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Leandro E. Garcia[1,2], Zitong Lin[1], Sophie Culos[3], M Catherine Muenker[1], Emily E. Johnson[1], Zheng Wang[4], Francesc Lopez-Giraldez[5], Alexandre Giraud-Gatineau[6], Angela Jackson[7], Mathieu Picardeau[6], David R. Goodlett[3,7], Jeffrey P. Townsend[4,8], Helena Pětrošová[3,7], Elsio A. Wunder Jr[1,2,9*].

[1]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, Connecticut, United States of America.

[2]Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, Connecticut, United States of America.

[3]Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

[4]Department of Biostatistics, Yale School of Public Health, New Haven, Connecticut, United States of America.

[5]Yale Center for Genomic Analysis, New Haven, Connecticut, United States of America.

[6]Institut Pasteur, Université Paris Cité, CNRS UMR 6047, Biology of Spirochetes Unit, Paris, France.

[7]University of Victoria Genome BC Proteomic Center, Victoria, BC, Canada

[8]Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut, United States of America.

[9]Gonçalo Moniz Institute, Oswaldo Cruz Foundation; Brazilian Ministry of Health; Salvador, Brazil.

***Correspondence**: elsio.wunder@uconn.edu (EAWJ)

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ABSTRACT

Pathogenic *Leptospira* species can survive and thrive in a wide range of environments. Distinct environments expose the bacteria to different temperatures, osmolarities, and amounts and sources of nutrition. However, leptospires are mostly cultured, in a laboratory setting under *in vitro* conditions that do not reflect natural environments. This constraint on laboratory cultures limits the applicability of *in vitro* studies to the understanding of even simple pathogenic processes. Here we report, investigate, and identify a medium and conditions that mimic the host environment during leptospirosis infection, expanding the available *in vitro* tools to evaluate leptospiral pathogenesis. We quantified genome-wide gene expression of pathogenic *Leptospira interrogans* cultured in different *in vitro* media compositions (EMJH, DMEM, EMEM, and HAN). Using EMJH as standard, we compared gene expression in these compositions to genome-wide gene expression gathered in a host environment: whole blood (WB) of hamsters after infection with pathogenic leptospires. Leptospires cultured in DMEM and EMEM media shared 40% and 47% of all differentially expressed genes (DEGs) of leptospires present within WB (FDR<0.01), while leptospires cultured in HAN media only shared 20% of DEGs with those from WB. Furthermore, gene and pathway expression of leptospires cultured on DMEM and EMEM media exhibited a better correlation with leptospires grown in WB, including promoting expression of a similar leptospiral lipid A profile to the one identified directly in host tissues. Taken together, these results indicate that commercial cell-culture media EMEM or DMEM are better surrogates for *in vivo* pathogenic studies than EMJH or HAN media in *Leptospira*. These alternative culture conditions, using media that are a standard supply worldwide, provide a reproducible and cost-effective approach that can accelerate research investigation and reduce the number of animal infections necessary for basic research of leptospirosis.

INTRODUCTION

The use of laboratory animals in research is a common and often highly informative practice. A diverse field of studies have been using research animals to better understand pathogenesis, evaluate vaccine candidates, or viability of new drugs and treatments. However, research animals often experience disagreeable symptoms and painful disease progression [1, 2]. Animal experiments can also have a distressing effect on personnel performing this work [3]. These drawbacks have spurred increased animal welfare and bioethical regimentation [4]. Indeed, the importance of animal use in research has been debated; legislation has been enforced to protect animals and maintain humane conditions while conducting such research. Efforts have been encouraged to explore alternatives to animal use, including the 3Rs: replacement, reduction, and refinement [3, 4]. One possible alternative to reduce animal use in research is the expansion of *in vitro* assays that mimic specific conditions, like temperature and nutrition, that are highly relevant factors in the host environment [5, 6], enabling the generation *in vivo* of essential information regarding pathogenesis without infecting animals.

Animal models such as guinea pigs, hamsters, mice, and rats are widely used for pathogenesis studies of leptospirosis [7], a zoonotic disease of global importance. Leptospirosis is caused by a diverse group of pathogenic spirochetes of the genus *Leptospira*, an imposes a significant burden on impoverished populations living in tropical climates [8]. It is estimated that the disease causes 1 million cases with a mortality rate of ~60,000 deaths per year worldwide [8]. A large spectrum of reservoir animals, from rats to wild animals, can survive infection and have their renal tubules colonized with leptospires. Live pathogenic *Leptospira* can then be excreted in urine, contaminating environments (soil, mud, and water) where the spirochete can survive for weeks, causing spillover infections to humans and other animals and contributing to the life cycle of the disease [9-11]. There are no effective diagnostics or vaccines for leptospirosis, hampering prevention and treatment efforts and leading to infections with lethality as high as 10–50% [12]. The burden of leptospirosis is believed to increase with the rise of the urban slum population worldwide [13, 14] and extreme weather events intensified by global climate change [15]. The lack of understanding of the molecular mechanisms of leptospirosis pathogenesis has curtailed the development of effective control measures for this important neglected disease [16].

The most common medium used and commercially available worldwide for culture of leptospires, EMJH, was developed by Ellinghausen and McCullough [17] and modified by Johnson and Harris [18]. The EMJH medium replaced previously used serum-containing media (e.g. Stuart and Korthof), which fostered leptospiral growth but caused phosphate precipitates complicating darkfield microscope visualization [19]. EMJH typically consists mostly of a salt base supplemented with bovine serum albumin (fraction V), Tween 80, chlorides, sulfides, vitamins, and rabbit serum, with some minor variations in formulation [20-22]. EMJH balanced and crafted composition based on nutritional and metabolic needs of the bacteria, makes it an ideal medium for the growth of several species of pathogenic and nonpathogenic *Leptospira*. Puzzlingly, *in vitro* growth of leptospires commonly occurs is in aerobic conditions at 29–30^{0}C—a temperature that is not consistent with host environments and physiologies. A medium that has a composition similar to the host and can be used to culture *Leptospira* in host-like conditions can induce patterns of protein expression that are similar to those observed during the natural infection process [23-25]. The balanced salt solution of media like Minimum Essential Medium Eagle (MEM), when added to EMJH, changed the osmolarity and affected leptospiral regulation [26]. Recently, Hornsby-Alt-Nally developed the HAN medium, a modified EMJH formulation for propagation of leptospires; HAN facilitated increased growth of leptospires directly from clinical samples [27]. The main features of the HAN medium are the use of hemin as an iron source and the addition of Ham's Nutrient mixture F12 (Dulbecco′s Modified Eagle′s Medium), which enable the culture and growth of leptospires at 37°C and 5% CO_2.

The specific nutrition formula and growth conditions of a medium can be crucial to the capability of individual leptospiral strains surviving or adapting to culture. However, little is known about the distinct transcriptomics and biological pathways that are altered by those different media formulations. To understand the adaptation of leptospires to culture conditions and nutrition, we analyzed their transcriptomic profiles. Using RNA-seq, we compared the transcriptome of leptospires during the process of infection and in response to cell culture media that are commonly used for the growth of leptospires or mammalian cells. We then applied mass spectrometry approaches to compare *Leptospira* lipid A profiles under these different culturing conditions and in host tissue. Comparing these profiles to those associated with leptospires in a whole-blood hamster model, we identified a culture medium and conditions that can be used as a surrogate for *in vivo* studies on leptospires. Use of these media for studies of leptospiral pathogenesis increases

the applicability of *in vitro* virulence studies, while also saving costs and research time and reducing the need for animal infections to elucidate basic properties of leptospiral pathogenesis.

RESULTS

Media composition and culturing conditions determine growth behavior in pathogenic leptospires without affecting their virulence. We evaluated the growth of pathogenic *Leptospira* strain Fiocruz L1-130 in different media. To establish the optimal timepoint for comparing transcriptomes during the mid- to late-log phase of bacterial growth, we characterized the growth curve. We observed environmental effects using a single strain: distinct behaviors in each medium (Fig 1A). Growth in DMEM and EMEM media (37°C) trended similarly: leptospires exhibited rapid exponential growth that peaked on day 4, followed by a longer stationary phase through day 10, with a decline on the number of cells after that. Culture in EMJH (29°C) and HAN (37°C) led to a long exponential phase with peaks at days 10 and 14, respectively. However, leptospires cultured on HAN media never reached the concentrations observed in EMJH (29°C), plateauing at similar concentrations as DMEM and EMEM. Finally, transcriptome analysis of *Leptospira* genome-wide gene expression on the EMJH medium at 37°C was not possible: these culture conditions were not conducive to the growth and multiplication of leptospires (Fig 1A). Previous studies that successfully used leptospires cells cultured in EMJH at 37°C either performed an overnight shift from 30°C or used higher inoculum doses compared to the low dose of inoculum (10^4 leptospires) used in our experiments [28-30]. Furthermore, we evaluated the virulence of our *Leptospira* strain and its ability to cause disease in the hamster model after *in vitro* culture using each medium. All animals were challenged using a dose of 10^8 leptospires by a conjunctival route and reached the endpoint criteria between 8- and 10-days post-challenge (Fig 1B). Similar to previous results obtained with *L. interrogans* cultured using HAN [31, 32], culture in these diverse media, temperatures, and conditions did not negatively affect the growth and virulence of the strain upon infection.

Whole blood as a surrogate for host environment in leptospirosis infection. Pathogenic leptospires can multiply and disseminate in the blood of hamsters. Dissemination that leads to colonization of significant organs and clinical symptoms [33]. Accordingly, we selected blood as

a surrogate for the host environment relevant for infection. We then determined the best component of the hamster blood (whole blood, plasma, or serum) to be used to assess the transcriptome of *Leptospira* during infection. With an inoculum dose of 10^8 leptospires of strain Fiocruz L1-130 by conjunctival route, the animals started presenting symptoms of severe leptospirosis between days 8–10 post-challenge [34], at which point the animals were euthanized. At this stage, leptospires in the blood are on their peak and antibodies start to circulate [35]. Therefore, for this study we collected blood from the animals on day seven after the challenge to represent leptospires in the host environment.

