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Original article

1 **Dual proteomic signature of immune cells and *Yersinia pestis* upon** 2 **blood infection**

3 Running title: Proteomics of *Yersinia pestis* bacteremia

4 **Authors**

5 Pierre Lê-Bury¹, Thibaut Douché², Quentin Gai Gianetto^{2,3}, Mariette Matondo², Javier Pizarro-
6 Cerdá^{1,4}, Olivier Dussurget¹

7 **Affiliations**

8 ¹Institut Pasteur, Université Paris Cité, CNRS UMR6047, *Yersinia* Research Unit, F-75015
9 Paris, France

10 ²Institut Pasteur, Université Paris Cité, CNRS UAR2024, Proteomic Platform, Mass Spec-
11 trometry for Biology Unit, F-75015 Paris, France

12 ³Institut Pasteur, Université Paris Cité, CNRS USR3756, Biostatistics and Bioinformatics
13 Hub, F-75015 Paris, France

14 ⁴Institut Pasteur, Université Paris Cité, *Yersinia* National Reference Laboratory, WHO Col-
15 laborating Research and Reference Centre for Plague FRA-140, F-75015 Paris, France

16 **Abstract**

17 Emerging and reemerging infectious diseases represent major public health concerns.
18 The urgent need for infection control measures requires deep understanding of molecular
19 pathogenesis. Global approaches to study biological systems such as mass-spectrometry
20 based proteomics benefited from groundbreaking physical and bioinformatical technologi-
21 cal developments over recent years. However, dual proteomic study of highly pathogenic
22 microorganisms and their hosts in complex matrices encountered during infection remains
23 challenging due to high protein dynamic range of samples and requirements imposed in
24 biosafety level 3 or 4 laboratories. Here, we constructed a dual proteomic pipeline of *Yersinia*
25 *pestis* in human blood and plasma, mirroring bacteremic phase of plague. We provide the
26 most complete *Y. pestis* proteome revealing a major reshaping of important bacterial path-
27 ways such as methionine biosynthesis and iron acquisition in human plasma. Remarkably,
28 proteomic profiling in human blood highlights a greater *Yersinia* outer proteins intoxication
29 of monocytes than neutrophils. Our study unravels global expression changes and points
30 to a specific pathogenic signature during infection, paving the way for future exploration of
31 proteomes in the complex context of host-pathogen interactions.

32 **Keywords**

33 Dual proteomics/human blood/monocytes/neutrophils/*Yersinia pestis*

34 **Subject Categories**

35 Microbiology, Virology and Host Pathogen Interaction, Proteomics

36 Introduction

37 Infectious diseases emerging and reemerging worldwide are major public health issues.
38 The understanding of microbial pathogenesis necessary for infection control immensely ben-
39 efits from technological progress. In particular, the current omics era shows spectacular
40 advances allowing affordable, fast and reliable genome sequencing and deep-coverage
41 of whole-genome transcriptomes. Simultaneously, whole-proteome exploration is quickly
42 gaining ground as the next step for functional studies in systems biology. Classically, pro-
43 teomics pipeline includes 3 steps: sample preparation, followed by data acquisition in mass-
44 spectrometry and *in silico* data analysis. In the last decade, many breakthroughs focused on
45 sample preparation, leading to more efficient protein extraction protocols such as Filter Aided
46 Sample Preparation (FASP) (Wiśniewski, Zougman, et al. 2009), Single-Pot Solid-Phase-
47 Enhanced Sample Preparation (SP3) (Hughes et al. 2014), Suspension Trapping (STrap)
48 (Zougman et al. 2014) and detergent-free method known as Sample Preparation by Easy
49 Extraction and Digestion (SPEED) (Doellinger et al. 2020). Meanwhile, mass-spectrometry
50 and data management constantly improved, supported by development of the Orbitrap tech-
51 nology (Hu et al. 2005) and impressive growth of computational power, allowing deeper and
52 quicker acquisition of proteomic data. However, complex samples are still challenging to
53 tackle. Culture-independent bacterial identification by proteo-typing in complex matrices
54 such as blood or urine using tandem mass-spectrometry for clinical diagnosis is in its in-
55 fancy (Roux-Dalvai et al. 2019; Kondori et al. 2021), and very few dual-proteomics research
56 studies investigating complex host-pathogen interactions have been published (Geddes-
57 McAlister et al. 2021; Ball et al. 2020; Willems et al. 2021; Leseigneur et al. 2022; Masson
58 et al. 2021). Additionally, research on highly virulent microorganisms is restricted by regula-
59 tory requirements for safety purposes. Mandatory sample treatment steps generally include
60 pathogen inactivation and pathogen DNA and RNA elimination in biosafety level (BSL) -3 or
61 -4 laboratories.

62 *Yersinia pestis* is the highly virulent bacillus responsible for plague, a quickly fatal human
63 disease if left untreated. This reemerging disease whose epidemics had a considerable
64 death toll in human history generally manifests as bubonic plague following bites from fleas
65 infected with *Y. pestis*. Primary pulmonary plague is the second form of the disease, which
66 occurs by inhalation of infected respiratory droplets or aerosols usually emitted by an infected
67 patient. In some cases, injection of *Y. pestis* by fleas directly into the bloodstream leads to so-
68 called septicemic plague (Perry et al. 1997). In the three forms of the disease, bacteria reach
69 the blood resulting in systemic infection. While plague pathogenesis has been investigated

70 for decades, the bacteremic phase of the disease remains to be fully deciphered.

71 Here, we report the design of experimental pipelines allowing characterization of human
72 and bacterial proteomes in plasma and blood infected with *Y. pestis*. We first applied protein
73 extraction methods to *Y. pestis* comparing the SPEED method (Doellinger et al. 2020) based
74 on trifluoroacetic acid (TFA) lysis, the standard bead-beating lysis in urea followed by the
75 FASP method (Wiśniewski, Zougman, et al. 2009), and sonication in sodium dodecyl sulfate
76 (SDS) followed by buffer exchange and the FASP method (Wiśniewski, Zougman, et al.
77 2009). SPEED surpassed the other methods, allowing quick, efficient and safe sample
78 preparation in BSL-3 environment with fully virulent *Y. pestis*. It was thus further used to
79 conduct the first dual proteomic study of a bacterial species in human plasma and human
80 blood, revealing the pathogenic proteomic signature of *Y. pestis*.

81 Results

82 **Comparison of urea-, SDS- and TFA-based methods for *Y. pestis* proteomics.** To
83 study the *Y. pestis* proteome, we first used the avirulent *Y. pestis* CO92 strain lacking pCD1
84 in a BSL-2 environment. Bacteria were grown on lysogeny broth with 0.002% pork hemin
85 (LBH) agar plates at 37°C for 24 hours. We compared the three following protein extraction
86 protocols: i) urea-based method: bacterial lysis by bead beating in 8M urea buffer, followed
87 by reduction-alkylation and digestion using the FASP method, ii) SDS-based method: bac-
88 terial resuspension in 4% SDS buffer, heating at 95°C and lysis by sonication, followed by
89 buffer exchange with 8M urea, reduction-alkylation and digestion using the FASP method,
90 and iii) TFA-based SPEED method: bacterial lysis upon TFA acidification, followed by neu-
91 tralization with 10 volume of 2M Tris base, reduction-alkylation and digestion.

92 Three protein quantification methods were compared to assess their compatibility with
93 urea, SDS or TFA-Tris buffers. Bradford (Bradford 1976), BCA (Smith et al. 1985) and Qubit
94 assays were only compatible with one buffer each, and not with 10% TFA-2M Tris base buffer
95 (Table 1). Consequently, we implemented protein quantification by tryptophan fluorescence
96 spectroscopy (Wiśniewski and Gaugaz 2015) which was compatible with the three buffers
97 (Table 1, Fig EV1A).

98 After data-dependent acquisition (DDA) analysis of triplicates using an Orbitrap mass-
99 spectrometer, we could detect a total of 1,587 proteins with at least one of the protocols,
100 1,390 bacterial proteins with the urea-based protocol, 1,444 proteins with the SDS-based
101 protocol and 1398 proteins with the TFA-based protocol (Fig EV1B, Table EV1). Overall

102 1,194 proteins were common to the three protocols, 30 proteins were only detected using
103 the urea-based protocol, 72 proteins were specifically detected using the SDS-based pro-
104 tocol and 34 proteins were exclusive to the TFA-based protocol (Fig EV1B). A total of 189
105 proteins were detected using urea- or SDS-based protocols but not by using the TFA-based
106 protocol. While the three protocols led to similar *Y. pestis* proteomes, the TFA-based protocol
107 surpassed both bead-beating and sonication methods, which required a fastidious centrifu-
108 gation step, by its quick implementation, high-throughput potential and application to very
109 small amount of biomass, which is important when dealing with precious purified fractions
110 from complex samples. Moreover, the minimal number of steps of the SPEED method re-
111 duced the risk of experimental errors and variability. These benefits led us to choose the
112 SPEED method to further investigate *Y. pestis* proteome in a BSL-3 environment.

113 **Inactivation of virulent *Y. pestis* using the SPEED method in BSL-3 environment.**

114 Mass spectrometric analysis of virulent *Y. pestis* proteome performed in BSL-1 platform re-
115 quires bacterial inactivation according to French regulatory protocols for “Micro-Organismes
116 et Toxines” (MOT) from the Agence Nationale de Sécurité du Médicament et des Produits
117 de Santé (ANSM). *Y. pestis* CO92 was grown on LBH agar plates at 37°C for 24 hours.
118 Bacterial proteins were extracted using the SPEED method. To validate absence of viable
119 bacteria, lysogeny broth (LB) was inoculated with TFA lysates before and after neutralization
120 and incubated under agitation at 28°C for 72 hours. Absence of growth measured by optical
121 density at 600 nm certified inactivation of viable bacteria in both samples. To assess ab-
122 sence of bacteriostatic effect of the inactivating agent, the LB samples were spiked with 1 to
123 2 bacteria and incubated 72 hours at 28°C. Growth measured at 600 nm revealed absence
124 of bacteriostatic effect of the inactivating agent. *Y. pestis* specific phage assay was used to
125 assess the absence of contamination of the spiked sample. To fulfill the MOT regulation,
126 bacterial DNA degradation was assessed by PCR on *Y. pestis* *caf1*, *yopM*, and *pla* genes
127 after a 30-minutes dialysis to remove inhibitory salts. While amplicons corresponding to the
128 three genes were detected when PCR was performed on *Y. pestis* DNA used as positive con-
129 trol, they could not be detected when PCR was performed on SPEED samples. Together,
130 our results indicate that the SPEED method allows bacterial inactivation and DNA degra-
131 dation directly after bacterial lysis and, consequently, that the following reduction, alkylation
132 and digestion steps and mass spectrometry can be performed in a BSL-1 environment.

133 **Proteomic reshaping of *Y. pestis* grown in human plasma.** To identify *Y. pestis* proteins
134 expressed in conditions mimicking the human bacteremic phase of plague, we first com-

135 pared the proteomes of bacteria grown in human plasma or in culture media. Proteomes
136 of the fully virulent *Y. pestis* CO92 strain were characterized in six BSL-3 culture conditions
137 (Fig 1A): i) inoculum grown overnight on LBH agar at 25°C (I25), ii) inoculum grown overnight
138 on LBH agar at 37°C (I37), iii) growth in chemically defined medium M9 at 37°C inoculated
139 with I25 (I25-M9), iv) growth in M9 at 37°C inoculated with I37 (I37-M9), v) growth in human
140 plasma at 37°C inoculated with I25 (I25-Plasma) and vi) growth in human plasma at 37°C
141 inoculated with I37 (I37-Plasma). The two different inoculum temperatures reflect the envi-
142 ronmental origin of bacteria entering the bloodstream: 25°C mimics direct transmission from
143 fleas in primary septicemic plague and 37°C mirrors hematogenous dissemination from the
144 bubo or lungs in secondary septicemic plague.

145 We identified 2,382 bacterial proteins in at least one sample across all conditions, from
146 a total of 3,915 coding sequences and small open reading frame-encoded proteins (Ta-
147 ble EV2) (Cao et al. 2021; Parkhill et al. 2001). Optimization of Orbitrap-based mass-
148 spectrometry in DDA mode led to a gain in the number of proteins detected compared to
149 our initial proteomic analysis comparing the extraction protocols, which identified only 1,587
150 proteins (see above). Between 2,025 to 2,205 proteins were identified in LBH and M9 sam-
151 ples (Fig 1B). The number of protein identifications was reduced to 1,825-1,878 in plasma
152 samples, presumably due to presence of human proteins (Fig 1B). The most extensive de-
153 tection was achieved in the LBH sample grown at 25°C in which 2,205 proteins were identi-
154 fied in the three replicates compared to the 2,033 proteins detected in LBH samples grown
155 at 37°C. Detection of proteins of lower abundance could have been prevented at 37°C by
156 the presence of highly produced proteins in the samples at that temperature, such as the
157 F1 pseudocapsule (Table 2) and components of the type three secretion system (T3SS)
158 (Demeure et al. 2019) (Table 3).

159 Comparison of differential abundances of the proteins between samples revealed exten-
160 sive proteome reshaping across conditions (absolute fold change > 2 and adjusted p-value
161 < 0.01 , Fig 1C). Expectedly, a shift in temperature and culture medium (I25 vs I25-M9, I25 vs
162 I25-Plasma) was associated with the highest number of differentially abundant proteins, with
163 439 and 444 proteins (taking into account only the proteins detected in both compared condi-
164 tions), respectively (Fig 1C). Temperature shift alone also induced major proteome changes
165 when I25 was compared to I37 (404 differentially abundant proteins). A change of culture
166 medium (I37 vs I37-M9, I37 vs I37-Plasma, I37-M9 vs I37-Plasma) led to slightly fewer differ-
167 entially abundant proteins with 375, 309, 247 proteins, respectively (Fig 1C). More modest
168 changes were observed when only shifting the inoculum temperature in M9 (117 proteins

169 for I25-M9 vs I37-M9) and in plasma (118 proteins for I25-Plasma vs I37-Plasma). A full de-
170 scription of numbers of differentially abundant proteins between each condition, as well as
171 numbers of proteins present in one condition but absent in another are indicated in Fig EV2.

