to the increase in virulence of this pathogen.

Additional files
Additional file 1: Table S1. Complete list of proteins differentially produced between the recovered and control condition of strain 1002_ovis. (XLSX 44 kb)
Additional file 2: Table S2. List of proteins identified in the exclusive proteome of control condition. (XLSX 12 kb)
Additional file 3: Table S3. Total list of peptide and proteins identified by LC-MS
together with all results. (XLSX 3 mb)
Additional file 4: Table S4. Proteins identified in the recovered condition detected in pathogenicity island. (XLSX 10 kb)

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Availability of data and materials
The datasets supporting the results of this article were then concatenated into a *.xltx file at peptide and protein level to fulfill the requirements and is available at supplemental material including sequence coverage and a number of identified peptides for each protein sequence identified. It also includes the native peptide information. In addition other data are included within the article.

Authors’ contributions
VA, WMS, and FAD designed the experiments. WMS, TLPC, and NS performed microbiological analyses and sample preparation for proteomic analysis. GHMFS and WMS conducted the proteomic analysis. WMS and SCS performed bioinformatics analysis of the data. YLL, AM, and YL participated substantially to data interpretation and revisions. VA, AS, and YL participated in all steps of the project as coordinators, and critically reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
All animals used in this study were provided by the Animal Care Facility of the Biological Sciences Institute from the Federal University of Minas Gerais and were handled by the guidelines of the UFMG Ethics Committee on Animal Testing (Permit Number: CETEA 103/2011).

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