Our qPCR analysis revealed statistically significant differences in the bacterial burden among the blood components. Leptospire concentration in whole blood reached 2.0×10^6 leptospires genome equivalents (GEq)/mL, which was significantly higher ($P < 0.05$) than the concentration reached in serum and plasma (7.9×10^3 and 2.4×10^3 leptospires GEq/mL, respectively; S1 Fig). Therefore, we selected whole blood seven days post-challenge as the comparator sample to evaluate media that approximate *Leptospira* in their host environment.

The transcriptome profile of pathogenic *Leptospira* is modulated by different culture conditions, with growth at 37 °C at 5% CO_2 eliciting gene expressions closely related to the host environment. We analyzed the complete transcriptomic data from different treatments using EMJH conditions as a baseline. The time for RNA extraction of leptospires on culture was defined based on the late-logarithmic stage under each media condition: day 4 for DMEM and EMEM and day 10 for HAN and EMJH at 29°C (Fig 1A). Principal component analysis (PCA) showed a strong association between our biological duplicates for each condition. Furthermore, a clear separation between EMJH and WB (62% of the variance in PC1) was observed, with EMEM and DMEM samples clustered closer to WB and HAN samples clustered closer to EMJH (Fig 2A). Differentially expressed genes (DEGs) were identified by a threshold False Discovery Rate (FDR) of less than 0.01 and an absolute log2 Fold Change (log2FC) ≥ 2. These thresholds established a total of 414 DEGs in DMEM, 505 DEGs in EMEM, 271 DEGs in HAN, and 740 DEGs in whole blood tissue (WB) of hamsters infected with strain L1-130 (Fig 2B), indicating that temperature and the composition of the medium exhibited significant effects on the transcriptome of pathogenic leptospires. Hierarchical clustering and heatmap analysis, using selected DEGs associated with virulence mechanisms [36-46], exhibited similarly distinct patterns of expression, with a clear

division of the EMJH cluster compared to other media and WB samples (Fig 2C). Also, in comparison to EMJH, we observed a similar gene expression pattern across WB with EMEM, DMEM, and HAN media (surrogate media). Genes regulating important virulence factors or regulatory networks (e.g. *hem0*, *ligA*, *ligB*, *sph2*, *colA*, *Tolc*, and *perB*) [42, 47-50] were upregulated in WB and surrogate media when compared to EMJH. In contrast, other virulence-related genes (e.g. *lsa27*, *lsa25*, *loa22*, *lipl41*, and *lipl45*) [48, 51], were significantly downregulated in WB and surrogate media compared to EMJH (Fig 2C). This observation indicates a high expression similarity between leptospiral gene expression in WB and in the surrogate *in vitro* media (EMEM, DMEM, and HAN) when compared to EMJH. This pattern was further supported by analysis of the top 10 DEGSs (up and down regulated) in WB samples (S1 Table).

We then investigated the influence of *in vitro* surrogate media on the bacterial cells compared to the host environment, performing an enrichment analysis of biological pathways using BioCyc tools [52] on DEGs between all treatments. We identified similar biological pathways related to metabolism in the surrogate *in vitro* media and WB (S1-S6 Tables). All surrogate *in vitro* media evaluated induced downregulation of genes involved in fatty-acid and lipid degradation while upregulating genes related to the biosynthesis of cobalamin (vitamin B12;. The DMEM medium induced higher expression levels for all analyzed genes in the vitamin B12 biosynthesis pathway, when compared with WB and other media (S2 Fig). Nevertheless, these results indicate that EMEM, DMEM, and HAN media, compared to EMJH, are good surrogates to study gene and pathway expressions that occurs during *in vivo* infection.

Quantitative real-time PCR of randomly selected genes (LIC10771, LIC11352, LIC11467, LIC11848, LIC20148, LIC_RS14805, and LIC_RS21185) in all conditions, corroborated our RNAseq results with a correlation coefficient of 0.70 (S3 Fig).

DMEM and EMEM media are an *in vitro* alternative that mimics the host environment. To identify the similarity between our treatments regarding gene expression, we performed a Pearson correlation of the log raw values of all DEGs (Fig 3A). We observed that WB samples exhibited the lowest correlation coefficients to EMJH (0.51-0.56) and HAN (0.67-072). Cultured on EMEM exhibited a positive correlation coefficient with cultures on DMEM (0.96 and 0.97), while cultures on both media shared the highest positive correlation values with WB samples (DMEM: 0.84–

0.86; EMEM: 0.84-0.87). We then performed a cluster of orthologous genes (COG) classification to categorize all differential expressed genes identified in WB (host environment) according to their predicted biological role (Fig 3B). Using EMJH as baseline, our analysis showed that EMEM and DMEM samples shared 164 and 132 DEGs with predicted biological function with the WB samples, respectively. In contrast, the HAN media induced only 64 COG-related DEGs shared with WB.

Furthermore, our DEGs analysis showed that leptospires cultured in EMEM and DMEM media shared more genes that were significantly up- (Fig 4A) and down-regulated (Fig 4B) in common with those in WB-cultured leptospires. DMEM- and EMEM-cultured leptospires shared 79% and 72% of their up-regulates genes, respectively, with those up-regulated in WB-cultured leptospires. Additionally, DMEM- and EMEM-cultured leptospires shared 65% of their leptospiral downregulated genes with those downregulated in WB-leptospires (S7 Table). Most importantly, when considering all DEGs identified in WB-leptospires, DMEM- and EMEM-leptospires shared 40% and 47% of them, respectively (S7 Table). In contrast, although cultured at 37°C with 5% CO_2, the HAN-leptospires shared only 18% of all the DEGs identified in WB-leptospires (Fig 4A–B and S7 Table). Those results indicate a higher similarity of DMEM- and EMEM-leptospires with the leptospiral gene expression occurring during *in vivo* infection.

***In vitro* culturing conditions at 37°C with 5% CO_2 induces a host-like expression of leptospiral lipid A biosynthesis genes.** To further evaluate the impact of growth conditions on *Leptospira* cells, we investigated their outer membrane components. Lipopolysaccharide (LPS) is an important virulence factor in pathogenic *Leptospira*. It consists of three parts: O-antigen, core antigen, and a hydrophilic anchor to the *Leptospira* outer membrane, lipid A [53]. *L. interrogans* derived from the host tissue presents with an altered O-antigen when compared to *L. interrogans* grown under the traditional conditions *in vitro* (EMJH) [54]. The precise structure of the complex *L. interrogans* O-antigen has not been elucidated to date. Therefore, we focused on the effect of the growth conditions on *Leptospira* lipid A. Bacterial cells have the ability to modify their lipid A structure to facilitate environmental adaptation or to evade host immune response [55]. Lipid A biosynthesis is a multistep process facilitated by a family of *lpx* genes. To evaluate the potential influence of the *in vivo* and *in vitro* environment on lipid A synthesis, we compared the expression of 8 genes related to lipid A biosynthesis among our different grow conditions. The expression

trend for most of those genes was similar between leptospires isolated from WB, HAN, EMEM, and EMEM compared to EMJH (S8 Table). The LpxC enzyme catalyzes a committed, energetically unfavorable, step-in lipid A biosynthesis [56]. The expression of *lpxC* was significantly upregulated (log2FC>2, $P < 0.05$) in WB-leptospires and upregulated with low expression values in leptospires cultured in EMEM, DMEM, and HAN (S8 Table). LpxK phosphorylates the lipid A precursor to form lipid IV_A in the last step of lipid A biosynthesis before adding sugar moieties to complete LPS [56]. Interesting, this gene was significantly upregulated in leptospires isolated from the host and leptospires cultured in DMEM, EMEM, and HAN. This result indicate that lipid A biosynthesis genes are altered in our *in vitro* media when compared to the EMJH medium, resulting in expression levels close related to the host environment.

***L. interrogans* lipid A profile in DMEM and EMEM media is similar to the one directly observed in the host environment.** Given the *in vitro* media-induced changes to expression of genes involved in biosynthesis of lipid A, we investigated the corresponding lipid A profiles. The lipid A of *L. interrogans* serovars Manilae and Copenhageni grown in EMEM, DMEM, HAN, and EMJH was extracted using the Fast Lipid Analysis Technique (FLAT) [57, 58] and analyzed using MALDI mass spectrometry (Fig 5A–E). Additionally, FLAT was used to investigate serum of *L. interrogans* serovar Copenhageni-infected hamsters. Lipid A profile of *L. interrogans* grown in EMJH at 30ºC corresponded to that published previously [43, 59-61]. The lipid A profile was complex, with several lipid A ion clusters separated by 28 Da, and a base peak at *m/z* 1722 [59, 60] (Fig 5A). Starting with a higher inoculum enabled us to obtain lipid A profile of *Leptospira* grown in the EMJH at 37 ºC with 5% CO_2. *L. interrogans* grown in EMJH and HAN at those conditions displayed an additional small lipid A ion cluster with a base peak at *m/z* 1542 (Fig 5B–C). Fragmentation *via* tandem MS analysis identified this cluster to consist of penta-acylated lipid A species. In comparison, lipid A profile of *L. interrogans* grown in EMEM and DMEM was significantly less complex (Fig 5D–E). The main lipid A cluster had a base peak at *m/z* 1748, corresponding to hexa-acylated lipid A species. The penta-acylated lipid A species with a base peak at *m/z* 1542 were also present (Fig 5D–E) under these conditions. *L. interrogans* serovars Copenhageni and Manilae share the same lipid A biosynthesis machinery, and we observed that their lipid A profiles were similar under different *in vitro* conditions (S4 Fig). No lipid A signal

was detected in the serum of infected hamsters, likely due to low *Leptospira* abundance in this blood component (S4 Fig).