172 To assess the influence of donors on the proteome of *Y. pestis* grown in plasma, we
173 compared proteomes of bacteria grown in the plasma of four different donors inoculated with
174 I25 and I37 in triplicates. For each donor, we identified between 1,486 and 1,696 proteins
175 common to the three replicates (Fig EV3, Table EV3), slightly different numbers than in our
176 initial proteome analysis (see above, Fig 1B). Comparison of samples derived from the four
177 different donors identified approximately 70 proteins (37°C inoculum) to 100 proteins (25°C
178 inoculum) which were differentially abundant, showing a good reproducibility of proteomes
179 when *Y. pestis* was grown in plasma of different donors.

180 Together, our results reveal a major *Y. pestis* protein expression remodeling upon growth
181 in human plasma at 37°C.

182 **Dual proteomics of *Y. pestis* in human blood.** To refine our model of bacteremia *ex*
183 *vivo*, we investigated *Y. pestis* grown in whole human blood. A previous proteotyping study
184 used blood cell lysis to deepen detection of bacterial peptides and identify *Staphylococcus*
185 *aureus*, *Escherichia coli* and *Candida albicans* for clinical diagnosis (Kondori et al. 2021).
186 Although this protocol enables proteomic analysis of bacteria in whole blood, it precludes
187 identification of the human proteome, which is useful to understand the host response to
188 infection. Thus, we set up an original pipeline allowing to analyze both bacterial and human
189 proteomes in whole blood. We first compared growth of *Y. pestis* in whole human blood using
190 ethylenediaminetetraacetic acid (EDTA), acid-citrate-dextrose (ACD) or heparin as anticoag-
191 ulant. We selected ACD, as *Y. pestis* grew the best in this blood compared to heparin- and
192 EDTA-anticoagulated blood, reaching 10^8 bacteria/mL after an 8 hour-incubation (Fig EV4).
193 Human blood is a highly complex biological fluid characterized by a high concentration of red
194 blood cells ($\approx 4.5\text{-}12 \cdot 10^9$ cells/mL), and lower concentrations of platelets ($\approx 150\text{-}475 \cdot 10^6$
195 cells/mL) and white blood cells ($\approx 5\text{-}10 \cdot 10^6$ cells/mL). Neutrophils constitute the vast major-
196 ity of white blood cells (40-60%), followed by lymphocytes (20-40%) and monocytes (2-8%),
197 eosinophils ranging from 1 to 4% and basophils 0.5 to 1%. The high number of human
198 cells compared to bacteria in our sample (10^8 bacteria/mL) and the vast dynamic range of
199 human proteins abundance ($\approx 10^{13}$), make characterization of dual proteomes very chal-
200 lenging. We thus set up a pipeline to enrich *Y. pestis* associated to relevant immune cells,
201 neutrophils and monocytes (Arifuzzaman et al. 2018; Pujol et al. 2005; Spinner et al. 2014;

202 Dudte et al. 2017; Osei-Owusu et al. 2019). After 8-hour incubation of bacteria in ACD-
203 anticoagulated human blood, we purified these cells by immunomagnetic separation (Fig 2).
204 Using CD66b neutrophil-specific antibodies and CD14 monocyte-specific antibodies, 2.10^5
205 viable neutrophils and 6.10^4 viable monocytes were recovered per milliliter of blood, which
206 account for around 10% of the total neutrophil and monocyte populations. Besides technical
207 issues, cell loss could be due to reduced lifespan of neutrophils *ex vivo* and *Y. pestis* capac-
208 ity to induce cell death (Marketon et al. 2005; Osei-Owusu et al. 2019). From 10^8 bacteria
209 per milliliter of whole blood, 4.10^6 bacteria were associated to the neutrophils fraction ($1/25^{\text{th}}$
210 of total bacteria), and 5.10^5 bacteria were associated to the monocyte fraction ($1/200^{\text{th}}$ of
211 total bacteria). This approach allowed to lower sample complexity and to switch from the
212 initial ratio of 10^8 bacteria to 5.10^9 human cells (1:50), to ratios of 4.10^6 bacteria to 2.10^5
213 neutrophils (20:1) and 5.10^5 bacteria to 6.10^4 monocytes (8:1). We then extracted proteins
214 using the SPEED method and analyzed samples by mass-spectrometry using a DIA mode
215 (Fig 2). To this end, we created two spectral libraries. The first library was constructed using
216 the antibody-mediated cell enrichments combined with a Nanotrap[®] enrichment, which had
217 previously been shown to concentrate *Y. pestis* bacteria from whole blood and bacterial pro-
218 teins from lysates (li et al. 2021). The second library was constructed from *Y. pestis* grown
219 in LB broth at 37°C to obtain the deepest peptide library. We identified 1,366 bacterial and
220 4,187 human proteins in the neutrophil fraction, and 877 bacterial and 3,969 human proteins
221 in the monocyte fraction (Table EV4). It is, to our knowledge, the first report of a pipeline
222 allowing semi-quantitative dual proteomics extracted from a complex matrix such as infected
223 whole blood, which could moreover be applied in a BSL-3 environment.

224 **Library depths and normalization strategies.** Nanotrap enrichment led to a good pep-
225 tide coverage of bacterial proteins that were very abundant in blood, such as MetE, MetF or
226 MetR, which were also enriched when *Y. pestis* was grown in human plasma (Table 4). Sim-
227 ilarly, T3SS proteins, such as YscX (Gurung et al. 2022; Day et al. 2000) and YopK, YopB,
228 YopD, YopT, YopH, whose secretion is induced in contact to host cells (Osei-Owusu et al.
229 2019) were identified. We could identify more than 2,800 bacterial proteins during the sec-
230 ondary library construction consisting in a DDA analysis of the fractionated pool of *Y. pestis*
231 grown in LB. To our knowledge, it is the most complete proteome of a *Yersinia* species. DIA
232 analysis led to identification of 1,366 bacterial proteins in the neutrophil fraction, 877 bac-
233 terial proteins in the monocyte fraction, 1,681 bacterial proteins in the Nanotrap enrichment
234 samples, and 2,335 bacterial proteins in *Y. pestis* grown in LB (Table 5). Hierarchical clus-

235 tering (Fig 3A) and principal component analysis (Fig 3B) based on MS peaks associated to
236 bacterial and human proteins showed an excellent clustering of the replicates as well as an
237 enrichment of bacterial and human proteins upon Nanotrap capture. The smaller number
238 of bacterial proteins identified in the monocyte fraction compared to the neutrophil fraction
239 (Table 5 and Fig 3A) are in line with the number of bacteria per cell, ranging from 8 bacteria
240 per cell in monocyte to 20 bacteria per cells for the neutrophils (see above).

241 We tested several normalization strategies to take into account the different ratios of
242 bacteria per cell in both fractions. These strategies are detailed in Table 6. The differ-
243 ential analysis was performed taking into account either the proteins identified in at least
244 one sample, strategies A (all), H (human), Ya (*Yersinia* all) and Yc (*Yersinia* per condition),
245 which allows to identify proteins which are detected in one condition but not the other, or in at
246 least two samples per condition, strategies A2 (all), H2 (human), Ya2 (*Yersinia* all) and Yc2
247 (*Yersinia* per condition), which reduces the number of protein considered in the analysis but
248 also reduces the rate of false positive identification. Most of the differential analysis below
249 will thus result from this second set of strategies.

250 The A2 strategy consisted in normalizing the intensities of both human and bacterial
251 proteins by equalizing the medians of the human proteins in the samples across the two
252 conditions, and to perform a differential analysis on proteins which were detected in at least
253 2 replicates per condition. When using this strategy, the lower ratio of bacteria per cells in
254 the monocyte fraction compared to the neutrophil fraction was in line with the analysis of the
255 differential abundance of the bacterial proteins. Indeed, a GSEA on biological processes GO
256 terms and KEGG pathways revealed an enrichment of translation and ribosomal processes
257 in the neutrophil fraction, considered here as house-keeping processes, and the associated
258 enriched protein $\log_2(\text{fold change})$ were centered around 2.5 (5.6 fold change) (Fig 4A and
259 B). This effect was also observed when we normalized only the bacterial proteins based
260 on the median of each conditions separately (strategy Yc2, Fig 4C), resulting in a volcano
261 plot where a large set of proteins mainly corresponding to ribosomal proteins were uniformly
262 upregulated in the neutrophil fraction (Fig 4C). The calculated 5.6-fold ratio based on riboso-
263 mal proteins was higher than the previous calculated ratio based on bacterial enumeration
264 (8 viable bacteria per monocyte and 20 viable bacteria per neutrophil, resulting in a 2.5-fold
265 ratio) which could be explained by the lower intracellular survival of *Y. pestis* in neutrophils
266 compared to monocytes (Arifuzzaman et al. 2018; Pujol et al. 2005; Spinner et al. 2014;
267 Dudte et al. 2017; Osei-Owusu et al. 2019). When normalizing only the bacterial proteins by
268 equalizing the median of the samples from the two conditions taken together (strategy Ya2),

269 we observed a shift of the ribosomal proteins to the non differentially abundant group when
270 performing an enrichment analysis on non differentially abundant proteins (Table 7). As the
271 number of identified proteins in the monocyte was much lower than in neutrophils, due to the
272 lower number of bacteria per cell and subsequent reduced sensitivity, most of the less abun-
273 dant proteins in the monocyte fraction could presumably not be detected. Only upregulated
274 proteins in the monocyte fraction, identified in both the monocyte and neutrophil fractions,
275 could be measured and compared, resulting in an asymmetrical volcano plot (Fig 4D).

276 **Proteomic signature of *Y. pestis* grown in human plasma.** To decipher bacterial adap-
277 tation to the host environment, a gene set enrichment analysis (GSEA) was performed on
278 the gene ontology (GO) biological process terms for the 9 comparisons of *Y. pestis* growths in
279 laboratory media and human plasma. Most of the enriched terms between samples grown in
280 different media were related to metabolism, such as amino acid processes, purine synthetic
281 processes such as inosine monophosphate (IMP) biosynthesis (depleted in I37 compared
282 to M9 and plasma), tricarboxylic acid cycle (enriched in the I25 and I37 inocula), or carbo-
283 hydrate transport (enriched in the I25 derived cultures) (Fig 5, Table 4). In particular, me-
284 thionine biosynthesis-related proteins such as MetE (5-methyltetrahydropteroyltriglutamate-
285 -homocysteine methyltransferase), MetR (HTH-type transcriptional regulator), MetF (methyl-
286 lenetetrahydrofolate reductase) or MetAS (homoserine O-succinyltransferase) were enriched
287 in plasma compared to M9 (Fig 5, 6A), consistent with adaptation to nutrient composition
288 of plasma and in line with transcriptomic observations in murine models of plague (Seb-
289 bane et al. 2006; Lathem et al. 2005). In addition, iron transport was enriched in plasma
290 samples compared to LBH or M9, represented by the proteins involved in biosynthesis of
291 the yersiniabactin siderophore and other iron uptake systems such as YfeAB (Fig 5, 6B,
292 Table 4), consistent with the low concentration of free iron in human plasma and extending
293 previous transcriptomic data acquired in our laboratory (Chauvaux et al. 2007). We also
294 observed higher levels of proteins encoded by the virulence plasmid pCD1, including T3SS
295 effectors, in M9 compared to plasma and LBH (Fig 5, 6C, Table 3). Low calcium is one of the
296 stimuli inducing T3SS expression and secretion (Perry et al. 1997). The low concentration
297 of calcium in M9, i.e., 0.1 mM, compared to 1 mM of free ionized calcium in human plasma
298 (Bisello et al. 2008), even in the presence of the calcium ion chelator acid-citrate-dextrose
299 (ACD) serving as anti-coagulant, may thus be a trigger. Another signal sensed by *Y. pestis*
300 RNA thermometers to activate expression of the T3SS regulon is the human host temper-
301 ature of 37°C (Perry et al. 1997). Accordingly, when I25 and the other proteomes derived

302 from 37°C cultures were compared, T3SS effector proteins were produced more abundantly
303 at 37°C than 25°C (Fig 5, 6D, Table 3). Similarly, the *caf* operon encoding *Y. pestis* pseudo-
304 capsule is highly upregulated at 37°C (Demeure et al. 2019). Expectedly, we detected high
305 levels of Caf1M, Caf1A, Caf1, and the regulator Caf1R when *Y. pestis* was grown at 37°C
306 (I37, I25-M9, I25-Plasma, I37-M9, I37-Plasma) but not at 25°C (I25) (Table 2). In addition,
307 pseudocapsule proteins were more abundant when cultures were started with a 37°C inocu-
308 lum (I37-M9 and I37-Plasma) than with a 25°C inoculum (I25-M9 and I25-Plasma) (Table 2).
309 Thus, *Y. pestis* growth in human plasma is characterized by a radical metabolic switch, in-
310 duction of iron uptake machineries and calcium- and temperature-dependent expression of
311 important virulence determinants.

312 **Proteomic signature of *Y. pestis* in whole blood fractions.** When analyzing the bac-
313 terial proteomes after incubation in human whole blood and immune cell purification, most
314 of the non-secreted pCD1 encoded proteins were not differentially abundant when using
315 the Ya2 normalization strategy. Such components include the T3S apparatus (T3SA) pro-
316 teins YscL (cytoplasmic complex), YscN (ATPase), YscJ (MS-ring forming proteins), YscC
317 (outer membrane secretin), YscW (pilotin), YscF (needle subunit), LcrV (needle tip), or reg-
318 ulators and chaperones such as LcrG (negative cytosolic regulator), LcrH/SycD (YopBD
319 chaperone), YscB (YopN chaperone), YPCD1.73c (chaperone), or YerA/SycE (YopE regu-
320 lator) and YscE and YscG (putative Yop translocation proteins) (Plano et al. 2013). On the
321 other hand, secreted proteins of the pCD1 plasmid such as the T3SS effectors (here, YopH,
322 YopE, YopJ, YopK, YpkA/YopO and YopM), the host-membrane pore-forming complex pro-
323 teins YopB and YopD, the secreted gatekeeper YopN, or the secreted protein YscX (Gurung
324 et al. 2022; Day et al. 2000) were interestingly more abundant in the monocyte fraction com-
325 pared to neutrophil fraction. It was also the case for the molecular ruler YscP, serving as a
326 needle length control protein, and the inner membrane platform protein LcrD/YscV, interact-
327 ing with YscX, although both proteins were never shown to be secreted in eukaryotic cells.
328 To validate the hypothesis that monocytes were more intoxicated with Yops than neutrophils,
329 we used the A2 normalization strategy and validated the higher abundance of Yops in the
330 monocyte fraction when normalized by human protein abundance (Table 8).