To reveal *Leptospira* lipid A profile directly from the host, liver of hamsters infected with *L. interrogans* serovar Manilae were investigated by MALDI mass spectrometry imaging. Given the similarity between the whole-genome and lipid A profile *in vitro* between serovars Copenhageni and Manilae, the result of the latter was determined as representative of *L. interrogans* species. Tissues were collected one-, two-, three-, and four-days post-challenge. Lipid A signal was detected on day three and reached maximum intensity on the experimental endpoint, at day four post-challenge (Fig 5F). The *Leptospira* lipid A profile in tissue (Fig 5G) resembled that identified in EMEM and DMEM media (Fig 5D–E). It was less complex than the lipid A profile in the EMJH and HAN media, with a base peak at *m/z* 1748.26 and additional small lipid A cluster around the *m/z* 1722.25 ion. To confirm that the observed ions were in fact lipid A molecules and not host lipids, the *m/z* 1748.26 ion was analyzed by on-tissue tandem spectrometry. The following diagnostic lipid A ions were identified: *m/z* 110.98 (representing a methylated phosphate), *m/z* 723.46 (B_1 ion resulting from fragmentation of the di-aminoglucose backbone), and *m/z* 1299.91 and 1524.09 (lipid A fragment ions resulting from the loss of 2'ε and 3'ε and 2'ε or 3' ε fatty acyl chains, respectively) [60], confirming *m/z* 1748.26 to be *Leptospira* lipid A (S5A Fig). Infected and control hamster liver tissues four days post-challenge were homogenized for lipid A microextraction [62, 63]. Lipid A signal was detected in 2/3 extracts from liver of *Leptospira*-infected animals (S5B-C Fig) and was not present in any of the four liver extracts from negative controls (S5D Fig). The fragmentation pattern of the extracted lipid A was identical to that obtained by on-tissue tandem mass spectrometry (S5A Fig), further validating our results. The penta-acylated lipid A species identified in EMEM- and DMEM-leptospires were not detected in tissue by either technique. However, it is possible that the abundance of these lipid A species was below the limit of detection of the technique used. Collectively, these results indicate that the lipid A profile of *L. interrogans* is highly influenced by culturing conditions and media components. Furthermore, the lipid A profile of *Leptospira* cultured in EMEM and DMEM media resembles *Leptospira* lipid A observed in the host, highlighting the potential of both media to be used as surrogates of *in vivo* conditions.

DISCUSSION

Leptospira is an environmentally transmitted spirochete that can survive for weeks in water or soil. Pathogenic species of the *Leptospira* genus have the ability to infect, disseminate, and colonize tissues of a wide range of mammal hosts [12]. Each of these environments has a different nutrition composition, osmolarity, and temperature. Previous studies have shown that shifting leptospires between different environments and conditions alters the bacterial transcriptome and proteome, as the spirochetes adapt to those changes [23, 64-70]. Despite recent efforts, the molecular mechanisms behind the pathogenesis and adaptation ability of leptospires to survive on this wide spectrum of environments are still unknown [71]. The lack of an *in vitro* media that can mimic host conditions has been a major barrier and a contributing factor to this knowledge gap.

In this study, temperature and media composition impacted the growth of pathogenic *Leptospira* without changing their pathogenicity. Leptospires are fastidious bacteria that require long chain fatty acids and ammonium ions as source of carbon and nitrogen, respectively, and the presence of select vitamins and nutritional supplements for optimal growth [21]. The most common medium used for *in vitro* culture and propagation of leptospires is the EMJH [20]. Although EMJH offers the bacteria all the nutrients necessary for survival and multiplication, it does not mimic host characteristics and conditions [46], leading to adaptation and potentially attenuation after high passages during *in vitro* culturing [72]. Moreover, the different recipes and variations of EMJH available create additional challenges with reproducibility of *in vitro* experiments [20, 73]. Recently, the HAN (Hornsby-Alt-Nally) medium has been proposed to culture pathogenic leptospires directly from host tissues [27]. Even though the components of the HAN medium are chemically defined, the major difference between HAN and EMJH is the source of iron and the addition of DMEM medium. Although the HAN media allowed the leptospires to thrive at 37 °C and 5% CO2, we observed a growth pattern very similar to EMJH at 29 °C, with cell growth peaking at day 10. In contrast, DMEM and EMEM media induced leptospires to a faster exponential growth curve, reaching their peak growth in four days, with similar cell numbers as the maximum peak observed in the HAN medium, but lower than in the EMJH medium. EMEM and DMEM use glucose as source of carbon, with the latter having a higher concentration of glucose, vitamins, and amino acids. Nevertheless, the ability of the *Leptospira* strain Fiocruz L1-130 to cause disease in the hamster model was not affected by the medium type, temperature, or CO_2 levels used for *in vitro* cultivation.

The transcriptome of virulent leptospires was highly affected under *in vitro* media that mimic the host environment, inducing gene expressions closely related to *in vivo* conditions. Previous studies have shown that individual conditions, similar to host environment, like osmolarity [26, 66, 67] or temperature shifts [24, 28-30, 69, 72, 74] can alter leptospiral gene and protein expression. Our results showed that exposing leptospires to a host-like environment based on temperature, osmolarity, and levels of CO_2, elicited a dramatic change of the leptospiral transcriptome in comparison with the traditional EMJH medium. Furthermore, our results indicated that the transcriptome shifts induced by *in vitro* host-like environment is similar to the shifts observed during the host infection. Previous studies have suggested that vitamin B12 (cobalamin) synthesis is critical for *in vivo* survival and dissemination of pathogenic leptospires, which is highlighted by its absence in saprophytic species of *Leptospira* [48, 75]. In our results, we identified a six-gene cluster involved in cob(II)yrinate a,c diamide biosynthesis I (early cobalt insertion; part of the cobalamin biosynthesis pathway) significantly activated in EMEM, DMEM, HAN, and WB samples compared to EMJH. In contrast, we observed that fatty acid and lipid degradation pathways were downregulated in WB, DMEM, and EMEM only, when compared to EMJH. This pathway is involved in the metabolism of fatty acids and lipids as a source of nutrients and energy for the cell. The upregulation of this pathway in EMJH and HAN media might be related to their high concentration of Tween, used as an energy source. When evaluating the expression of virulence factors, we observed that important genes encoding leptospiral immunoglobulin-like proteins (*ligA* and *ligB)* [26], sphingomyelinase hemolysin (*sph2*) [38, 66, 76], and collagenase (*colA*) [77, 78] where all upregulated on EMEM, DMEM, HAN, and WB samples compared to EMJH. Finally, the gene *lipL36*, which encodes a lipoprotein (LipL36) expressed when *Leptospira* grows *in vitro* at 30 °C but not *in vitro* at 37°C or *in vivo* [79], was downregulated in WB, HAN, EMEM, and DMEM.

The tissue culture media DMEM and EMEM are promising candidates as *in vitro* surrogates for *in vivo* experiments that evaluate leptospiral gene expression and pathogenesis. Heavy animal use in research has been raising ethical concerns. Leptospiral studies frequently rely on laboratory animal models, even though there is no consensus correlating experimental infection studies with natural infection [7]. A recent study used intraperitoneal dialysis membrane chambers (DMC) that replicated conditions of a natural leptospirosis infection. The authors identified several essential genes modulated *in vivo*, but the method involves animal experiments and surviving

surgery [64]. In our experiments, analyzing the transcriptome of hamster blood infected with leptospires, we identified more than 500 differentially expressed genes (DEGs) that do not appear in the DMC approach. When considering the number of DEGs, the correlation of modulated genes, and genes orthologous classification, our analysis showed that DMEM and EMEM media are comparable to WB samples, and thus to *in vivo* conditions. DMEM and EMEM conditions lead to a gene expression that shared 40% and 47%, respectively, of all differentially regulated genes on the host (WB). In contrast, HAN and DMC shared only 18% and 9%, respectively. Most pathogenesis studies focus on host-pathogen interactions with different animal models, resulting in many animals experiencing painful disease progression. Each animal has a variable reaction to infectious agents based on genetic and environmental conditions and reproducibility is required as the foundation of conducting the experiment [80]. Repeating animal experiments always involves extra cost, time, and other resources. Compassion burnout in technicians can also present a challenge to proper animal care [81]. Thus, minimizing or reducing animal use and meeting animal welfare requirements are desirable and regulated under the federal government.

The growth media composition and *in vitro* culturing conditions also impacted the *Leptospira* lipid A profile. Many bacteria species, including *Leptospira*, maintain the integrity of their membranes by modifying lipid A structure in response to environmental cues (*e.g.* temperature shift and presence of antimicrobials) [43, 82, 83]. Growth in DMEM and EMEM media reduced heterogeneity of *Leptospira* lipid A and induced incorporation of longer secondary fatty acyl chains and the occurrence of penta-acylated lipid A species. *Leptospira* cannot synthesize fatty acids *de novo* and has only a limited capacity to modify them [84]. Therefore, one possible explanation of the observed lipid A diversity is a variation in the availability of the individual fatty acids in different media. Both HAN and EMJH use Tween 80 as a major source of fatty acids (50-70% oleic acid, balanced with linoleic, palmitic, and stearic acids). EMEM and DMEM source of fatty acids is exclusively from the addition of rabbit serum, which overall is composed of palmitic acid (~40%), linoleic acid derivatives (~20%), and oleic acid (~20%) [85]. Nevertheless, changes in the abundances of transcripts of genes involved in lipid A biosynthesis were detected between the growth conditions, suggesting that modifications to lipid A were also regulated on the gene expression level. Further studies to better understand the effect of the source of fatty acids on the leptospiral lipid A profile should be considered.