331 **Human proteome signature upon *Y. pestis* infection of whole blood fractions.** We
332 could identify 4,187 human proteins in at least one replicate of the neutrophil fraction and
333 3,969 human proteins in at least one replicate of the monocyte fraction (Table EV4). Among
334 the most abundant human proteins in monocytes based on PG quantities, we identified

335 the monocyte differentiation antigen CD14, annexin A2, interleukin-1 beta (induced in non-
336 intoxicated macrophages by YopJ-mediated inflammasome activation in neighboring intox-
337 icated cells (Orning et al. 2018)), the galectin-1 (shown to interact with YopJ/YopP to re-
338 duce nitric oxide production in macrophages (Jofre et al. 2021)), or the gamma-interferon-
339 inducible lysosomal thiol reductase (Table EV4). Among the most abundant human proteins
340 in neutrophils, we identified the carcinoembryonic antigen-related cell adhesion molecule
341 (CEACAM) 1, 6 and 8 (also respectively known as CD66a, CD66c and CD66b, specific for
342 granulocytes and neutrophils), the protein S100-A12, S100-A8 and S100-A9 (the most abun-
343 dant proteins in neutrophils (Tardif et al. 2015)), S100-P, annexin A3 (induced in neutrophils
344 during sepsis (Toufiq et al. 2020)) or galectin-10. The most abundant human proteins in
345 both fractions were the histone proteins (H2B type 1-O, H2A type 1-B, type 3, type 1-C, H4,
346 H3.1, H1.3), cytoskeleton proteins (actin cytoplasmic 1 and 2, profilin), hemoglobin and al-
347 bumin, but most interestingly we could find many neutrophil components such as S100-A8
348 and S100-A9, and granule contents such as neutrophil defensin 3, cathepsin G, neutrophil
349 elastase, lactoferrin, azurocidin, lysozyme C or myeloperoxidase.

350 Using the H and H2 normalization strategies and performing a GSEA on biological pro-
351 cess GO term, we identified neutrophil-specific terms in the most enriched processes in the
352 neutrophil fraction, such as "neutrophil activation" or "leukocyte degranulation", as well as
353 terms related to actin cytoskeleton and motility, or carbohydrates metabolism (Fig 7A). On
354 the other hand, enriched terms in monocytes were mainly related to translation and metabolic
355 pathways linked to aerobic and mitochondrial respiration (Fig 7A). Among the proteins only
356 identified in the neutrophil fraction, an enrichment of lipid metabolism-related proteins was
357 observed. The proteins only identified in the monocyte fraction were again related to transla-
358 tion and mitochondria, as well as major histocompatibility complex class II (MHC II) (Fig 7B).
359 These data are in line with the enrichment of the specific cell types we targeted. Indeed, in
360 addition to cell-specific terms such as neutrophil granule or the monocyte MHCII, neutrophils
361 are known to mainly rely on a glycolytic metabolism (Kumar et al. 2019; Injarabian, Devin,
362 et al. 2019; Jeon et al. 2020), which could explain the enrichment of aerobic metabolism in
363 the monocytes and a higher carbohydrate metabolism in the neutrophils.

Discussion

Dual proteomics to study bacterial host-interaction in *ex vivo* or *in vivo* conditions is challenging due to the high complexity of the host proteome and the high dynamic range between bacterial and host protein abundance. Moreover, application of protocols in a BSL-3 environment are more difficult to implement and perform due to safety procedures and regulation.

Our pipeline, based on bacterial enrichment from blood, pure culture for spectral library preparation, host cell purification for sample complexity reduction, and a safe and efficient TFA-based protein extraction on minute amount of samples, could overcome all these problems. Using a *Y. pestis* model of bacteremia, this pipeline allowed us to work in a BSL3 environment fulfilling the French regulation on MOT agents, and to perform a dual proteomic study in a highly-complex matrix, human whole blood. First, we benchmarked and validated the TFA-based protein extraction SPEED method in a BSL-3 environment, confirming bacterial inactivation upon protein extraction, and allowing comparison of bacterial protein abundances after incubation in laboratory media or human plasma. We validated and extended previous transcriptomic data (Chauvaux et al. 2007) by obtaining more sensitive and functionally relevant data. We then developed a new and affordable pipeline based on the SPEED method to study bacteria incubated in human whole blood and identified more than 5,500 proteins in the cellular fractions, composed of around 4/5 of human proteins and 1/5 of bacterial proteins, validating the possibility to use this pipeline for dual proteome studies. Cellular enrichment was performed under a hood in a BSL-3 environment before bacterial inactivation upon protein extraction, eliminating the need for expensive cell-sorting instrument or fixation step. Differential analysis of bacterial protein abundances showed a higher injection of *Y. pestis* T3SS effectors in monocytes than in neutrophils, suggesting a preferential targeting of monocytes, or a higher survival of bacteria in contact with monocytes compared to neutrophils. The normalization method was validated by the fact that most of the translation-associated proteins such as the ribosomal proteins, and metabolic pathway proteins, were not differentially abundant between both fractions. Human proteome analysis confirmed the good purification of CD14+ and CD66b+ cells, based on cell-specific GO terms and cell metabolic pathways. Strikingly, a very high abundance of neutrophil components was observed in both fractions. This could suggest degranulation of neutrophils and phagocytosis or macropinocytosis of granules and/or dead neutrophils by monocytes. Furthermore, it was recently shown that A100A8/A9 were not released along with degranulation but upon formation of neutrophil extracellular traps (NETs), suggesting activation of NETosis in our infection model (Sprenkeler et al. 2022), as S100-A8 and A9 were not only

398 found abundantly in neutrophils but also in monocytes.

399 Based on our methodology, future studies could consist in comparing fraction proteomes
400 of infected and non-infected blood samples, to better understand the immune response to
401 infection in a relevant *ex vivo* environment, and to expand fractionation to other cell types
402 such as platelets, T-cells or red blood cells, depending on the relevance for the pathogen
403 under study. Our pipeline, using a small amount of blood, could be applied to blood of bac-
404 teremic animals in *in vivo* conditions. Further improvement in sensitivity could be achieved
405 by implementing hybrid spectral libraires based on DDA and DIA runs (Willems et al. 2021),
406 as well as using the sensitive bacterial proteome first obtained in human plasma to enhance
407 the spectral library used in whole blood.

408 Challenges remain to better characterize bacteria in the bacteremic phase of diseases.
409 One of them is the comparison of the bacterial proteome between high-complexity and low-
410 complexity samples. We could indeed compare bacterial protein abundance between bac-
411 teria associated to neutrophils and monocytes as the host proteome background was very
412 similar. Similarly, we could compare the proteomes of bacteria incubated in LB or human
413 plasma, as a simple centrifugation and successive washes could separate bacteria from
414 plasma. However, it remains difficult to compare protein abundance of cell-associated bac-
415 teria with that of a pure bacterial culture in laboratory media. One solution could be to add a
416 host protein background to the bacterial pure culture during protein extraction to mimic sam-
417 ple complexity without altering protein expression. Another challenge is the study of free
418 bacteria, as our technique relies on cell enrichment. A way to solve this issue could be to
419 use bacteria-specific antibodies coupled to magnetic beads, such as antibodies against the
420 F1 pseudocapsule for *Y. pestis*, and enrich bacteria from the blood. However, this approach
421 would presumably also enrich cell-associated bacteria, and a second orthogonal separation
422 method such as differential centrifugation would be required to separate cell-free and cell-
423 associated bacteria. Moreover, the short life span of neutrophils once blood is drawn from
424 donors at atmospheric level of oxygen, greatly limits truly relevant models of bacteremia
425 (Monceaux et al. 2016; Injarabian, Skerniskyte, et al. 2021).

426 We believe that our methodological development successfully applied in challenging en-
427 vironments, such as BSL-3 laboratories, validates the proof of concept of studying by pro-
428 teomics host-pathogen interactions of highly pathogenic bacteria in complex human sam-
429 ples.

430 **Methods**

431 **Strains, culture media and human material.** The avirulent *Y. pestis* CO92 pCD1- strain
432 was used for protein extraction setup in BSL2 environment. The fully virulent *Y. pestis* CO92
433 was used for validation in a BSL3 environment, and incubation in human plasma and whole
434 blood. Bacteria were routinely grown on LB agar plates (BD Difco, reference 244520) sup-
435 plemented with 0.002% pork haemin (Acros Organics, reference 345960010) (LBH plate),
436 or in LB (BD Difco, reference 244620) under agitation at 180 rpm, at 25°C or 37°C. M9 was
437 prepared with 47.8 mM Na₂HPO₄ (Sigma, reference S7907), 22 mM KH₂PO₄ (Sigma, ref-
438 erence P0662), 8.6 mM NaCl (Sigma, reference 31434), 18.7 mM NH₄Cl (Sigma, reference
439 09718), 2 mM MgSO₄, 0.1 mM CaCl₂, 1% glucose, 1% casamino acid and 1 mM thiamine-
440 HCl. Frozen human plasma and fresh human blood anticoagulated with citrate dextrose
441 were provided by the Etablissement Français du Sang (EFS). Whole blood was inoculated
442 with a 25°C preculture of *Y. pestis* grown on LBH plate. For bacterial enumeration, bacte-
443 ria were serially diluted in phosphate-buffered saline (PBS) and plated on LBH agar plates,
444 which were incubated at 28°C for 48 h. When enumerating bacteria after incubation in hu-
445 man whole blood, 0.1% triton X100 was added in the first dilution to lyse human cells. Viable
446 purified cells were counted in a Malassez chamber after addition of 0.1% Trypan blue at a
447 1:1 ratio.

448 **Urea-based protein extraction.** After culture, bacteria were centrifuged and the pellet
449 was washed 3 times with cold PBS. Bacteria were resuspended in 1 mL of urea lysis buffer
450 composed of 8M urea, 100 mM TrisHCl pH 8.5, vortexed, transferred in a 2 mL tube with
451 0.1 mm glass beads (Micro-organism lysing VK01, Bertin Corp), and lysed in a Precellys24
452 (Bertin Corp) at 4°C for 90 seconds at 5,500 rpm. Lysates were centrifugated 5 minutes
453 at 15,000 *g* and 500 µL of supernatant were concentrated using an Amicon® Ultra 30 kDa
454 filter (Merck Millipore) following centrifugation at 14,000 *g* for 20 minutes. Proteins were
455 resuspended in 450 µL 8M urea, 100mM TrisHCl pH 8.5, before addition of 50 µL of tris(2-
456 carboxyethyl)phosphine (TCEP) 100 mM and 2-chloroacetamide (CAA) 400 mM and incu-
457 bated for 5 min at 95°C. Samples were centrifugated at 14,000 *g* for 10 minutes, and 300
458 µL of ammonium bicarbonate (ABC) 100 mM pH 8.0 were added. Following centrifugation
459 3 times at 14, 000 *g* for 10 minutes, proteins were resuspended in 350 µL ABC 100 mM and
460 quantified as explained below.

461 **SDS-based protein extraction.** After culture, bacteria were centrifuged and the pellet
462 was washed 3 times with cold PBS. Bacteria were resuspended in 1 mL of a SDS lysis
463 buffer composed of 4% SDS, 100mM TrisHCl, 10 mM TCEP, 40 mM CAA, incubated for
464 5 minutes at 95°C and sonicated for 10 seconds. Samples were centrifuged at 16,000 *g*
465 for 5 minutes, supernatants were concentrated with an Amicon[®] Ultra 30 kDa filter (Merck
466 Millipore) following centrifugation at 14,000 *g* for 20 minutes. Proteins were resuspended in
467 450 μ L 8M urea, 100 mM TrisHCl pH 8.5, followed by centrifugation 2 times at 14,000 *g* for
468 20 minutes. 100 μ L 8M urea, 100 mM TrisHCl pH 8.5 were added, followed by centrifugation
469 3 times at 14,000 *g* for 15 minutes. Then 300 μ L ABC were added, followed by centrifugation
470 3 times at 14,000 *g* for 10 minutes. Proteins were resuspended in 350 μ L ABC 100 mM, pH
471 8.0 and quantified as explained below.

472 **TFA-based protein extraction.** After culture, bacteria were centrifuged and the pellet was
473 washed 3 times with cold PBS. Bacteria were resuspended in 5 volumes of TFA compared
474 to the pellet (20 μ L of TFA for a pellet of 4 μ L) incubated for 10 minutes and transferred to a
475 sterile 1.5 mL protein low-binding tube. 10 volumes, compared to the TFA volume (200 μ L)
476 of 2M Tris were added, then 24 μ L of TCEP 100mM, CAA 400 mM (for a final concentration
477 of TCEP at 10 mM and CAA at 40 mM). Samples were incubated at 95°C for 5 minutes and
478 proteins were quantified as explained below. Volumes were adjusted to achieve the desired
479 quantity of proteins to digest, and 5 volumes of water were added.

480 **Protein quantification.** Bradford, BCA and Qubit assays were performed as described
481 by the supplier. Bovine serum albumin (BSA) standard resuspended in 8M urea , 100 mM
482 TrisHCl pH 8.5 or 4% SDS, 100 mM TrisHCl pH 8.5 or 10% TFA- 2M Tris was used to
483 assess buffer compatibility with the different assays. Tryptophan standard was performed
484 using pure L-tryptophan dissolved in distilled water. 50 μ L of samples were aliquoted in black
485 96-well (or alternatively 384-well) plates and measured with a Xenius spectrophotometer (or
486 alternatively Synergy H1M microplate reader - BioTek) with an excitation wavelength of 280
487 nm and an emission wavelength of 360 nm.

488 **Protein digestion and desalting.** For the comparison of urea-based, SDS-based and
489 TFA-based protein extractions, proteins were digested using Sequencing Grade Modified
490 Trypsin (Promega - V5111) with a 1:50 ratio (enzyme:protein) at 37°C for 12 h before stop-
491 ping the digestion by addition of TFA to reach a final pH lower than 2. Digested peptides were
492 desalted on 50 mg Sep-Pak C18 cartridge (Waters - WAT054955). Peptides were eluted

493 twice with a acetonitrile (ACN) 50%, formic acid (FA) 0.1% buffer and once with an ACN
494 80%, FA 0.1% buffer. Finally, the peptide solutions were speed-vac dried and resuspended
495 in ACN 2%, FA 0.1% buffer. For each sample, absorbance at 280 nm was measured with a
496 Nanodrop™ 2000 spectrophotometer (Thermo Scientific) to inject an equivalent of DO = 1.

497 For *Y. pestis* incubation in human plasma and in human whole blood, digested peptides
498 were desalted using the Stage-Tips method (Rappsilber et al. 2007) using C18 Empore disc
499 and eluted with ACN 80%, FA 0.1%. Finally, the peptide solutions were speed-vac dried
500 and resuspended in ACN 2%, FA 0.1% buffer. For each sample, absorbance at 280 nm
501 was measured with a Nanodrop™ 2000 spectrophotometer (Thermo Scientific) to inject an
502 equivalent of DO = 1.

503 **Peptide mixing and pre-fractionation.** A pool of 100 µg of all SPEED digested samples
504 was used to obtain a spectral library for DIA. To do this, equivalent amounts of each digested
505 sample were pooled before proceeding to a peptide fractionation using a manual workflow
506 based on Stage-Tips or an automatic workflow with the AssayMAP Bravo (Agilent).

507 Manual workflow was performed with a poly(styrene-divinylbenzene) reverse phase sul-
508 fonate (SDB-RPS) Stage-Tips method as described in (Rappsilber et al. 2007; Kulak et al.
509 2014). The pooled sample (20 µg) was loaded into 3 SDB-RPS (Empore™, 66886-U) discs
510 stacked on a P200 tip and 8 serial elutions were applied as following: elution 1 (Ammonium
511 formate (AmF) 60 mM, ACN 20%, FA 0.5%), elution 2 (AmF 80 mM, ACN 30%, FA 0.5%),
512 elution 3 (AmF 95 mM, ACN 40%, FA 0.5%) , elution 4 (AmF 110 mM, ACN 50%, FA 0.5%),
513 elution 5 (AmF 130 mM, ACN 60%, FA 0.5%), elution 6 (AmF 150 mM, ACN 70%, FA 0.5%)
514 and elution 7 (ACN 80%, ammonium hydroxide 5%).

515 Automatic workflow was performed using the AssayMAP Bravo with the Fractionation
516 v1.1 protocol. The dried pooled sample (80 µg) was resuspended in 20 mM ammonium
517 formate, pH 10 before a high pH reverse phase fractionation. RPS cartridge (Agilent Tech-
518 nologies, 5 µL bead volume, G5496-60033) were primed with 100 µL ACN 80%, FA 0.1%
519 and equilibrated with 70 µL 20 mM AmF, pH 10. Samples were loaded at 5 µL/min followed
520 by an internal cartridge wash and cup wash with 50 µL of 20 mM AmF, pH 10 at 5 µL/min.
521 Step elution was performed with 60 µL of ACN 10%, 20%, 30%, 40%, 50%, and 80% in 20
522 mM AmF, pH 10 at 5 µL/min. A preexisting volume of 20 µL containing the same elution
523 buffer was present in the collection plates upon elution.

524 All fractions were speed-vac dried and resuspended with ACN 2%, FA 0.1% before injec-
525 tion. For all fractions, iRT peptides were spiked as recommended by Biognosys (Biognosys

526 - Ki-3002-1).

527 **LC-MS/MS for DDA and spectral libraries creation.** In all proteomic analyses, a nanochro-
528 matographic system (Proxeon EASY-nLC 1200 - Thermo Scientific) was coupled online with
529 a Q Exactive™ HF mass spectrometer (Thermo Scientific).

530 For the comparison of urea-based, SDS-based and TFA-based protein extractions, 1 µg
531 of peptides was injected into a reverse phase column (EASY-Spray™ - ES902 - Thermo
532 Scientific: 25cm x 75 µm ID, 2.0 µm particles, 100 Å pore size,) after an equilibration step
533 in 100% solvent A (H₂O, FA 0.1%).

534 For comparisons of *Y. pestis* protein abundances after growth in human plasma or lab-
535 oratory media, 1 µg of peptides was injected into a reverse phase column (home-made
536 column, 45 cm x 75 µm ID, 1.9 µm particles, 100 Å pore size, ReproSil-Pur Basic C18 -
537 Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) after an equilibration step in 100%
538 solvent A (H₂O, 0.1% FA). For creation of the spectral library, an equivalent volume of each
539 fraction was injected into the same reverse phase column.

540 Peptides were eluted with a multi-step gradient from 2 to 7% buffer B (ACN 80% / FA
541 0.1%) in 5 min, 7 to 23% buffer B in 70 min, 23 to 45% buffer B in 30 min and 45 to 95% buffer
542 B in 5 min at a flow rate of 250 nL/min for up to 132 min. Column temperature was set to 60°C.
543 Mass spectra were acquired using Xcalibur software using a data-dependent Top 10 method
544 with a survey scans (300-1700 m/z) at a resolution of 60,000 and MS/MS scans (fixed first
545 mass 100 m/z) at a resolution of 15,000. The AGC target and maximum injection time for
546 the survey scans and the MS/MS scans were set to 3.0×10^6 , 100 ms and 1.0×10^5 , 45 ms
547 respectively. The isolation window was set to 1.6 m/z and normalized collision energy fixed
548 to 28 for HCD fragmentation. We used a minimum AGC target of 2.0×10^3 for an intensity
549 threshold of 4.4×10^4 . Unassigned precursor ion charge states as well as 1, 7, 8 and >8
550 charged states were rejected and peptide match was disable. Exclude isotopes was enabled
551 and selected ions were dynamically excluded for 45 seconds.

552 **LC-DIA-MS for DIA** For the *Y. pestis* incubation in human blood, two libraries for DIA were
553 constructed from i) CD14 and CD66b cell enrichments + Nanotrap®-enriched *Y. pestis* and
554 ii) pure culture of *Y. pestis* in LB at 37°C. Mass spectra were acquired in DIA mode with
555 the XCalibur software using the same nanochromatographic system coupled on-line to a Q
556 Exactive™ HF Mass Spectrometer. For each sample, 1 µg of peptides was injected into
557 a reverse phase column (home-made column, 45 cm x 75 µm ID, 1.9 µm particles, 100 Å
558 pore size, ReproSil-Pur Basic C18 - Dr. Maisch GmbH, Ammerbuch-Entringen, Germany)

559 after an equilibration step in 100% solvent A (H₂O, 0.1% FA). Peptides were eluted using
560 the same multi-step gradient and temperature. MS data was acquired using the Xcalibur
561 software with a scan range from 295 to 1170 m/z. The DIA method consisted in a succes-
562 sion of one MS scan at a resolution of 60,000 and 36 MS/MS scans of 1 Da overlapping
563 windows (isolation window = 25 m/z) at 30,000 resolution. The AGC (Automatic Gain Con-
564 trol) target and maximum injection time for MS and MS/MS scans were set to 3.0x10⁶, 60
565 ms and 5.0x10⁵, auto respectively. The normalized collision energy was set to 28 for HCD
566 fragmentation.

567 **Bioinformatic analyses** MaxQuant analyses: DDA raw files were processed using Max-
568 Quant software version 1.6.10.43 (Cox and Mann 2008) with Andromeda search engine
569 (Cox, Neuhauser, et al. 2011). The MS/MS spectra were searched against a UniProt *Y. pestis*
570 database (3,909 entries the 25/04/2019). Variable modifications (methionine oxidation and
571 N-terminal acetylation) and fixed modification (cysteine carbamidomethylation) were set for
572 the search and trypsin with a maximum of two missed cleavages was chosen for searching.
573 The minimum peptide length was set to 7 amino acids and the false discovery rate (FDR)
574 for peptide and protein identification was set to 0.01. The main search peptide tolerance
575 was set to 4.5 ppm and to 20 ppm for the MS/MS match tolerance. Second peptides was
576 enabled to identify co-fragmentation events. The “match between runs” feature was applied
577 for samples having the same experimental condition with a maximal retention time window
578 of 0.7 minute. One unique peptide to the protein group was required for the protein identifi-
579 cation. Quantification was performed using the XIC-based LFQ algorithm with the Fast LFQ
580 mode as described in Cox, Hein, et al. 2014. Unique and razor peptides included modified
581 peptides, with at least 2 ratio count were accepted for quantification.

582 Spectral libraries generation: DDA raw data were directly loaded into Pulsar and spectral
583 library generation was done using an in-house *Y. pestis* CO92 database containing 3,991
584 entries, including 3,915 unique proteins from the initial CO92 Sanger sequencing (Parkhill
585 et al. 2001), reannotated by RefSeq (Accession GCF_000009065.1_ASM906, accessed in
586 2020) and 76 small open frame (sORF) encoded peptides (SEP) recently identified (Cao
587 et al. 2021) and the UniProt Homo sapiens database (20,361 entries the 03/03/2023). We
588 applied the default BGS Factory Settings for both Pulsar Search and Library Generation.
589 Briefly, full tryptic digestion allowing two missed cleavages, carbamidomethylation as a fixed
590 modification on all cysteines, oxidation of methionines, and protein N-terminal acetylation as
591 dynamic modifications were set. We used the search archive properties to create specific

592 *Y. pestis* and human libraries as well as a combined library with a control of the FDR.

593 Spectronaut analyses: Spectronaut v. 16.0.220606 (Biognosys AG) (Bruderer et al.
594 2015) was used for DIA-MS data analyses. Data extraction was performed using the de-
595 fault BGS Factory Settings. Briefly, for identification, both precursor and protein FDR were
596 controlled at 1%. For quantification, peptides were grouped based on stripped sequences
597 and Qvalue was used for precursor filtering. MaxLFQ was used with no imputation and no
598 cross run normalization strategies.

599 **Statistical analysis.** Protocol comparison and plasma analysis: To find the proteins more
600 abundant in one condition than another, the LFQ intensities quantified using MaxQuant were
601 compared. Only proteins identified with at least one peptide that was not common to other
602 proteins in the FASTA file used for the identification (at least one "unique" peptide) were kept.
603 Additionally, only proteins with at least two intensity values in one of the two compared con-
604 ditions were kept for further statistics. Proteins absent in a condition and present in another
605 are put aside. These proteins can directly be assumed differentially abundant between the
606 conditions. After this filtering, intensities of the remaining proteins were first log-transformed
607 (log₂). Next, intensity values were normalized by median centering within conditions (Giai
608 Gianetto 2023). Missing values were imputed using the `impute.mle` function of the R pack-
609 age `imp4p` (Giai Gianetto et al. 2020). Statistical testing was conducted using a `limma` t-test
610 thanks to the R package `limma` (Ritchie et al. 2015). An adaptive Benjamini-Hochberg pro-
611 cedure was applied on the resulting p-values thanks to the function `adjust.p` of the `cp4p`
612 R package (Giai Gianetto et al. 2016) using the robust method described in Pounds et al.
613 2006 to estimate the proportion of true null hypotheses among the set of statistical tests.
614 The proteins associated to an adjusted p-value inferior to a FDR (false discovery rate) level
615 of 1% and an absolute log₂(fold-change) superior to 1 have been considered as significantly
616 differentially abundant proteins. Finally, the proteins of interest were therefore those which
617 emerged from this statistical analysis supplemented by those which were considered to be
618 present from one condition and absent in another.

619 Whole blood analysis: To find the proteins more abundant in one condition than in an-
620 other, the intensities quantified using Spectronaut were compared. Only proteins identified
621 with at least one peptide that is not common to other proteins in the FASTA file used for
622 the identification (at least one "unique" peptide) were kept. Depending on the normaliza-
623 tion and differential analysis strategy used, only proteins with at least one intensity values
624 in one of the two compared conditions, or at least two intensity values in both compared

625 conditions were kept for further statistics. In the first case, proteins absent in a condition
626 and present in another are put aside. These proteins can directly be assumed differentially
627 abundant between the conditions. After this filtering, intensities of the remaining proteins
628 were first log-transformed (\log_2). Next, depending on the normalization strategy, intensity
629 values only for bacterial or human proteins were normalized either by median centering
630 within conditions or by median centering on all conditions (Giai Gianetto 2023). This method
631 consists in estimating the median of intensities in a sample j (med_j), to subtract this me-
632 dian to all the intensities x in the sample j and to add the average of median intensities
633 ($y = x - med_j + average(med_j)$). Missing values were imputed using the `impute.slsa`
634 function of the R package `imp4p` (Giai Gianetto et al. 2020). Statistical testing was con-
635 ducted using a `limma` t-test thanks to the R package `limma` (Ritchie et al. 2015). An adaptive
636 Benjamini-Hochberg procedure was applied on the resulting p-values thanks to the function
637 `adjust.p` of the `cp4p` R package (Giai Gianetto et al. 2016) using the robust method de-
638 scribed in Pounds et al. 2006 to estimate the proportion of true null hypotheses among the
639 set of statistical tests. The proteins associated to an adjusted p-value inferior to a FDR (false
640 discovery rate) level of 1% and an absolute \log_2 (fold-change) superior to 1 have been con-
641 sidered as significantly differentially abundant proteins. Finally, the proteins of interest were
642 therefore those which emerged from this statistical analysis supplemented by those which
643 were considered to be present from one condition and absent in another.

644 We used the R package `clusterProfiler` v4.6.2 for enrichment and gene set enrichment
645 analysis (Wu et al. 2021). For the bacterial proteome in plasma, the `GSEA` function was used
646 based on UniProt accession number and mapped to an in-house annotation file extracted
647 from the CO92 Gene Ontology terms downloaded from the QuickGo website accessed in
648 April 2023 (Binns et al. 2009). The `enrichKEGG` function was used with the online KEGG
649 dataset for the ype organism accessed in April 2023, based on the CO92 loci. Analysis were
650 performed with the detected proteins as statistical background. For the human proteome
651 analysis, the `gseGo` was used with the `org.Hs.eg.db` v3.16.0 as annotation database and
652 statistical background, and the `gseKEGG` was used with the online annotation for the `hsa`
653 organism accessed in April 2023 as annotation database and statistical background.

654 **Comparison of bacterial proteome after growth in human plasma or laboratory media.**

655 The fully virulent CO92 strain was cultivated overnight for 20 h on LBH plate at 25°C or 37°C.
656 Frozen human plasma (Donor A) was thawed, centrifuged at 8,600 g for 10 minutes and
657 filtered on 0.22 μm filter to remove precipitates. Bacteria were resuspended in 3 mL PBS and

658 the optical density at 600 nm (OD600) was adjusted to 0.25 for the 25°C preculture and 0.67
659 for the 37°C preculture. 2 mL of preheated human plasma or 2 mL of preheated M9 media
660 were inoculated with 200 µL of each suspension and incubated at 37°C for 8 h under agitation
661 at 150 rpm in 14 mL polypropylene tubes with round bottom, in 3 replicates. Bacteria grown
662 for 8 hours and the inocula were pelleted by centrifugation and washed 3 times with cold
663 PBS, followed by centrifugation at 10,000 *g* for 10 min at 4°C. Bacteria were then lysed with
664 10 µL TFA for the inocula and bacteria grown in M9, and 20 µL TFA for bacteria grown in
665 plasma and processed as explained above in “TFA-based protein extraction”. To assess
666 reproducibility of protein expression between different plasma donors, the same experiment
667 was performed with donor A and 3 other donors (B, C and D).