EMEM- and DMEM-grown *Leptospira* induced lipid A profile more closely to the bacteria found *in vivo*. Detection of lipid A by MALDI mass spectrometry requires mild-acid hydrolysis that disrupts the covalent bond between the molecule and the rest of the LPS; lipid A otherwise does not ionize [57]. Curiously, *Leptospira* lipid A was readily detectable in the liver of infected hamsters without the need for on-tissue derivatization that ordinarily facilitates the hydrolysis [86]. The direct detection suggests that a portion of lipid A is present in the *Leptospira* membrane in a free form, not bound to LPS. The only other bacterium described to date with free lipid A is *Francisella*. *Francisella* lipid A is also detectable in tissue without needing of acid hydrolysis and its LPS has similar biochemical properties to *Leptospira* LPS [63, 87, 88]. Lipid A activates the innate immune cascades by binding to the Toll-like receptor 4-myeloid differentiation protein 2 complex (TLR4-MD2). Variation in the number, length, and saturation of lipid A acyl chains affects the binding efficiency, modifying the downstream TLR-induced signalling and the recruitment of immune responses [89-95]. Overall, the *Leptospira* lipid A profile in tissue differed from that of the EMJH-grown *Leptospira* traditionally used to study host immune responses and to generate bacterins for animal vaccines against leptospirosis. In the future, experiments to determine activation of TLR4-MD2 by individual lipid A extracts will be essential to better understand media composition and lipid A structure on immune responses against leptospirosis.

In summary, in this work, we characterize the *in vitro* growth of virulent *Leptospira* sp. using different media and conditions commonly used in various mammalian cell types. Our growth analysis indicated that EMEM or DMEM are good alternative media to grow leptospires in a host-like environment and circumvent the issue of fastidiousness when culturing pathogenic *Leptospira*. After the identification of different profile signatures obtained in each medium, we also observed that commercial media DMEM and EMEM elicit similar gene expressions as the *in vivo* environment represented here by whole blood of infected hamsters. Furthermore, *Leptospira* lipid A was, for the first time, characterized directly from the infected tissue and our results showed that DMEM and EMEM media induces a lipid A profile that is more closely related to that *in vivo* when compared to traditional growth conditions. Although the HAN media has been developed and used as an alternative for EMJH to isolate leptospires, HAN is closer to EMJH than the other media used here when considering the induced transcriptomic analysis and lipid A structure. Our results are limited by using a minimal number of replicates (n=2). Therefore, we used conservative cut-off values for our analysis and validated our findings using additional methods and approaches.

Our choice for host-condition sample (7-days post-infection blood) has established biological relevance. However, we do not have a clear understanding of the transcriptomic profile of leptospires in different tissues and times of the infection process. Additional experiments are in progress in appropriate media to address those differences.

Recently, the leptospiral field has seen an increased on the number of novel *Leptospira* species identified [96, 97], coupled with an emergence of cases around the world, including in developed countries [98]. In parallel, there have been major advances in development of genetic tools to manipulate leptospires cells [99] and important discoveries on *Leptospira* biology and pathogenicity [71]. Both DMEM and EMEM media can be found worldwide at lower costs compared to EMJH and HAN and have a defined composition eliminating the necessity of in-house media production and variability. The prospect of a commercially available *in vitro* media that can be used as a surrogate for the host environment is critical and essential to reduce animal use, simplify the culture of leptospires, and facilitate research, thus allowing for new discoveries on this important neglected disease.

METHODS

Ethics Statement

The protocol of animal experimentation was prepared and approved according to the guidelines of the Institutional Committee for the Use of Experimental Animals at Yale University (protocol # 2023–11424) and Institute Pasteur (protocol # 220016). All animal procedures carried out at Institute Pasteur were performed in accordance with the European Union legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). Animals were monitored twice daily for endpoints, including signs of disease and death, up to 21-days post-infection. Surviving animals at the end of the experiment (7-days post-challenge) or moribund animals showing difficulty in moving, breathing, and/or signs of bleeding or seizure were immediately sacrificed by inhalation of CO_2.

Bacteria culture, media, and growth conditions

A low-passage of *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 was cultured in four different media (EMJH, EMEM, DMEM, and HAN). We used Ellinghausen–

McCullough–Johnson–Harris (EMJH) [18] supplemented with 1% rabbit serum (Sigma-Aldrich) at 30˚C in a shaking incubator (100 rpm). EMEM medium was prepared from Eagle's Minimum Essential Medium (EMEM; ATCC 30-2003) with 5% rabbit serum and 100 µL 0.036 mM $FeSO_4$. DMEM medium was prepared from Dulbecco's modified Eagle medium (DMEM) supplemented with 5% rabbit serum and 100 µl 0.036 mM FeSO4, and HAN medium was prepared following the methods previously described by the authors [27]. Leptospires were enumerated by dark-field microscopy in a Petroff-Hausser chamber (Thermo Fisher Scientific, Waltham, MA, USA). Each media was inoculated with 10^4 *L. interrogans* serovar Copenhageni strain L1-130 and incubated at 37 °C under 5% CO_2 (EMJH 37 °C, EMEM, DMEM, and HAN) and at 29 °C (EMJH 29 °C). The growth curve was evaluated by counting the cells every other day for 14 days. The experiment was repeated twice for reproducibility.

Animal challenge

Golden Syrian Hamsters (*Mesocricetus auratus*) were used as the animal model. Hamsters are highly susceptible to leptospirosis, are the model of choice for acute leptospirosis, and emulate the natural history and clinical presentation of severe leptospirosis in humans [100, 101]. Two groups of six 12-week-old male hamsters were infected with a dose of 10^8 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 by conjunctival route (Wunder et al., 2016). After 7-days post-challenge, five hamsters were euthanized, and one hamster was left for confirmation of virulence. During the euthanasia, 3 mL of blood was taken by cardiac puncture from each animal for DNA and RNA extraction. Blood was collected with and without EDTA for DNA extraction to obtain sera and plasma. For RNA extraction, blood was collected using only EDTA tubes.

For the virulence studies, four groups of three 12-week-old male hamsters were infected with a dose of 10^8 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 by conjunctival route as described above. Each group was challenged with leptospires cultured under different media and conditions: HAN, EMEM, and DMEM culture at 37 °C under 5% CO_2, and EMJH cultured aerobically at 29 °C as described above.

For the mass spectrometry imaging experiment, four groups of four 12-week-old male hamsters were inoculated intraperitoneally with 10^8 of low-passage *L. interrogans* serovar Manilae strain L495 grown in EMJH medium to mid-logarithmic phase. Hamsters were sacrificed at Day 1, 2, 3 and 4 post-infections (n=4 per group). One out of sixteen hamsters succumbed to infection

before the experimental end point, and was, therefore, not included in the analysis. Control group (n = 4) was inoculated intraperitoneally with EMJH medium only and sacrificed at Day 4 post-injection. Liver was collected and immediately frozen by floating in vapors of liquid nitrogen. Tissues were stored protected from moisture at −80°C until processing.

DNA and RNA extraction

Sera and plasma were obtained by centrifugation of clotted blood and whole blood with EDTA at 1,000 × g for 15 min at room temperature. DNA was extracted from 250 µL of hamsters' serum, plasma, and whole blood using Maxwell® 16 tissue DNA purification kit (Promega), following the manufacturer's instructions and using a 200-elution volume.

We extracted RNA for two biological replicates of each culture condition and hamster blood. A volume of 20 mL of leptospires cultured on different media at late-logarithmic phase and EDTA tubes containing ~3 mL of whole blood from each hamster were centrifuged at 6000 x g for 15 minutes. Trizol (Invitrogen) was added to the pellets for RNA stabilization (3:1 *v/v*). RNA isolation was performed on 400 µL of the mix using Direct-zol RNA MiniPrep (Zymo Research), following manufacturer's instructions. TURBO DNA-free kit (Invitrogen) was used to eliminate DNA residuals. RNA concentration, purity, and quality were verified using Qubit and NanoDrop instruments (Thermo Fisher Scientific). Samples were stored at −80°C until downstream analysis.

RNA-Sequencing and Data Analysis

Samples were sent to Yale Center for Genome Analysis (YCGA) for bioanalyzer, to check integrity and concentration. Only samples with RIN > 7 were chosen for mRNA library construction using TruSeq standard mRNA Library Prep kit (Illumina) and sequenced on NovaSeq (Illumina) with 100 base pair paired end reads. To increase the RNA alignment quantity from *Leptospira* extracted from bacterial cells using blood of hamsters, we supplied multi-targeted primers (MTPs) [102] to the reverse transcription to specifically generate cDNA from *Leptospira* genomic mRNA: degenerated primers were sorted and scored for their binding preferences against the genome of *Leptospira* strain Fiocruz L1-130 and the host hamster. The primer pool with the highest MTP score was used for the cDNA libraries preparation. Trimmed raw reads were aligned to the reference genome of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 from NCBI

(267671) using HISAT2 v2.1 [103]. Alignments with a quality score below 20 were excluded from further analysis. Reads were counted for each gene with StringTie v1.3.3 [103], and differentially expression analysis was performed in DESeq2 [104]. *P*-values were corrected for multiple testing with Benjamini-Hochberg correction chosen with thresholds of $P < 0.01$ and log2FC ± 2. PCA plots showed samples clustering by different culture medium.

qPCR and RT-qPCR for Target Gene Identification and RNA Seq Data Validation

Using the extracted DNA, the concentration of leptospires was quantified by a TaqMan-based quantitative-PCR assay using an ABI 7500 system (Thermo Fisher Scientific) and Platinum Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific). The qPCR reaction was performed using *lipL32* primers and probes as previously described [34]. Based on a standard curve, the bacterial quantification was calculated and expressed as the number of leptospires per milliliter. For the RT-PCR, the High-capacity cDNA reverse transcription kit (Applied Biosystems) was employed to convert total RNA to single-stranded cDNA. The RT-PCR was performed on 7500 fast real-time PCR (Applied Biosystems) using iQTM SYBRR Green SuperMix (Bio-Rad) according to the manufacturer's instructions. For the endogenous control, we used the 23S rRNA gene. The thermal cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. The specificity of the SYBR green PCR signal was confirmed by melt curve analysis. A relative quantification analysis was performed using the comparative CT method, and the relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method [105].

EGGNOG classification and Enrichment of biological pathways

We assessed the cluster of orthologous genes (COG) classification system to extract important information from our gene lists according to their homologous relationships [106]. We use the Pathway tool from BioCyc to analyze biological pathways with an enriched list of differentially expressed genes between treatments[52].