668 **Comparison of monocyte- and neutrophil-associated bacterial proteome.** The fully
669 virulent CO92 strain was grown overnight for 20 h on LBH plate at 25°C. Bacteria were
670 resuspended in 3 mL PBS and the OD600 was adjusted to 0.25. 200 µL of the inoculum
671 were added to 2 mL of preheated human blood at 37°C, and 2 mL of preheated LB in 14
672 mL polypropylene tubes with round bottom, in 5 replicates. Suspensions were incubated at
673 37°C for 8 h under agitation at 180 rpm.

674 For immune cell purifications, 1 mL and 3 mL of the blood cultures were aliquoted, filtered
675 on 30 µm mesh to remove clogs and aggregates, in 5 replicates. StraightFrom® Whole
676 Blood kits (Miltenyi Biotec) were used to selectively enrich either monocytes (CD14 beads)
677 or neutrophils (CD66b beads). 50 µL of CD66b beads were added to 1 mL of blood culture
678 and 150 µL CD14 beads were added to 3 mL of blood culture, incubated for 15 minutes
679 at 4°C and transferred in conditioned Whole Blood Columns (Miltenyi Biotec) following the
680 manufacturer’s instructions. After 2 washes with PBS, 2 mM EDTA, 0.5% BSA, cells were
681 eluted with 4 mL Whole Blood Column Elution Buffer (Miltenyi Biotec) and centrifuged at
682 500 *g* for 10 minutes at 4°C. The supernatants were removed, cells and associated bacteria
683 were lysed with 80 µL TFA and proteins were processed as explained above in “TFA-based
684 protein extraction”.

685 For the first library construction, bacteria enriched from whole blood with magnetic Nanotrap®
686 Microbiome A particles (Ceres Nanosciences, Inc) after the 8 h-incubation, in 5 replicates.
687 In 15 mL tubes, 1 mL of Nanotrap® beads were added to 4 mL of blood culture, briefly vor-
688 texed, and incubated at room temperature for 30 min with regular quick vortexing. After the
689 addition of 6 mL of PBS, samples were vortexed and put on a magnetic rack for 10 minutes.
690 Supernatants were discarded, the tubes were removed from the magnetic rack, 1 mL of PBS

691 was added and samples were vortexed. The mix of beads and cells were then transferred
692 to a sterile 1.5mL protein low-binding tube and pelleted on a magnetic rack for 10 minutes.
693 The supernatant was discarded, and the pellet was washed a second time with 1 mL PBS.
694 After a third magnetic pelleting, the cell pellet was lysed with 100 μ L TFA and processed as
695 explained above in “TFA-based protein extraction”.

696 For the second library construction, 4 mL of the 8 h-incubation in LB were pelleted by
697 centrifugation at 4,000 *g* for 15 minutes at 4°C and washed 3 times with 4 mL cold PBS, in
698 5 replicates. The pellet was then lysed with 80 μ L TFA and processed as explained above
699 in “TFA-based protein extraction”.

700 **Data availability**

701 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
702 Consortium via the PRIDE partner repository (Perez-Riverol et al. 2022) with the dataset
703 identifier PXD042837.

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715 We declare no conflict of interest.

716 **Author contributions**

717 **Pierre Lê-Bury**: conceptualization ; investigation ; formal analysis ; visualization ; writing
718 – original draft ; **Thibaut Douché**: investigation ; formal analysis ; visualization ; **Quentin**

719 **Giai Gianetto**: investigation ; formal analysis ; visualization ; **Mariette Matondo**: formal
720 analysis ; writing – original draft ; funding acquisition ; **Javier Pizarro-Cerdá**: formal analysis
721 ; writing – original draft ; funding acquisition ; **Olivier Dussurget**: conceptualization ; formal
722 analysis ; writing – original draft ; funding acquisition. All the authors contributed to editing
723 the original manuscript before submission.

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Figures

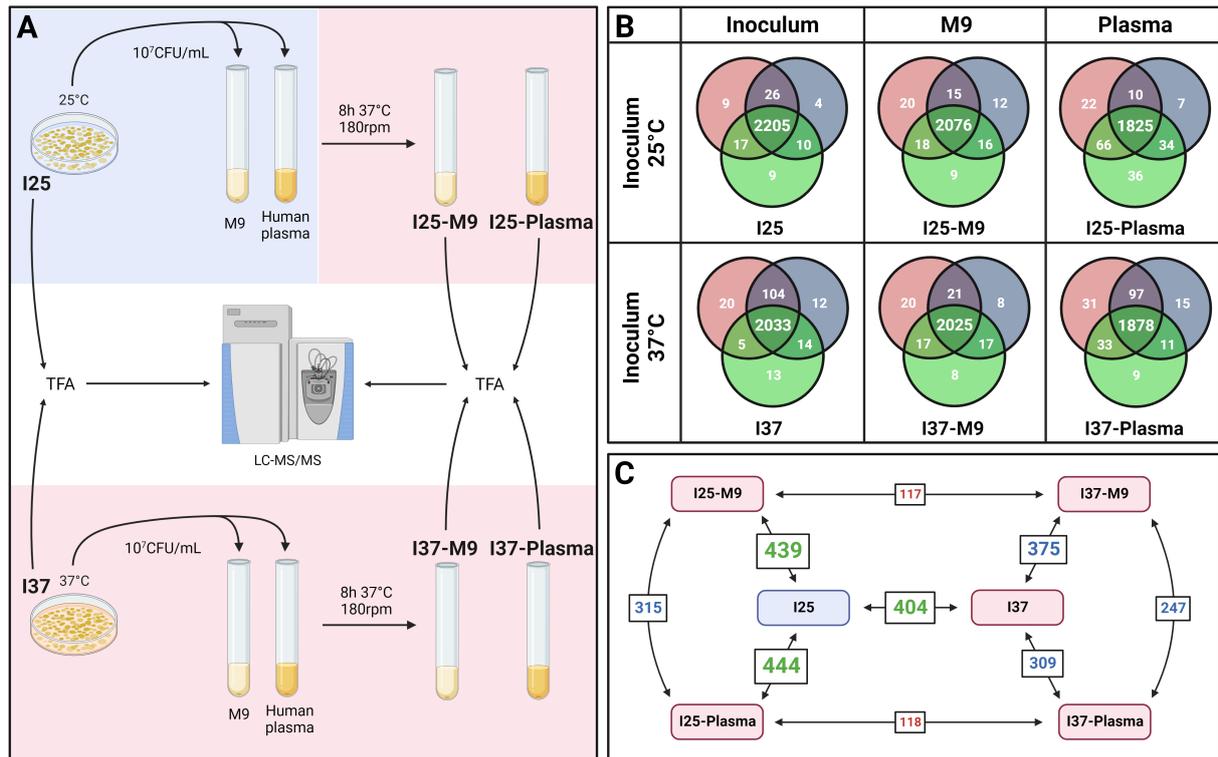


Figure 1. (A) Protocol comparing *Y. pestis* grown on LBH plate at 2 different temperatures and after growth in M9 or human plasma at 37°C. (B) Venn diagrams of the number of identified proteins across replicates in the same condition, for samples of *Y. pestis* grown on LBH, in M9 or human plasma at different temperatures. Each circle in the Venn diagrams corresponds to a replicate. (C) Number of differentially abundant proteins between samples after *Y. pestis* growth on LBH, in M9 or human plasma at different temperatures. These numbers only consider the proteins detected in both conditions. Condition in the blue rectangle is grown at 25°C and conditions in the red rectangles are grown at 37°C. Numbers in green are associated with change in temperature culture, numbers in blues are associated with a change in media grown at 37°C, and numbers in red are associated with a change in inoculum temperature in the same medium.

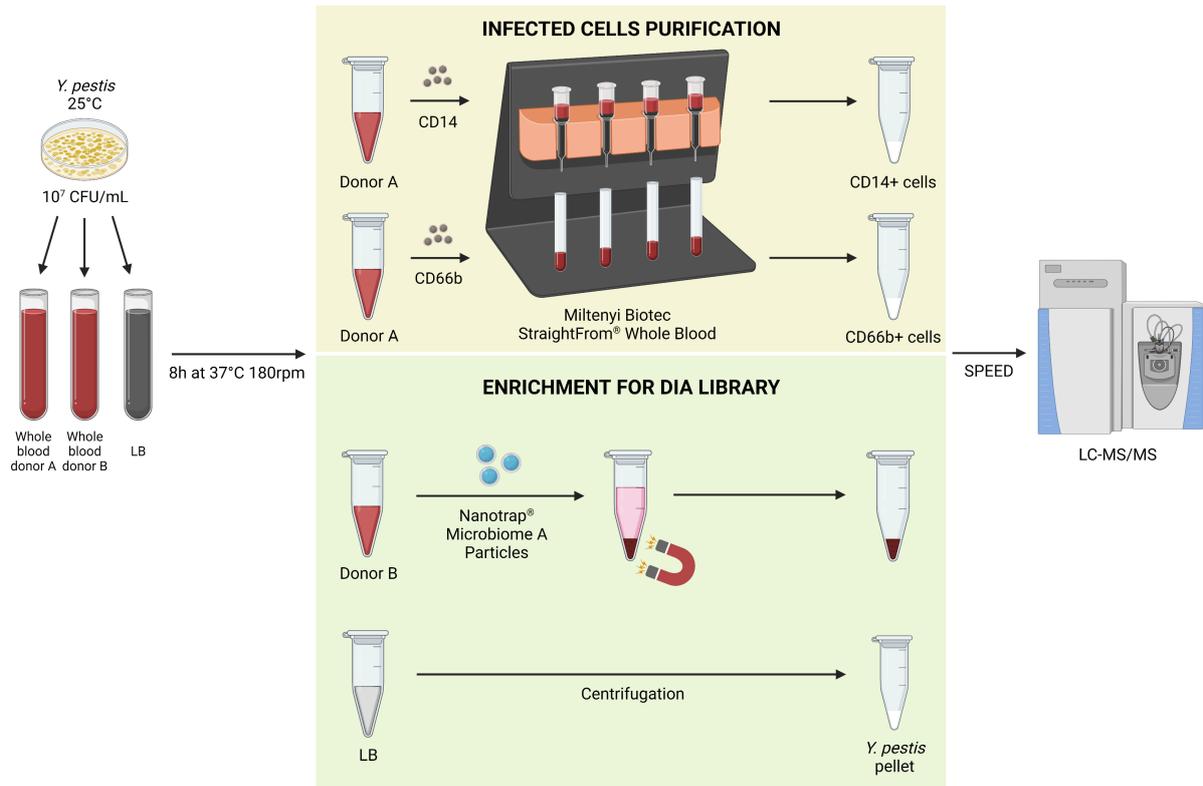


Figure 2. Workflow for enrichment of cell-associated *Y. pestis* from human whole blood, and library construction based on Nanotrap enrichment and pure culture in LB.

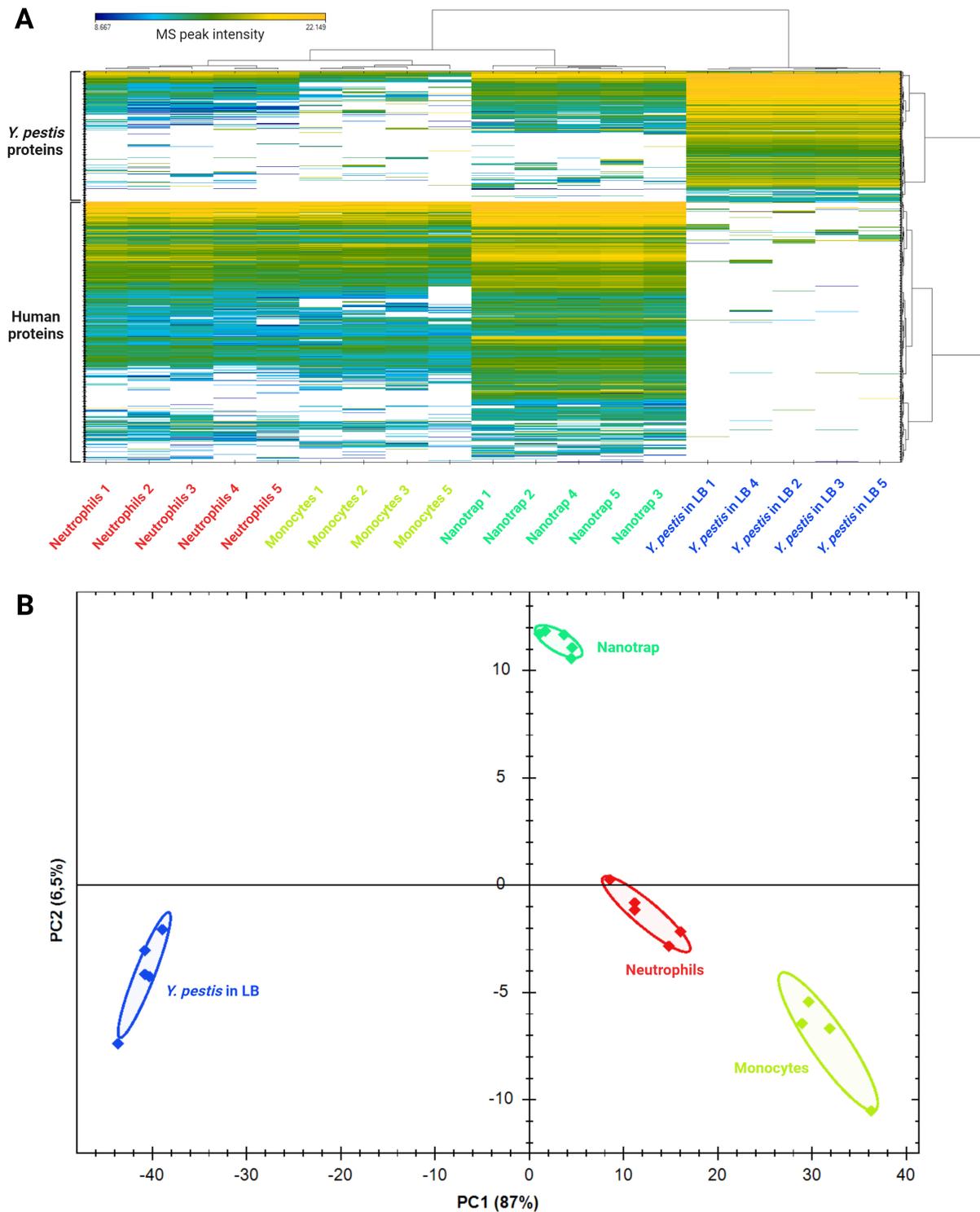


Figure 3. (A) Heatmap and (B) principal component analysis (PCA) based of the MS peak intensities for the bacterial and the human proteins on the neutrophil fraction, the monocyte fraction, the Nanotrap enrichment and the *Y. pestis* pure culture in LB. The replicate 4 of the monocyte fraction was removed due to a contamination with red blood cells during cell purification.

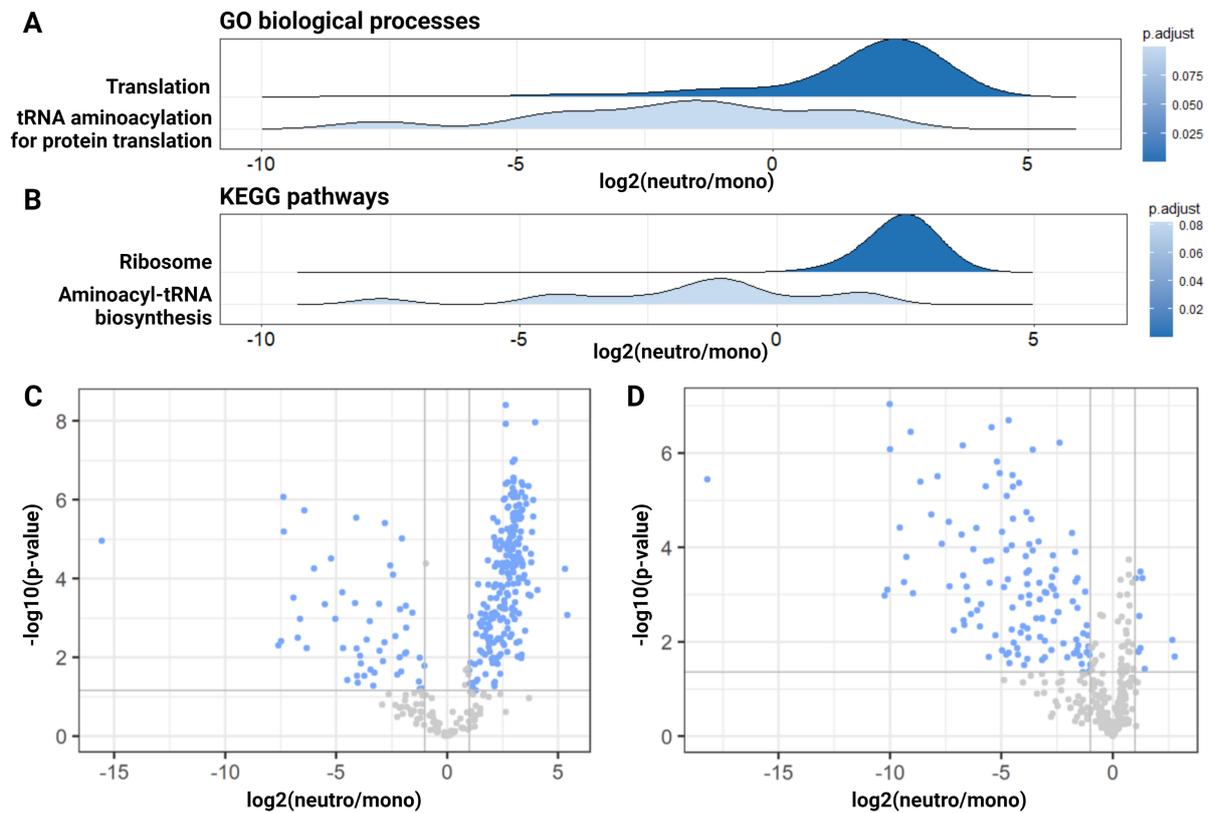


Figure 4. (A-B) Ridgeplots of enriched (A) GO biological processes or (B) KEGG pathways after performing a GSEA on *Y. pestis* protein differential abundances between the neutrophil and monocyte fractions using the A2 normalization strategy. The curve represents the density of proteins in the enriched terms depending on the fold change of these proteins. (C-D) Volcano plots showing differentially abundant *Y. pestis* proteins between the neutrophil fraction and the monocyte fraction using (C) the Yc2 strategy and (D) the Ya2 strategy. In blue are the proteins which are significantly differentially expressed, considering a threshold of 1 for the absolute value of the $\log_2(\text{foldchange})$, and a value of 1% for the unadjusted p-value.

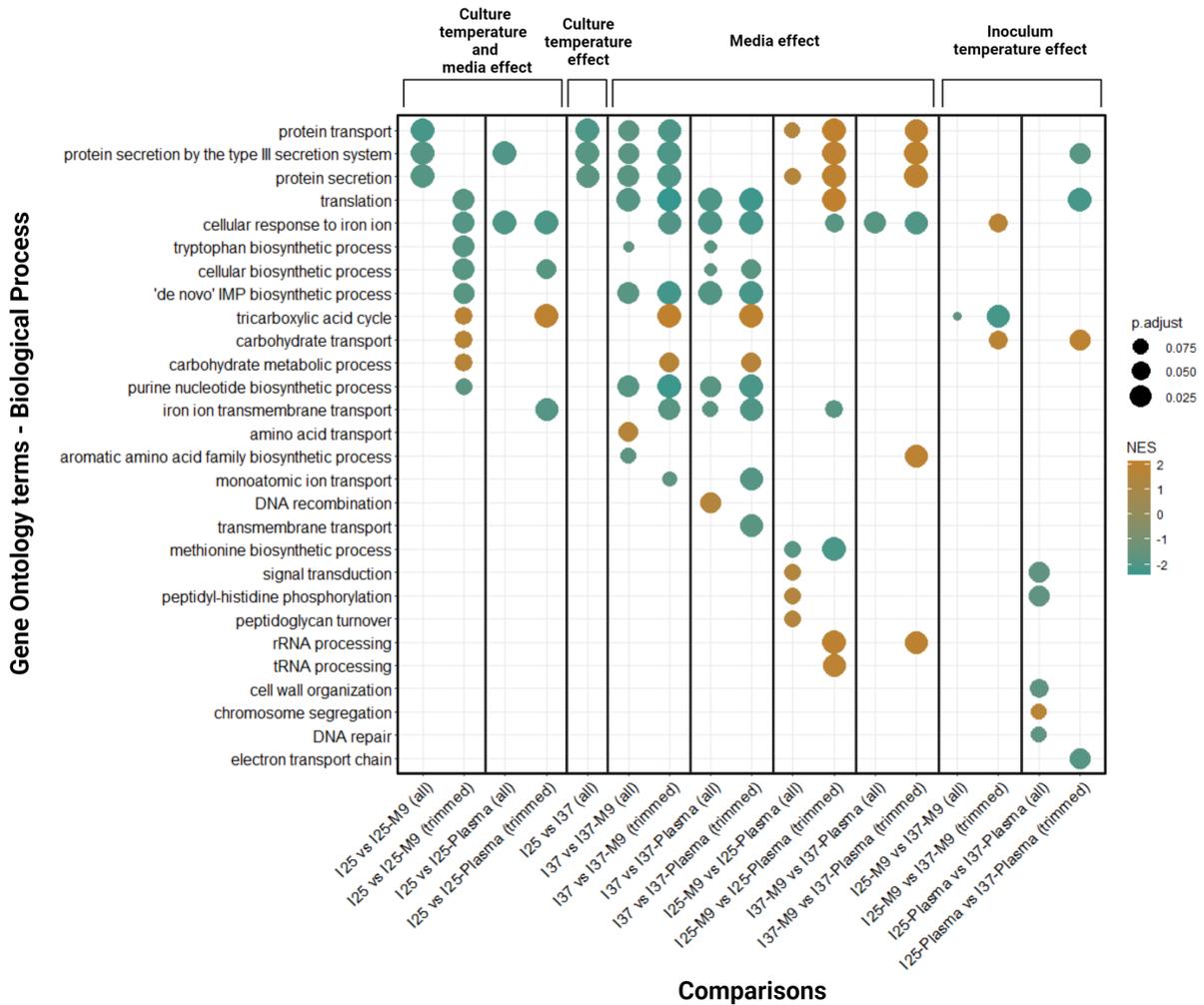


Figure 5. Dot plot representing the gene set enrichment analysis (GSEA) of the biological process terms from the gene ontology (GO) for the 9 proteomic comparisons. Each comparison is analyzed twice: i) with the whole data set comprising the proteins only present in one or the other conditions (marked "all") and ii) with the proteins detected only in both conditions (marked "trimmed"). The dot color represents the normalized enrichment score (NES) of the GO term in the GSEA. The dot size represents the adjusted p-value of the enriched GO term (control of the false discovery rate by the Benjamini-Hochberg correction).

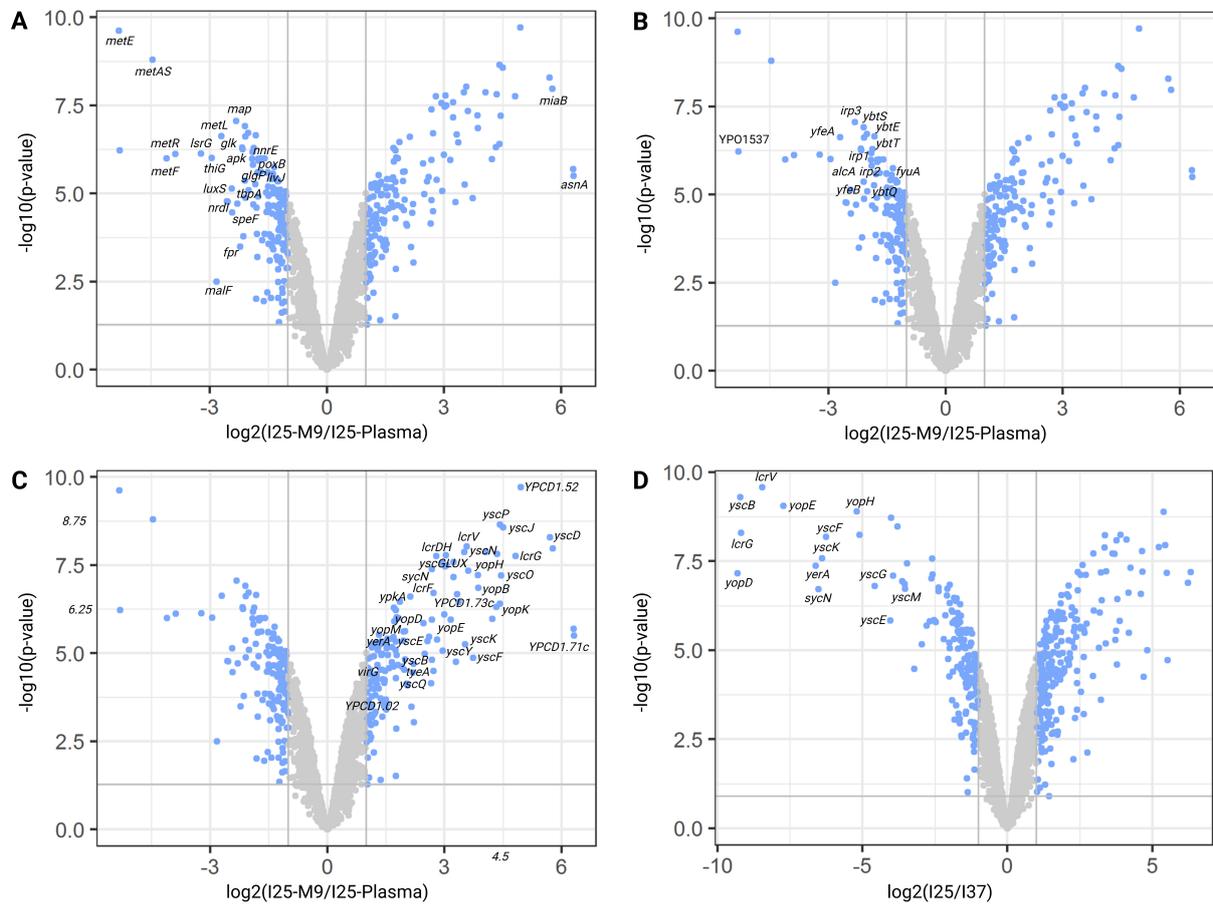


Figure 6. Volcano plots showing differentially abundant proteins between *Y. pestis* grown in different conditions. The displayed dots represent proteins detected in both conditions of the comparison. In blue are the proteins which are significantly differentially expressed, considering a threshold of 1 for the absolute value of the $\log_2(\text{foldchange})$, and a value of 1% for the unadjusted p-value. (A-C) M9 at 37°C with a 25°C inoculum (I25-M9) versus *Y. pestis* grown in human plasma at 37°C with a 25°C inoculum (I25-Plasma). (A) Focus on regulated metabolism proteins and the corresponding annotated genes. (B) Focus on regulated iron acquisition proteins and the corresponding annotated genes. (C) Focus on T3SS regulated proteins and the corresponding annotated genes. (D) 25°C inoculum on LBH plate (I25) versus 37°C inoculum on LBH plate (I37), with a focus on T3SS proteins and the corresponding annotated genes.