***Leptospira* lipid A extraction**

Cultured cells. *L. interrogans* serovars Manilae strain L495 [107] and serovar Copenhageni strain Fiocruz L1-130 were grown in triplicates to a mid-logarithmic growth phase (approximate density of 5×10^8 cells/mL; as determined by dark field microscopy). Five milliliters

of culture were pelleted *via* centrifugation at 4,000× g for 15 minutes. Pellets were washed twice with 1 mL of phosphate buffered saline (Sigma Aldrich) and reconstituted with 200 µL of MS-grade water (Fisher Chemical). Lipid A was isolated from the resulting cell suspension by the Fast Lipid Analysis Technique (FLAT^n) [57, 58]. Briefly, one microliter of the sample was spotted in triplicate on a disposable Matrix-Assisted Laser Desorption/Ionization (MALDI) plate (MFX µFocus plate 12×8 c 2,600 µm, Hudson Surface Technology) and dried. One microliter of citric acid buffer (0.2M citric acid, 0.1M trisodium citrate, Fisher Chemical) was then deposited over the samples. Plates were incubated over in a glass humidifying chamber filled with 4 mL of MS grade water at 110 °C for 30 minutes, allowed to cool, and washed with ~10–15 mL deionized water.

Infected tissues. Lipid A was extracted from tissues according to the lipid A microextraction protocol with modifications [62, 63]. Briefly, approximately 50 mg of *Leptospira*-infected or uninfected (control) hamster liver tissue was homogenized using a Retsch MM 400 homogenizer (Retsch) at 28Hz for 2 × 2min. Samples were cooled in liquid nitrogen vapor between the cycles. The homogenized tissue was resuspended in 1.2 mL of isobutyric acid: 1 M ammonium hydroxide (5:3, *v/v*; Sigma Aldrich) and incubated for 2 h at 100 °C with occasional vortexing. Samples were cooled on ice and centrifuged at 2000× g for 15 min at room temperature. Pellets were washed twice with methanol. The methanol-insoluble lipid A was then extracted from the pellet using chloroform: methanol: water (3: 1.5: 0.25, *v/v*; Fisher Chemical). Samples were centrifuged for 1.5 min at 6000× g, and lipid A was re-extracted with chloroform: methanol (2:1, *v/v*). Both lipid A extracts were combined in an autosampler vial with a glass insert and brought to dryness under a stream of heated nitrogen gas (35 ºC). Finally, samples were rehydrated in 10 µL of chloroform: methanol (2:1, *v/v*), and 1.5 µL was spotted on a disposable MALDI plate.

Mass Spectrometry Analysis of extracted *Leptospira* lipid A

Lipid A extracts were overlaid with one microliter of norharmane matrix (Sigma Aldrich) dissolved to 10mg/mL in MS grade chloroform: methanol (2:1, *v/v*). Data was acquired on a timsTOF flex MALDI-2 (Bruker) in the negative ion mode with 1 burst of 1,000 shots per spot at 5,000 Hz. The following settings were used: scan range *m/z* 600–2300, collision RF 4,000 Vpp, pre-pulse storage 15.0 ms, and transfer time 150 ms. Lipid A ions were analyzed by tandem mass spectrometry, using a 1,000 shots per spot, *m/z* 4 isolation width and variable collision energy

(100–120V), collision RF (700–4,000 Vpp), pre-pulse storage (7.0–15.0 ms), and transfer time (70–150 ms) to generate a full mass fragment signature. Mass spectra were analyzed using Compass Data Analysis v6.0 (Bruker) and mMass v5.5.0 [108].

Mass Spectrometry Imaging

Liver tissues were sectioned to 10 µm thickness on a C1950 cryostat (Leica Biosystems); chamber temperature was set to −17 °C, specimen head cooling was turned off. Sections were thaw-mounted on indium tin oxide slides (Delta Technologies), dried in a nitrogen box for 30 minutes and stored protected from moisture at −80 °C until processing. Samples were taken out of the freezer in randomized order, thawed sealed at room temperature, and dried in a nitrogen box for 30 minutes. One microliter of SPLASH LIPIDOMIX Mass Spec Standard (Croda) in MS-grade methanol (Fisher Chemical) was deposited on the slide separated from the tissue for quality control purposes. Norharmane matrix (7 mg/ml; Sigma Aldrich) in 2:1 (v/v) MS-grade chloroform and methanol (Fisher Chemical) was deposited on tissue sections using an automated pneumatic HTX M5 sprayer (HTX Technologies). Spraying conditions were as follows: 12 passes with 30 s drying time between them, 30 °C nozzle temperature, 0.12 ml/min flow rate, 1200 mm/min velocity, 3 mm track spacing, crisscross pattern, 10 psi pressure, 2 l/min gas flow rate, 40 mm nozzle height and heated tray temperature at 25 °C [109]. Slides were sprayed in batches of three and kept in the nitrogen box until processing. Optical images were acquired using an office scanner (Epson) and regions for imaging were outlined in Flex Imaging (Bruker): tissue, matrix only (negative control), and an area with the SPLASH LIPIDOMIX standard (quality control). A timsTOF flex MALDI-2 instrument was calibrated in the electrospray mode using a direct infusion of the Agilent Tuning Mix (Agilent Technologies). Mass spectra were acquired using the following settings: custom laser with a single smart beam (46 × 46 µm; resulting in 50µm pixel size), scan range *m/z* 600–2300, 250 shots per spot at 5,000 Hz, collision RF 4,000 Vpp, pre-pulse storage 15.0 ms, and transfer time 150 ms. The following ions were used for online calibration: *m/z* 515.1620 (norharmane matrix ion) *m/z* 885.5497 (phosphatidylinositol, PI 38:4), *m/z* 1447.9650 (cardiolipin, CL 72:8) and *m/z* 1449.9806 (cardiolipin, CL 72:7). On-tissue tandem mass spectrometry analysis of lipid A was performed using the same parameters as described above for on-plate tandem mass spectrometry. Raw data were processed in SCiLS Lab v2024a Pro (Bruker) and ion images were created upon normalization to total ion count.

607

608 **Statistical Analysis**

609 GraphPad Prism (Prism Mac 5.0) was used to analyze *in vivo* and *in vitro* assays
610 statistically. Real-time qRT-PCR data were analyzed using one-way ANOVA with Bonferroni post
611 hoc test at $P < 0.05$. Benjamini-Hochberg FDR was use as cut off for RNA seq data. Pearson
612 correlation was used to compare RNA expression between different treatments. Deseq2 Package
613 was used to perform differential expressed analysis [104].

614

615 **Data Availability**

616 RNA-Seq reads are available in the NCBI GEO data base. Mass spectrometry imaging data
617 were converted to the imzML format in SCiLS (centroid, no normalization). The resulting imzML
618 files can be accessed *via* METASPACE [110] using the following link:
619 https://metaspace2020.eu/project/Leptospira_lipidA_liver.

620

621 **COMPETING INTERESTS**

622 DRG is a co-founder and a vice president of Patagain, a company that develops mass
623 spectrometry-based microbiology tests to identify pathogens and determine antimicrobial
624 resistance. The company was not involved on the development of this work or analysis and
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627

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REFERENCES

1. Baamonde A, Menendez L. Experiences and reflections about behavioral pain assays in laboratory animals. J Neurosci Methods. 2023;386:109783. Epub 20230104. doi: 10.1016/j.jneumeth.2023.109783. PubMed PMID: 36610617.
2. Freires IA, Morelo DFC, Soares LFF, Costa IS, de Araujo LP, Breseghello I, et al. Progress and promise of alternative animal and non-animal methods in biomedical research. Arch Toxicol. 2023;97(9):2329-42. Epub 20230702. doi: 10.1007/s00204-023-03532-1. PubMed PMID: 37394624.
3. Goodman J, Chandna A, Roe K. Trends in animal use at US research facilities. J Med Ethics. 2015;41(7):567-9. Epub 20150225. doi: 10.1136/medethics-2014-102404. PubMed PMID: 25717142.
4. Robinson NB, Krieger K, Khan FM, Huffman W, Chang M, Naik A, et al. The current state of animal models in research: A review. Int J Surg. 2019;72:9-13. Epub 20191015. doi: 10.1016/j.ijsu.2019.10.015. PubMed PMID: 31627013.
5. Wang Z, Miguel-Rojas C, Lopez-Giraldez F, Yarden O, Trail F, Townsend JP. Metabolism and Development during Conidial Germination in Response to a Carbon-Nitrogen-Rich Synthetic or a Natural Source of Nutrition in Neurospora crassa. mBio. 2019;10(2). Epub 20190326. doi: 10.1128/mBio.00192-19. PubMed PMID: 30914504; PubMed Central PMCID: PMCPMC6437048.
6. Last A, Maurer M, A SM, M SG, Hube B. In vitro infection models to study fungal-host interactions. FEMS Microbiol Rev. 2021;45(5). doi: 10.1093/femsre/fuab005. PubMed PMID: 33524102; PubMed Central PMCID: PMCPMC8498566.
7. Gomes-Solecki M, Santecchia I, Werts C. Animal Models of Leptospirosis: Of Mice and Hamsters. Frontiers in immunology. 2017;8:58. doi: 10.3389/fimmu.2017.00058. PubMed PMID: 28270811; PubMed Central PMCID: PMCPMC5318464.
8. Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS neglected tropical diseases. 2015;9(9):e0003898. doi: 10.1371/journal.pntd.0003898. PubMed PMID: 26379143; PubMed Central PMCID: PMCPMC4574773.
9. Casanovas-Massana A, Pedra GG, Wunder EA, Jr., Diggle PJ, Begon M, Ko AI. Quantification of Leptospira interrogans Survival in Soil and Water Microcosms. Applied and environmental microbiology. 2018;84(13). Epub 2018/04/29. doi: 10.1128/AEM.00507-18. PubMed PMID: 29703737; PubMed Central PMCID: PMCPMC6007094.
10. Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD, Jr., Riley LW. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. Lancet. 1999;354(9181):820-5. PubMed PMID: 10485724.
11. Ko AI, Goarant C, Picardeau M. Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nature reviews Microbiology. 2009;7(10):736-47. doi: 10.1038/nrmicro2208. PubMed PMID: 19756012; PubMed Central PMCID: PMC3384523.
12. Wunder EA, Adhikarla H, Hamond C, Owers Bonner KA, Liang L, Rodrigues CB, et al. A live attenuated-vaccine model confers cross-protective immunity against different species of

the Leptospira genus. Elife. 2021;10. Epub 2021/01/27. doi: 10.7554/eLife.64166. PubMed PMID: 33496263; PubMed Central PMCID: PMCPMC7837694.