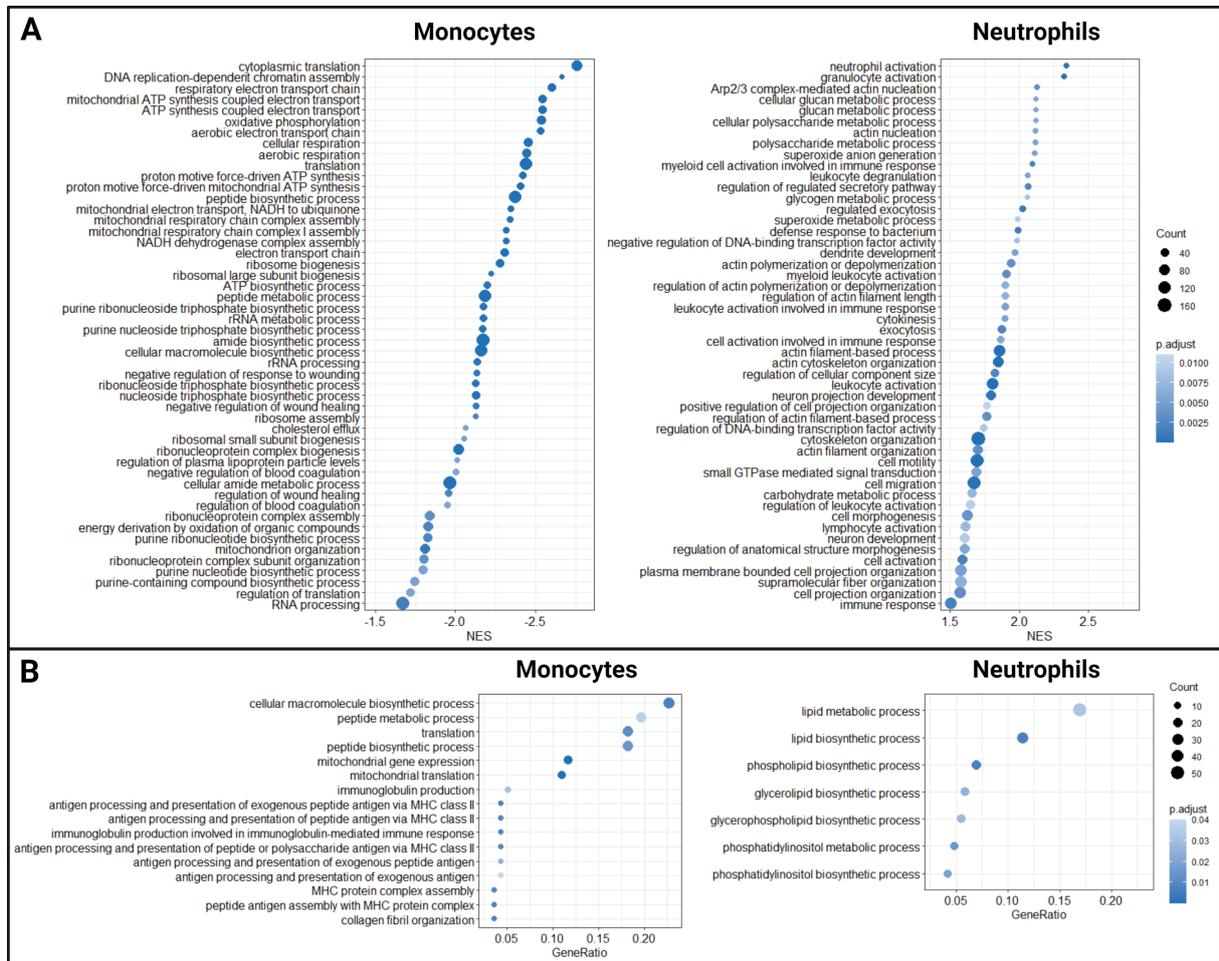
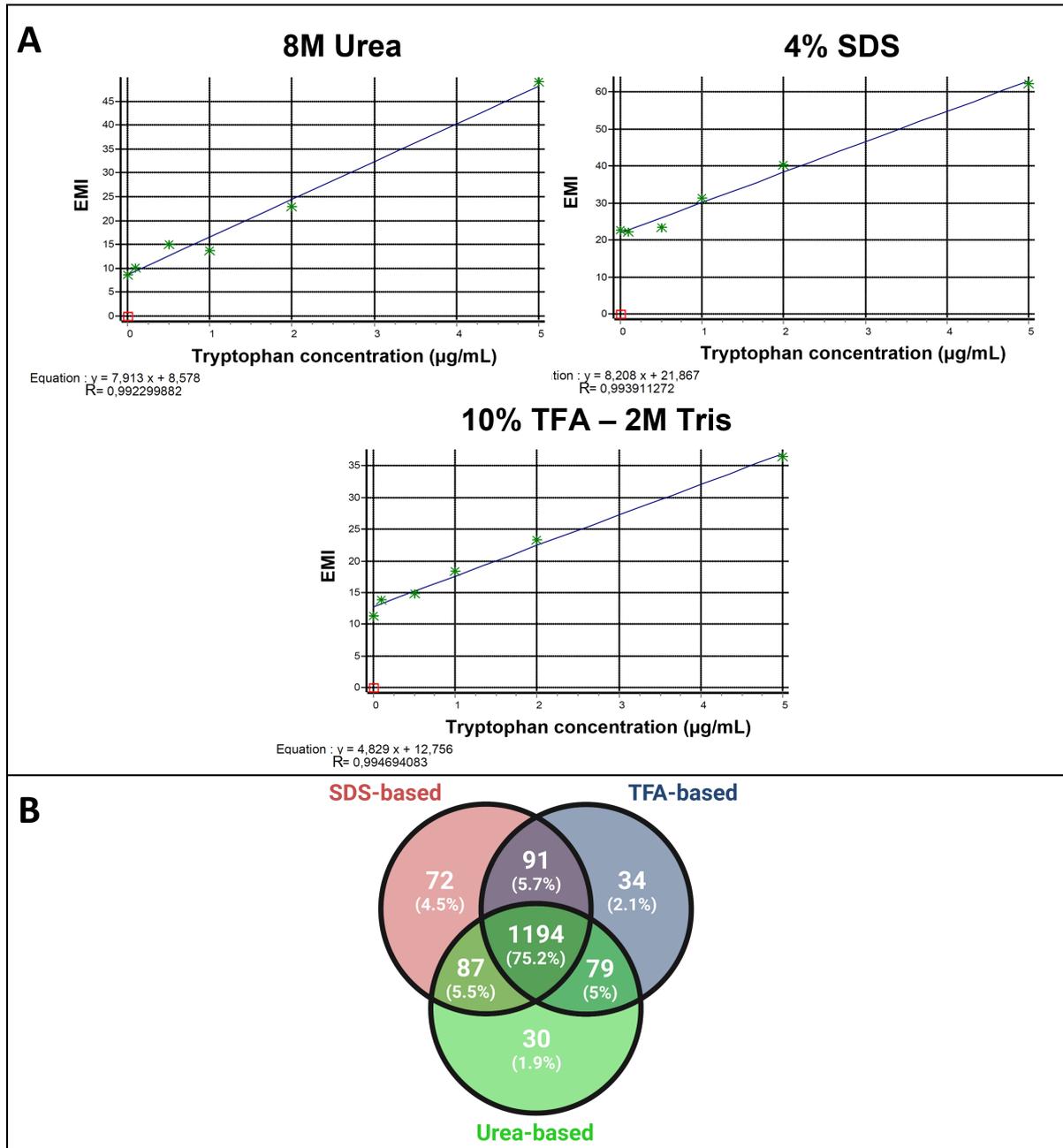


Figure 7. (A) Dotplots of enriched gene ontology (GO) biological processes after performing a gene set enrichment analysis (GSEA) on human protein differential abundances between the neutrophil and monocyte fractions using the H2 normalization strategy, with a focus on the 50 most enriched GO terms in monocytes (left) or in neutrophils (right). Data are ordered by normalized enrichment score (NES). (B) Dotplots of enriched GO biological processes after performing an enrichment analysis on human proteins only detected in monocytes (left) or in neutrophils (right) with the H normalization strategy. The dot size represents the number of proteins counted in this GO term. Terms are sorted by gene ratio. The dot color represents the adjusted p-value of the enriched GO term (control of the false discovery rate by the Benjamini-Hochberg correction).

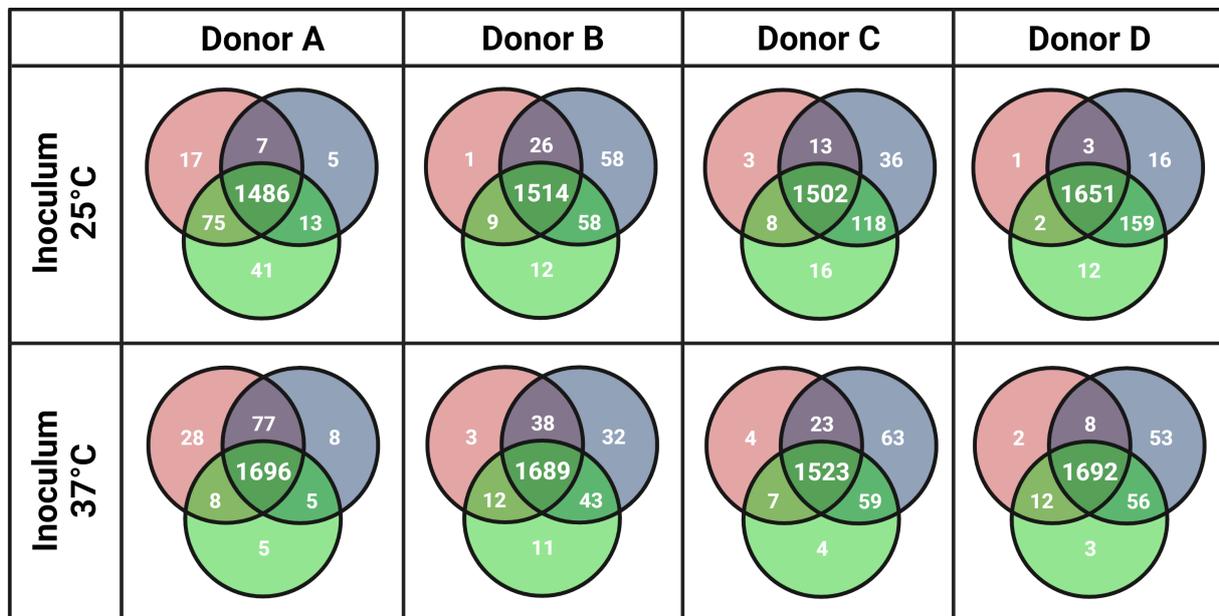
Expanded View Figures



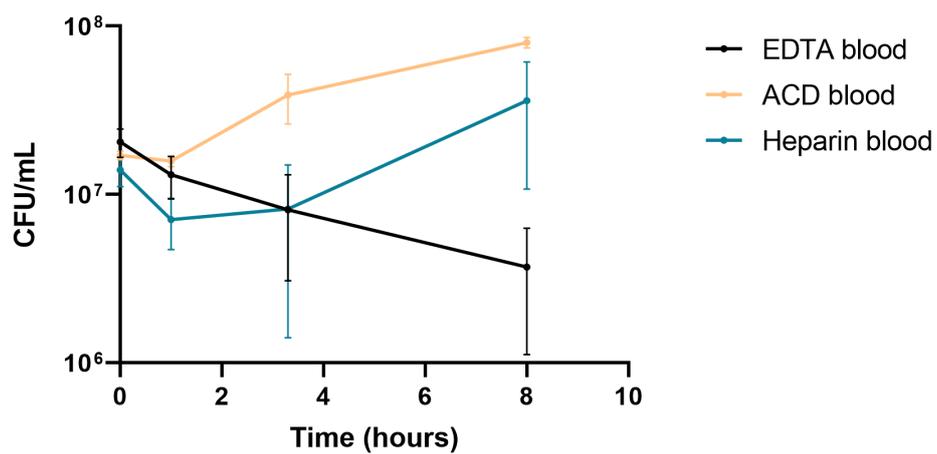
Expanded View Figure 1. (A) Tryptophan standard measured by fluorescence (excitation: 280nm, emission: 360nm) in a plate reader for the three lysis buffers: 8M urea, 4% SDS and 10% TFA - 2M Tris. (B) Venn diagram of identified proteins repartition between the urea-based, SDS-based and TFA-based protocols.

A B	I25	I37	I25-M9	I37-M9	I25-Plasma	I37-Plasma
I25		86 175	94 166		63 182	
I37	194 229			63 154		37 123
I25-M9	246 273			38 46	40 150	
I37-M9		129 221	66 71			37 129
I25-Plasma	387 262		203 165			90 67
I37-Plasma		182 186		125 118	49 51	

Expanded View Figure 2. Differentially abundant proteins and proteins present and absent in the different comparisons. Each cell corresponds to the comparison A versus B (column versus row). In green are the number of proteins present in A and absent in B, in blue are the numbers of proteins present in both conditions and more abundant in A versus B.



Expanded View Figure 3. Venn diagram of number of detected proteins in replicates after *Y. pestis* growth in plasma of different donors.



Expanded View Figure 4. Growth of *Y. pestis* CO92 in human blood anticoagulated with EDTA, ACD or heparin.

Tables

Table 1. Compatibility of Bradford, BCA, Qubit and tryptophan fluorescent assay with 8M Urea, 4% SDS and 10% TFA - 2M Tris lysis buffer for protein quantification.

		Extraction method		
		8M Urea	4% SDS	10% TFA - 2M Tris
Measure method	Bradford	Compatible	Incompatible	Incompatible
	BCA	Incompatible	Compatible	Incompatible
	Qubit	Compatible	Incompatible	Incompatible
	Tryptophan fluorescence	Compatible	Compatible	Compatible

Table 2. Value of \log_{10} (LFQ) for the *caf* operon encoding *Y. pestis* pseudocapsule. ND: Not Detected.

Protein	Sample					
	I25	I37	I25-M9	I25-Plasma	I37-M9	I37-Plasma
Caf1R	ND	8.52	ND	ND	7.69	8.35
Caf1M	ND	10.4	8.88	9.41	10.1	10.4
Caf1A	ND	9.66	8.14	8.41	9.43	9.70
Caf1	ND	10.1	8.98	9.26	10.5	10.0

Table 3. Log₂(fold change) of protein abundance for proteins involved in the type 3 secretion system (T3SS). A value of "15" is indicated for proteins only detected in the first condition and a value of "-15" is indicated for proteins only detected in the second condition.

Locus	Protein	Comparison				
		I25	I37	I37	I25-M9	I37-M9
		vs I37	vs I37-M9	vs I37-Plasma	vs I25-Plasma	vs I37-Plasma
YPCD1.05c	SycE	-6.6	-1.1	0.1	1.7	1.2
YPCD1.06	YopE	-7.7	-2.0	-0.1	2.8	1.9
YPCD1.19c	YopK	-15.0	-2.2	0.7	4.3	2.9
YPCD1.20	YopT	-15.0	-2.2	15.0	0.0	15.0
YPCD1.21	SycT	0.0	-15.0	0.0	0.0	15.0
YPCD1.26c	YopM	-15.0	-0.8	1.2	2.0	2.0
YPCD1.28c	YopD	-9.3	-1.3	0.8	2.5	2.2
YPCD1.29c	YopB	-15.0	-1.6	1.0	3.9	2.7
YPCD1.30c	LcrH	-15.0	-1.4	0.5	2.8	1.9
YPCD1.31c	LcrV	-8.5	-1.3	0.9	3.6	2.2
YPCD1.32c	LcrG	-9.2	-1.5	1.6	4.8	3.1
YPCD1.33c	LcrR	-15.0	-2.0	15.0	15.0	15.0
YPCD1.34c	LcrD	-15.0	-2.0	0.9	3.0	2.9
YPCD1.35c	YscY	-15.0	-1.7	1.4	3.0	3.1
YPCD1.36c	YscX	-15.0	-2.0	0.8	3.1	2.9
YPCD1.37c	SycN	-6.5	-2.5	0.0	2.7	2.5
YPCD1.38c	TyeA	-15.0	-1.6	0.3	2.7	1.9
YPCD1.39c	YopN	-15.0	-0.1	0.8	0.9	0.9
YPCD1.40	YscN	-15.0	-1.7	1.0	3.5	2.7
YPCD1.41	YscO	-15.0	-2.5	1.8	4.5	4.3
YPCD1.42	YscP	-15.0	-1.5	1.4	4.4	2.9
YPCD1.43	YscQ	-15.0	-0.9	1.3	2.7	2.2
YPCD1.44	YscR	0.0	-15.0	0.0	15.0	15.0
YPCD1.46	YscT	0.0	-15.0	0.0	15.0	15.0
YPCD1.47	YscU	-15.0	-2.2	1.0	3.2	3.2
YPCD1.48	YscW	-15.0	-0.8	-0.3	1.2	0.5
YPCD1.49	VirF	-15.0	-1.1	0.2	2.7	1.3
YPCD1.51	YscB	-9.2	-1.7	0.7	2.7	2.4
YPCD1.52	YscC	-15.0	-2.6	0.7	5.0	3.4
YPCD1.53	YscD	-15.0	-2.8	0.9	5.7	3.6
YPCD1.54	YscE	-4.0	-2.2	-0.2	2.6	2.0
YPCD1.55	YscF	-6.2	-2.5	0.3	3.7	2.8
YPCD1.56	YscG	-3.9	-2.3	-0.1	3.2	2.2
YPCD1.57	YopR	-15.0	-2.6	0.7	15.0	3.4
YPCD1.58	LcrO	-15.0	-3.7	1.6	15.0	5.1
YPCD1.59	YscJ	-15.0	-3.4	0.3	4.5	3.6
YPCD1.60	YscK	-6.4	-1.7	0.8	3.5	2.5
YPCD1.61	YscL	-15.0	-1.6	0.5	3.0	2.1
YPCD1.62	LcrQ	-3.5	-0.5	0.0	-1.4	0.5
YPCD1.67c	YopH	-5.2	-1.8	1.4	3.6	3.2
YPCD1.71c	YopJ	-15.0	-1.2	1.4	6.3	2.6
YPCD1.72c	YopO	-15.0	-0.6	1.0	2.1	1.5
YPCD1.95c	SycH	-1.5	1.0	0.5	-0.6	-0.5

Table 4. Log₂(fold change) of protein abundance for proteins involved in metabolism and iron uptake. A value of "15" is indicated for proteins only detected in the first condition and a value of "-15" is indicated for proteins only detected in the second condition.

Locus	Protein	Comparison				
		I25 vs I37	I37 vs I37-M9	I37 vs I37-Plasma	I25-M9 vs I25-Plasma	I37-M9 vs I37-Plasma
Purine biosynthesis						
YPO1636	PurB	0.8	-0.2	-0.5	0.4	-0.2
YPO1775	PurT	0.7	-1.3	-1.6	0.4	-0.2
YPO2387	PurR	0.1	-0.3	-0.5	0.3	-0.1
YPO2772	PurF	0.7	-1.2	-1.6	0.5	-0.3
YPO2828	PurM	1.3	-1.3	-1.3	0.4	0.0
YPO2829	PurN	0.9	-1.6	-1.0	0.8	0.6
YPO2870	GuaA	0.6	-0.1	-0.7	-0.2	-0.6
YPO2871	GuaB	0.7	-0.6	-0.6	-0.2	0.0
YPO2921	PurL	1.4	-1.9	-1.8	0.7	0.0
YPO3076	PurE	1.8	-2.5	-2.3	0.7	0.1
YPO3077	PurK	3.0	-4.3	-3.6	1.4	0.7
YPO3728	PurH	1.1	-1.6	-1.6	1.0	-0.1
YPO3729	PurD	0.9	-1.8	-1.5	1.0	0.2
Iron uptake and metabolism						
YPO0281	HmuT	2.3	-2.9	-3.3	0.0	-0.5
YPO0282	HmuS	0.7	-1.7	-1.1	0.6	0.6
YPO0283	HmuR	-0.9	-2.0	-2.3	-0.7	-0.3
YPO0955		1.9	-2.1	-2.2	1.4	-0.1
YPO0956		0.5	-1.8	-1.6	1.1	0.2
YPO1537		15.0	0.0	0.0	-5.3	0.0
YPO1753	FcuA	1.7	-2.1	-1.7	1.4	0.4
YPO1906	FyuA	-2.0	-0.7	-2.6	-1.3	-1.9
YPO1907	YbtE	-1.8	0.0	-2.2	-1.9	-2.1
YPO1908	YbtT	-1.6	-0.8	-3.0	-1.9	-2.2
YPO1909	YbtU	-1.7	0.2	-2.2	-2.1	-2.4
YPO1910	Irp1	-1.6	-0.2	-2.6	-1.8	-2.4
YPO1911	Irp2	-2.1	-0.2	-2.5	-1.8	-2.2
YPO1912	YbtA	0.0	0.0	-15.0	-1.1	-15.0
YPO1913	YbtP	-1.6	-0.8	-2.9	-1.2	-2.0
YPO1914	YbtQ	-3.0	-1.2	-3.4	-1.5	-2.3
YPO1916	YbtS	-2.6	0.3	-2.2	-2.0	-2.5
YPO1941		0.3	-0.5	-0.4	-0.1	0.1
YPO2439	YfeA	1.2	-1.0	-4.1	-2.7	-3.1
YPO2440	YfeB	0.5	-0.7	-2.6	-2.1	-1.9
YPO2650	NrdI	0.0	-2.2	-2.4	-2.3	-0.6
YPO2960	FbpC	0.3	-0.6	-0.5	-0.5	0.0
Tricarboxylic acid cycle						
YPO0348	AspA	-0.7	1.4	2.7	1.1	1.2
YPO0359		-1.6	0.9	1.5	0.0	0.6
YPO0897	SdhE	0.5	-0.9	0.0	0.6	0.9
YPO1108	GltA	-0.4	0.9	0.9	0.0	-0.1

YPO1109	SdhC	-1.2	15.0	15.0	0.0	0.0
YPO1110	SdhD	-0.3	15.0	15.0	0.0	0.0
YPO1111	SdhA	-0.7	0.9	1.6	1.1	0.7
YPO1113	SucA	-0.3	1.4	1.9	0.9	0.4
YPO1114	SucB	-0.3	0.9	1.1	0.7	0.2
YPO1115	SucC	0.1	0.8	1.2	0.8	0.4
YPO1116	SucD	-0.2	1.1	1.4	0.6	0.3
YPO1641	Icd	-0.1	0.9	0.5	0.1	-0.4
YPO2221	AcnA	-1.3	1.8	2.4	2.0	0.6
YPO2264	FumC	0.3	0.8	-0.3	-1.5	-1.0
YPO3415	AcnB	-0.3	1.1	1.7	1.1	0.6
YPO3418	AceF	0.2	-0.9	-0.1	0.7	0.8
YPO3419	AceE	-0.1	-0.7	0.1	0.6	0.7
YPO3724	AceK	1.7	2.3	6.2	-1.2	4.5
YPO3725	AceA	0.5	1.4	1.5	-0.8	0.1
YPO3726	AceB	1.1	1.7	0.9	-1.3	-0.8
Amino acid						
YPO0035	GlhS	0.5	15.0	-0.8	0.0	0.0
YPO0584	SstT	0.8	15.0	-0.6	-0.1	-15.0
YPO1318	MetN2	1.2	15.0	15.0	0.0	0.0
YPO1321		0.5	15.0	-0.3	1.0	-15.0
YPO1846	TcyJ	-1.0	2.1	1.4	0.1	-0.6
YPO1853	PutP	0.7	15.0	15.0	0.0	0.0
YPO4110		-15.0	15.0	15.0	0.0	0.0
YPO4111		1.6	2.2	3.8	3.0	1.6
Methionine						
YPO0114	MetJ	-0.1	0.3	-0.9	0.3	-1.2
YPO0117	MetF	15.0	-15.0	-15.0	-4.1	-7.2
YPO0287		3.7	1.0	2.1	-0.7	1.1
YPO0681	MetC	0.6	0.6	0.0	-0.7	-0.5
YPO0931	MetK	0.5	0.1	-1.1	-0.4	-1.2
YPO1073	MetN	-0.2	-1.0	-1.7	-0.8	-0.7
YPO1522	MetG	0.2	0.4	0.1	0.2	-0.3
YPO3722	MetH	1.4	-0.1	1.9	0.6	2.0
YPO3727	MetA	0.3	-0.6	-5.1	-4.5	-4.5
YPO3788	MetE	-0.6	1.3	-2.0	-5.3	-3.3
YPO3789	MetR	0.0	0.0	-15.0	-3.9	-15.0
YPO3949	Asd	1.0	0.1	-0.6	-0.6	-0.7
Tryptophan						
YPO0157	TrpS	0.6	0.3	0.2	0.0	-0.1
YPO0169	PabA	1.2	-0.4	-2.1	0.0	-2.3
YPO0453	TrpR	-0.7	-1.9	-0.7	1.4	1.2
YPO1773	PabB	-0.3	-1.1	-0.5	1.0	0.6
YPO2203	TrpA	-0.2	-0.5	-0.1	0.9	0.5
YPO2204	TrpB	-0.4	-0.6	0.2	0.6	0.7
YPO2205	TrpCF	1.2	-2.5	-0.4	2.2	2.1
YPO2522	YbiB	0.5	0.7	-0.5	-0.5	-1.1

Table 5. Number of bacterial proteins identified after the DIA analysis of the blood fractions, the Nanotrap enrichment and the pure culture in LB. The replicate 4 of the monocyte fraction was removed due to a contamination with red blood cells during cell purification.

		Sample			
		Neutrophils	Monocytes	Nanotrap	Pure culture
Identified in	Replicate 1	944	440	1,215	2,280
	Replicate 2	992	532	1,223	2,270
	Replicate 3	939	483	1,146	2,275
	Replicate 4	844	N/A	1,162	2,270
	Replicate 5	784	277	1,306	2,279
	At least 1 replicate	1,366	877	1,681	2,335
	At least 2 replicates	1,011	446	1,346	2,326
	All replicates	575	148	857	2,176

Table 6. Normalization and differential analysis strategies for whole blood fraction analysis. The number of samples where peptides were found (last column) was only taken into account during the differential analysis and not during normalization, which was based on all peptides. Yp: *Y. pestis*. Hsa: *Homo sapiens*. Spl: sample. Cond: biological condition (monocyte or neutrophil fraction).

Name	Description	Dataset	Median normalization	Normalized on	Prot. quantified in at least 2 spl
A	All	Hsa+Yp	on all spl	Hsa	in a cond
A2	All (2 spl)	Hsa+Yp	on all spl	Hsa	in each cond
H	Hsa	Hsa	on all spl	Hsa	in a cond
H2	Hsa (2 spl)	Hsa	on all spl	Hsa	in each cond
Ya	Yp all	Yp	on all spl	Yp	in a cond
Ya2	Yp all (2 spl)	Yp	on all spl	Yp	in each cond
Yc	Yp by cond	Yp	by cond	Yp	in a cond
Yc2	Yp by cond (2 spl)	Yp	by cond	Yp	in each cond

Table 7. Enrichment analysis of KEGG pathways for bacterial proteins with an absolute \log_2 (fold change) less than 1 between neutrophil and monocyte fraction after Ya or Ya2 normalization strategy.

KEGG ID	Description	Gene ratio	Background ratio	p-value	adjusted p-value
Ya normalization					
ype03010	Ribosome	47/142	54/557	7.3E ⁻²⁴	3.0E ⁻²²
ype01200	Carbon metabolism	26/142	65/557	4.5E ⁻³	6.3E ⁻²
ype00020	Citrate cycle	10/142	17/557	3.1E ⁻³	6.3E ⁻²
Ya2 normalization					
ype03010	Ribosome	46/121	50/229	3.4E ⁻¹¹	5.1E ⁻¹⁰

Table 8. Differential abundance of the pCD1 plasmid-encoded proteins between neutrophil and monocyte fractions, with Ya2 and A2 normalization strategies. \log_2 (fold change) less than -1 are represented in bold. T3SA: type 3 secretion apparatus.

Locus	Protein	Role	\log_2 (fold change) Norm. Ya2	\log_2 (fold change) Norm. A2
YPCD1.05c	YerA/SycE	regulator	-0.5	1.7
YPCD1.06	YopE	effector	-3.8	-1.6
YPCD1.19c	YopK	effector	-2.5	-0.3
YPCD1.26c	YopM	effector	-3.8	-1.6
YPCD1.28c	YopD	translocon	-4.1	-1.9
YPCD1.29c	YopB	translocon	-4.7	-2.5
YPCD1.30c	LcrH/SycD	chaperone	0.9	3.1
YPCD1.31c	LcrV	T3SA	0.9	3.1
YPCD1.32c	LcrG	regulator	0.2	2.4
YPCD1.34c	LcrD/YscV	T3SA	-9.3	-6.7
YPCD1.36c	YscX	secreted	-2.4	-0.3
YPCD1.39c	YopN	secreted regulator	-3.3	-1.1
YPCD1.40	YscN	T3SA	-0.1	1.9
YPCD1.42	YscP	T3SA	-9.0	-4.3
YPCD1.48	YscW	T3SA	-1.5	0.8
YPCD1.51	YscB	chaperone	-0.3	2.0
YPCD1.52	YscC	T3SA	0.4	2.6
YPCD1.54	YscE	T3SA	-0.4	1.8
YPCD1.55	YscF	T3SA	0.3	2.5
YPCD1.56	YscG	T3SA	-0.8	1.5
YPCD1.59	YscJ	T3SA	-0.7	1.2
YPCD1.61	YscL	T3SA	-0.9	1.2
YPCD1.67c	YopH	effector	-3.1	-0.9
YPCD1.71c	YopJ	effector	-4.5	-2.3
YPCD1.72c	YpkA/YopO	effector	-3.3	-1.1
YPCD1.73c		chaperone	-0.4	1.7