13. Khalil H, Santana R, de Oliveira D, Palma F, Lustosa R, Eyre MT, et al. Poverty, sanitation, and Leptospira transmission pathways in residents from four Brazilian slums. PLoS Negl Trop Dis. 2021;15(3):e0009256. Epub 20210331. doi: 10.1371/journal.pntd.0009256. PubMed PMID: 33788864; PubMed Central PMCID: PMCPMC8041187.

14. Vinetz JM, Glass GE, Flexner CE, Mueller P, Kaslow DC. Sporadic urban leptospirosis. Annals of internal medicine. 1996;125(10):794-8. PubMed PMID: 8928985.

15. Weinberger D, Baroux N, Grangeon JP, Ko AI, Goarant C. El Nino Southern Oscillation and leptospirosis outbreaks in New Caledonia. PLoS neglected tropical diseases. 2014;8(4):e2798. Epub 2014/04/20. doi: 10.1371/journal.pntd.0002798. PubMed PMID: 24743322; PubMed Central PMCID: PMCPMC3990495.

16. Barbosa AS, Patarakul K, Isaac L. Editorial: Leptospirosis: pathogenesis, clinical and epidemiological aspects. Front Cell Infect Microbiol. 2023;13:1210178. Epub 20230509. doi: 10.3389/fcimb.2023.1210178. PubMed PMID: 37228663; PubMed Central PMCID: PMCPMC10203697.

17. Ellinghausen HC, Jr., McCullough WG. Nutrition of Leptospira Pomona and Growth of 13 Other Serotypes: Fractionation of Oleic Albumin Complex and a Medium of Bovine Albumin and Polysorbate 80. Am J Vet Res. 1965;26:45-51. PubMed PMID: 14266934.

18. Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic letospires. I. Growth at low temperatures. Journal of bacteriology. 1967;94(1):27-31. PubMed PMID: 6027998; PubMed Central PMCID: PMCPMC251866.

19. Adler B. History of leptospirosis and leptospira. Current topics in microbiology and immunology. 2015;387:1-9. doi: 10.1007/978-3-662-45059-8_1. PubMed PMID: 25388129.

20. Guedes IB, Souza GO, Castro JFP, Cavalini MB, Filho AFS, Aizawa J, et al. Improvement of the enrichment used in the EMJH medium (Ellinghausen-McCullough-Johnson-Harris) for the cultivation of Leptospira spp. Revista Argentina de microbiologia. 2022;54(2):95-9. Epub 20210601. doi: 10.1016/j.ram.2021.03.002. PubMed PMID: 34083031.

21. Cameron CE. Leptospiral structure, physiology, and metabolism. Current topics in microbiology and immunology. 2015;387:21-41. doi: 10.1007/978-3-662-45059-8_3. PubMed PMID: 25388131.

22. Ellis WA. Animal leptospirosis. Current topics in microbiology and immunology. 2015;387:99-137. doi: 10.1007/978-3-662-45059-8_6. PubMed PMID: 25388134.

23. Nally JE, Grassmann AA, Planchon S, Sergeant K, Renaut J, Seshu J, et al. Pathogenic Leptospires Modulate Protein Expression and Post-translational Modifications in Response to Mammalian Host Signals. Frontiers in cellular and infection microbiology. 2017;7:362. doi: 10.3389/fcimb.2017.00362. PubMed PMID: 28848720; PubMed Central PMCID: PMCPMC5553009.

24. Nally JE, Timoney JF, Stevenson B. Temperature-regulated protein synthesis by Leptospira interrogans. Infect Immun. 2001;69(1):400-4. doi: 10.1128/IAI.69.1.400-404.2001. PubMed PMID: 11119530; PubMed Central PMCID: PMCPMC97896.

25. Nally JE, Whitelegge JP, Bassilian S, Blanco DR, Lovett MA. Characterization of the outer membrane proteome of Leptospira interrogans expressed during acute lethal infection. Infection and immunity. 2007;75(2):766-73. doi: 10.1128/IAI.00741-06. PubMed PMID: 17101664; PubMed Central PMCID: PMC1828474.

26. Matsunaga J, Sanchez Y, Xu X, Haake DA. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun. 2005;73(1):70-8. doi: 10.1128/IAI.73.1.70-78.2005. PubMed PMID: 15618142; PubMed Central PMCID: PMCPMC538979.
27. Hornsby RL, Alt DP, Nally JE. Isolation and propagation of leptospires at 37 degrees C directly from the mammalian host. Sci Rep. 2020;10(1):9620. Epub 20200615. doi: 10.1038/s41598-020-66526-4. PubMed PMID: 32541841; PubMed Central PMCID: PMCPMC7296004.
28. Qin JH, Sheng YY, Zhang ZM, Shi YZ, He P, Hu BY, et al. Genome-wide transcriptional analysis of temperature shift in L. interrogans serovar lai strain 56601. BMC Microbiol. 2006;6:51. Epub 20060609. doi: 10.1186/1471-2180-6-51. PubMed PMID: 16762078; PubMed Central PMCID: PMCPMC1534042.
29. Lo M, Bulach DM, Powell DR, Haake DA, Matsunaga J, Paustian ML, et al. Effects of temperature on gene expression patterns in Leptospira interrogans serovar Lai as assessed by whole-genome microarrays. Infection and immunity. 2006;74(10):5848-59. doi: 10.1128/IAI.00755-06. PubMed PMID: 16988264; PubMed Central PMCID: PMC1594916.
30. Cullen PA, Cordwell SJ, Bulach DM, Haake DA, Adler B. Global analysis of outer membrane proteins from Leptospira interrogans serovar Lai. Infection and immunity. 2002;70(5):2311-8. PubMed PMID: 11953365; PubMed Central PMCID: PMC127947.
31. Richard L Hornsby DPA, Jarlath E Nally. Isolation and propagation of leptospires at 37 °C directly from the mammalian host. Scientific Reports. 2020;10. doi: 10.1038/s41598-020-66526-4.
32. Hamond C, LeCount K, Browne AS, Anderson T, Stuber T, Hicks J, et al. Concurrent colonization of rodent kidneys with multiple species and serogroups of pathogenic Leptospira. Appl Environ Microbiol. 2023;89(10):e0120423. Epub 20231011. doi: 10.1128/aem.01204-23. PubMed PMID: 37819079; PubMed Central PMCID: PMCPMC10617434.
33. Haake DA. Hamster model of leptospirosis. Current protocols in microbiology. 2006;Chapter 12:Unit 12E 2. doi: 10.1002/9780471729259.mc12e02s02. PubMed PMID: 18770576; PubMed Central PMCID: PMC2667198.
34. Wunder EA, Jr., Figueira CP, Santos GR, Lourdault K, Matthias MA, Vinetz JM, et al. Real-Time PCR Reveals Rapid Dissemination of Leptospira interrogans after Intraperitoneal and Conjunctival Inoculation of Hamsters. Infect Immun. 2016;84(7):2105-15. Epub 20160623. doi: 10.1128/IAI.00094-16. PubMed PMID: 27141082; PubMed Central PMCID: PMCPMC4936353.
35. Wunder EA, Jr., Figueira CP, Santos GR, Lourdault K, Matthias MA, Vinetz JM, et al. Real-Time PCR Reveals Rapid Dissemination of Leptospira interrogans after Intraperitoneal and Conjunctival Inoculation of Hamsters. Infection and immunity. 2016;84(7):2105-15. doi: 10.1128/IAI.00094-16. PubMed PMID: 27141082; PubMed Central PMCID: PMCPMC4936353.
36. Schuler EJA, Patel DT, Marconi RT. The leptospiral OmpA-like protein (Loa22) is a surface-exposed antigen that elicits bactericidal antibody against heterologous Leptospira. Vaccine X. 2023;15:100382. Epub 20230901. doi: 10.1016/j.jvacx.2023.100382. PubMed PMID: 37727366; PubMed Central PMCID: PMCPMC10506094.
37. Lai WY, Wong Z, Chang CH, Samian MR, Watanabe N, Teh AH, et al. Identifying Leptospira interrogans putative virulence factors with a yeast protein expression screen. Appl

Microbiol Biotechnol. 2022;106(19-20):6567-81. Epub 20220916. doi: 10.1007/s00253-022-12160-1. PubMed PMID: 36112204.

38. Ashaiba A, Arun AB, Prasad KS, Tellis RC. Leptospiral sphingomyelinase Sph2 as a potential biomarker for diagnosis of leptospirosis. J Microbiol Methods. 2022;203:106621. Epub 20221112. doi: 10.1016/j.mimet.2022.106621. PubMed PMID: 36375539.

39. Wunder EA, Jr., Slamti L, Suwondo DN, Gibson KH, Shang Z, Sindelar CV, et al. FcpB Is a Surface Filament Protein of the Endoflagellum Required for the Motility of the Spirochete Leptospira. Front Cell Infect Microbiol. 2018;8:130. Epub 20180508. doi: 10.3389/fcimb.2018.00130. PubMed PMID: 29868490; PubMed Central PMCID: PMCPMC5953323.

40. Adhikarla H, Wunder EA, Jr., Mechaly AE, Mehta S, Wang Z, Santos L, et al. Lvr, a Signaling System That Controls Global Gene Regulation and Virulence in Pathogenic Leptospira. Frontiers in cellular and infection microbiology. 2018;8:45. Epub 2018/03/31. doi: 10.3389/fcimb.2018.00045. PubMed PMID: 29600195; PubMed Central PMCID: PMCPMC5863495.

41. Evangelista KV, Lourdault K, Matsunaga J, Haake DA. Immunoprotective properties of recombinant LigA and LigB in a hamster model of acute leptospirosis. PloS one. 2017;12(7):e0180004. doi: 10.1371/journal.pone.0180004. PubMed PMID: 28704385; PubMed Central PMCID: PMCPMC5509140.

42. Narayanavari SA, Lourdault K, Sritharan M, Haake DA, Matsunaga J. Role of sph2 Gene Regulation in Hemolytic and Sphingomyelinase Activities Produced by Leptospira interrogans. PLoS neglected tropical diseases. 2015;9(8):e0003952. Epub 20150814. doi: 10.1371/journal.pntd.0003952. PubMed PMID: 26274394; PubMed Central PMCID: PMCPMC4537108.

43. Eshghi A, Henderson J, Trent MS, Picardeau M. Leptospira interrogans lpxD Homologue Is Required for Thermal Acclimatization and Virulence. Infection and immunity. 2015;83(11):4314-21. Epub 20150817. doi: 10.1128/IAI.00897-15. PubMed PMID: 26283339; PubMed Central PMCID: PMCPMC4598399.

44. Zhang K, Murray GL, Seemann T, Srikram A, Bartpho T, Sermswan RW, et al. Leptospiral LruA is required for virulence and modulates an interaction with mammalian apolipoprotein AI. Infection and immunity. 2013;81(10):3872-9. doi: 10.1128/IAI.01195-12. PubMed PMID: 23918777; PubMed Central PMCID: PMC3811782.

45. Mendes RS, Von Atzingen M, de Morais ZM, Goncales AP, Serrano SM, Asega AF, et al. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. Infect Immun. 2011;79(11):4657-67. Epub 20110815. doi: 10.1128/IAI.05583-11. PubMed PMID: 21844229; PubMed Central PMCID: PMCPMC3257903.

46. Patarakul K, Lo M, Adler B. Global transcriptomic response of Leptospira interrogans serovar Copenhageni upon exposure to serum. BMC microbiology. 2010;10:31. doi: 10.1186/1471-2180-10-31. PubMed PMID: 20113507; PubMed Central PMCID: PMC2841595.

47. Murray GL, Srikram A, Henry R, Puapairoj A, Sermswan RW, Adler B. Leptospira interrogans requires heme oxygenase for disease pathogenesis. Microbes and infection / Institut Pasteur. 2009;11(2):311-4. doi: 10.1016/j.micinf.2008.11.014. PubMed PMID: 19114124.

48. Fouts DE, Matthias MA, Adhikarla H, Adler B, Amorim-Santos L, Berg DE, et al. What Makes a Bacterial Species Pathogenic?:Comparative Genomic Analysis of the Genus Leptospira.

816 PLoS neglected tropical diseases. 2016;10(2):e0004403. doi: 10.1371/journal.pntd.0004403.
817 PubMed PMID: 26890609; PubMed Central PMCID: PMCPMC4758666.
818 49. Eshghi A, Cullen PA, Cowen L, Zuerner RL, Cameron CE. Global proteome analysis of
819 Leptospira interrogans. Journal of proteome research. 2009;8(10):4564-78. doi:
820 10.1021/pr9004597. PubMed PMID: 19663501; PubMed Central PMCID: PMC2757032.
821 50. Grassmann AA, Zavala-Alvarado C, Bettin EB, Picardeau M, Benaroudj N, Caimano MJ.
822 The FUR-like regulators PerRA and PerRB integrate a complex regulatory network that
823 promotes mammalian host-adaptation and virulence of Leptospira interrogans. PLoS pathogens.
824 2021;17(12):e1009078. Epub 20211202. doi: 10.1371/journal.ppat.1009078. PubMed PMID:
825 34855918; PubMed Central PMCID: PMCPMC8638967.
826 51. de Assis Noman G, Lacerda de Moura BE, Vieira ML. Leptospiral LipL45 lipoprotein
827 undergoes processing and shares structural similarities with bacterial sigma regulators.
828 Biochemical and biophysical research communications. 2024;717:150057. Epub 20240504. doi:
829 10.1016/j.bbrc.2024.150057. PubMed PMID: 38718568.
830 52. Karp PD, Midford PE, Billington R, Kothari A, Krummenacker M, Latendresse M, et al.
831 Pathway Tools version 23.0 update: software for pathway/genome informatics and systems
832 biology. Brief Bioinform. 2021;22(1):109-26. doi: 10.1093/bib/bbz104. PubMed PMID:
833 31813964; PubMed Central PMCID: PMCPMC8453236.
834 53. Srikram A, Zhang K, Bartpho T, Lo M, Hoke DE, Sermswan RW, et al. Cross-protective
835 immunity against leptospirosis elicited by a live, attenuated lipopolysaccharide mutant. The
836 Journal of infectious diseases. 2011;203(6):870-9. doi: 10.1093/infdis/jiq127. PubMed PMID:
837 21220775; PubMed Central PMCID: PMC3071135.
838 54. Nally JE, Chow E, Fishbein MC, Blanco DR, Lovett MA. Changes in lipopolysaccharide
839 O antigen distinguish acute versus chronic Leptospira interrogans infections. Infection and
840 immunity. 2005;73(6):3251-60. doi: 10.1128/IAI.73.6.3251-3260.2005. PubMed PMID:
841 15908349; PubMed Central PMCID: PMC1111870.
842 55. Simpson BW, Trent MS. Pushing the envelope: LPS modifications and their
843 consequences. Nature reviews Microbiology. 2019;17(7):403-16. doi: 10.1038/s41579-019-
844 0201-x. PubMed PMID: 31142822; PubMed Central PMCID: PMCPMC6913091.
845 56. Raetz CR, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in gram-
846 negative bacteria. Annu Rev Biochem. 2007;76:295-329. doi:
847 10.1146/annurev.biochem.76.010307.145803. PubMed PMID: 17362200; PubMed Central
848 PMCID: PMCPMC2569861.
849 57. Sorensen M, Chandler CE, Gardner FM, Ramadan S, Khot PD, Leung LM, et al. Rapid
850 microbial identification and colistin resistance detection via MALDI-TOF MS using a novel on-
851 target extraction of membrane lipids. Sci Rep. 2020;10(1):21536. Epub 20201209. doi:
852 10.1038/s41598-020-78401-3. PubMed PMID: 33299017; PubMed Central PMCID:
853 PMCPMC7725828.
854 58. Yang H, Smith RD, Chandler CE, Johnson JK, Jackson SN, Woods AS, et al. Lipid A
855 Structural Determination from a Single Colony. Anal Chem. 2022;94(21):7460-5. Epub
856 20220516. doi: 10.1021/acs.analchem.1c05394. PubMed PMID: 35576511; PubMed Central
857 PMCID: PMCPMC9392460.
858 59. Novak A, Pupo E, Van't Veld E, Rutten V, Broere F, Sloots A. Activation of Canine,
859 Mouse and Human TLR2 and TLR4 by Inactivated Leptospira Vaccine Strains. Front Immunol.
860 2022;13:823058. Epub 20220321. doi: 10.3389/fimmu.2022.823058. PubMed PMID: 35386703;
861 PubMed Central PMCID: PMCPMC8978998.

60. Petrosova H, Mikhael A, Culos S, Giraud-Gatineau A, Gomez AM, Sherman ME, et al. Lipid A structural diversity among members of the genus Leptospira. Front Microbiol. 2023;14:1181034. Epub 20230525. doi: 10.3389/fmicb.2023.1181034. PubMed PMID: 37303810; PubMed Central PMCID: PMCPMC10248169.
61. Que-Gewirth NL, Ribeiro AA, Kalb SR, Cotter RJ, Bulach DM, Adler B, et al. A methylated phosphate group and four amide-linked acyl chains in leptospira interrogans lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. J Biol Chem. 2004;279(24):25420-9. Epub 20040324. doi: 10.1074/jbc.M400598200. PubMed PMID: 15044492; PubMed Central PMCID: PMCPMC2556802.
62. El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res. 2005;46(8):1773-8. Epub 20050601. doi: 10.1194/jlr.D500014-JLR200. PubMed PMID: 15930524.
63. Scott AJ, Flinders B, Cappell J, Liang T, Pelc RS, Tran B, et al. Norharmane Matrix Enhances Detection of Endotoxin by MALDI-MS for Simultaneous Profiling of Pathogen, Host, and Vector Systems. Pathog Dis. 2016;74(8). Epub 20160919. doi: 10.1093/femspd/ftw097. PubMed PMID: 27650574; PubMed Central PMCID: PMCPMC8427938.
64. Caimano MJ, Sivasankaran SK, Allard A, Hurley D, Hokamp K, Grassmann AA, et al. A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by Leptospira interrogans serovar Copenhageni. PLoS pathogens. 2014;10(3):e1004004. doi: 10.1371/journal.ppat.1004004. PubMed PMID: 24626166; PubMed Central PMCID: PMC3953431.
65. Matsui M, Soupe ME, Becam J, Goarant C. Differential in vivo gene expression of major Leptospira proteins in resistant or susceptible animal models. Appl Environ Microbiol. 2012;78(17):6372-6. Epub 20120622. doi: 10.1128/AEM.00911-12. PubMed PMID: 22729538; PubMed Central PMCID: PMCPMC3416592.
66. Matsunaga J, Lo M, Bulach DM, Zuerner RL, Adler B, Haake DA. Response of Leptospira interrogans to physiologic osmolarity: relevance in signaling the environment-to-host transition. Infect Immun. 2007;75(6):2864-74. Epub 20070319. doi: 10.1128/IAI.01619-06. PubMed PMID: 17371863; PubMed Central PMCID: PMCPMC1932867.
67. Matsunaga J, Medeiros MA, Sanchez Y, Werneid KF, Ko AI. Osmotic regulation of expression of two extracellular matrix-binding proteins and a haemolysin of Leptospira interrogans: differential effects on LigA and Sph2 extracellular release. Microbiology (Reading). 2007;153(Pt 10):3390-8. doi: 10.1099/mic.0.2007/007948-0. PubMed PMID: 17906138.
68. Putz EJ, Fernandes LGV, Sivasankaran SK, Bayles DO, Alt DP, Lippolis JD, et al. Some like it hot, some like it cold; proteome comparison of Leptospira borgpetersenii serovar Hardjo strains propagated at different temperatures. J Proteomics. 2022;262:104602. Epub 20220506. doi: 10.1016/j.jprot.2022.104602. PubMed PMID: 35526804.
69. Putz EJ, Sivasankaran SK, Fernandes LGV, Brunelle B, Lippolis JD, Alt DP, et al. Distinct transcriptional profiles of Leptospira borgpetersenii serovar Hardjo strains JB197 and HB203 cultured at different temperatures. PLoS Negl Trop Dis. 2021;15(4):e0009320. Epub 20210407. doi: 10.1371/journal.pntd.0009320. PubMed PMID: 33826628; PubMed Central PMCID: PMCPMC8055020.
70. Xue F, Dong H, Wu J, Wu Z, Hu W, Sun A, et al. Transcriptional responses of Leptospira interrogans to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane. PLoS Negl Trop Dis. 2010;4(10):e857. Epub 20101026. doi:

908 10.1371/journal.pntd.0000857. PubMed PMID: 21049008; PubMed Central PMCID:
909 PMCPMC2964297.
910 71. Coburn J, Picardeau M, Woods CW, Veldman T, Haake DA. Pathogenesis insights from
911 an ancient and ubiquitous spirochete. PLoS pathogens. 2021;17(10):e1009836. Epub 20211021.
912 doi: 10.1371/journal.ppat.1009836. PubMed PMID: 34673833; PubMed Central PMCID:
913 PMCPMC8530280.
914 72. Fraser T, Brown PD. Temperature and Oxidative Stress as Triggers for Virulence Gene
915 Expression in Pathogenic Leptospira spp. Front Microbiol. 2017;8:783. doi:
916 10.3389/fmicb.2017.00783. PubMed PMID: 28536558; PubMed Central PMCID:
917 PMCPMC5423269.
918 73. Zuerner RL. Laboratory maintenance of pathogenic Leptospira. Current protocols in
919 microbiology. 2005;Chapter 12:Unit 12E 1. doi: 10.1002/9780471729259.mc12e01s00. PubMed
920 PMID: 18770554.
921 74. Murray GL, Srikram A, Hoke DE, Wunder EA, Jr., Henry R, Lo M, et al. Major surface
922 protein LipL32 is not required for either acute or chronic infection with Leptospira interrogans.
923 Infection and immunity. 2009;77(3):952-8. doi: 10.1128/IAI.01370-08. PubMed PMID:
924 19103763; PubMed Central PMCID: PMCPMC2643616.
925 75. Kurilung A, Keeratipusana C, Horiike T, Suriyaphol P, Hampson DJ, Prapasarakul N.
926 Chronology of emergence of the genus Leptospira and over-representation of gene families
927 enriched by vitamin B2, B12 biosynthesis, cell adhesion and external encapsulating structure in
928 L. interrogans isolates from asymptomatic dogs. Infect Genet Evol. 2019;73:7-12. Epub
929 20190408. doi: 10.1016/j.meegid.2019.04.005. PubMed PMID: 30974265.
930 76. Wang H, Wu Y, Ojcius DM, Yang XF, Zhang C, Ding S, et al. Leptospiral hemolysins
931 induce proinflammatory cytokines through Toll-like receptor 2-and 4-mediated JNK and NF-
932 kappaB signaling pathways. PLoS One. 2012;7(8):e42266. Epub 20120801. doi:
933 10.1371/journal.pone.0042266. PubMed PMID: 22870312; PubMed Central PMCID:
934 PMCPMC3411626.
935 77. Kassegne K, Hu W, Ojcius DM, Sun D, Ge Y, Zhao J, et al. Identification of collagenase
936 as a critical virulence factor for invasiveness and transmission of pathogenic Leptospira species.
937 J Infect Dis. 2014;209(7):1105-15. Epub 20131125. doi: 10.1093/infdis/jit659. PubMed PMID:
938 24277745.
939 78. Matsushita O, Koide T, Kobayashi R, Nagata K, Okabe A. Substrate recognition by the
940 collagen-binding domain of Clostridium histolyticum class I collagenase. J Biol Chem.
941 2001;276(12):8761-70. Epub 20001219. doi: 10.1074/jbc.M003450200. PubMed PMID:
942 11121400.
943 79. Haake DA, Martinich C, Summers TA, Shang ES, Pruetz JD, McCoy AM, et al.
944 Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated
945 with late-log-phase growth and mammalian infection. Infection and immunity. 1998;66(4):1579-
946 87. PubMed PMID: 9529084; PubMed Central PMCID: PMC108091.
947 80. Voelkl B, Altman NS, Forsman A, Forstmeier W, Gurevitch J, Jaric I, et al.
948 Reproducibility of animal research in light of biological variation. Nat Rev Neurosci.
949 2020;21(7):384-93. Epub 20200602. doi: 10.1038/s41583-020-0313-3. PubMed PMID:
950 32488205.
951 81. Mukerjee M. Trends in animal research. Sci Am. 1997;276(2):86-93. doi:
952 10.1038/scientificamerican0297-86. PubMed PMID: 9000767.

82. Leung LM, McElheny CL, Gardner FM, Chandler CE, Bowler SL, Mettus RT, et al. A Prospective Study of Acinetobacter baumannii Complex Isolates and Colistin Susceptibility Monitoring by Mass Spectrometry of Microbial Membrane Glycolipids. Journal of clinical microbiology. 2019;57(3). Epub 20190227. doi: 10.1128/JCM.01100-18. PubMed PMID: 30567747; PubMed Central PMCID: PMCPMC6425172.
83. Gunn JS, Ernst RK. The structure and function of Francisella lipopolysaccharide. Annals of the New York Academy of Sciences. 2007;1105:202-18. Epub 20070329. doi: 10.1196/annals.1409.006. PubMed PMID: 17395723; PubMed Central PMCID: PMCPMC2742961.
84. Stern N, Shenberg E, Tietz A. Studies on the metabolism of fatty acids in Leptospira: the biosynthesis of delta 9- and delta 11-monounsaturated acids. Eur J Biochem. 1969;8(1):101-8. doi: 10.1111/j.1432-1033.1969.tb00501.x. PubMed PMID: 5781264.
85. Diascro DD, Jr., Vogel RL, Johnson TE, Witherup KM, Pitzenberger SM, Rutledge SJ, et al. High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells. J Bone Miner Res. 1998;13(1):96-106. doi: 10.1359/jbmr.1998.13.1.96. PubMed PMID: 9443795.
86. Yang H, Chandler CE, Jackson SN, Woods AS, Goodlett DR, Ernst RK, et al. On-Tissue Derivatization of Lipopolysaccharide for Detection of Lipid A Using MALDI-MSI. Anal Chem. 2020;92(20):13667-71. Epub 20200928. doi: 10.1021/acs.analchem.0c02566. PubMed PMID: 32902263; PubMed Central PMCID: PMCPMC8717242.
87. Wang X, Ribeiro AA, Guan Z, McGrath SC, Cotter RJ, Raetz CR. Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in Francisella tularensis subsp. novicida. Biochemistry. 2006;45(48):14427-40. doi: 10.1021/bi061767s. PubMed PMID: 17128982; PubMed Central PMCID: PMCPMC2569856.
88. Bonhomme D, Werts C. Purification of LPS from Leptospira. Methods in molecular biology. 2020;2134:53-65. doi: 10.1007/978-1-0716-0459-5_6. PubMed PMID: 32632859.
89. Ohto U, Fukase K, Miyake K, Shimizu T. Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(19):7421-6. Epub 20120424. doi: 10.1073/pnas.1201193109. PubMed PMID: 22532668; PubMed Central PMCID: PMCPMC3358893.
90. Ernst O, Khan MM, Oyler BL, Yoon SH, Sun J, Lin FY, et al. Species-Specific Endotoxin Stimulus Determines Toll-Like Receptor 4- and Caspase 11-Mediated Pathway Activation Characteristics. mSystems. 2021;6(4):e0030621. Epub 20210803. doi: 10.1128/mSystems.00306-21. PubMed PMID: 34342534; PubMed Central PMCID: PMCPMC8407122.
91. Gauthier AE, Chandler CE, Poli V, Gardner FM, Tekiau A, Smith R, et al. Deep-sea microbes as tools to refine the rules of innate immune pattern recognition. Sci Immunol. 2021;6(57). doi: 10.1126/sciimmunol.abe0531. PubMed PMID: 33712473; PubMed Central PMCID: PMCPMC8367048.
92. Facchini FA, Zaffaroni L, Minotti A, Rapisarda S, Calabrese V, Forcella M, et al. Structure-Activity Relationship in Monosaccharide-Based Toll-Like Receptor 4 (TLR4) Antagonists. J Med Chem. 2018;61(7):2895-909. Epub 20180312. doi: 10.1021/acs.jmedchem.7b01803. PubMed PMID: 29494148.
93. Bonhomme D, Werts C. Host and Species-Specificities of Pattern Recognition Receptors Upon Infection With Leptospira interrogans. Frontiers in cellular and infection microbiology.

2022;12:932137. Epub 20220722. doi: 10.3389/fcimb.2022.932137. PubMed PMID: 35937697; PubMed Central PMCID: PMCPMC9353586.

94. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature. 2009;458(7242):1191-5. Epub 20090301. doi: 10.1038/nature07830. PubMed PMID: 19252480.

95. Scott AJ, Oyler BL, Goodlett DR, Ernst RK. Lipid A structural modifications in extreme conditions and identification of unique modifying enzymes to define the Toll-like receptor 4 structure-activity relationship. Biochim Biophys Acta Mol Cell Biol Lipids. 2017;1862(11):1439-50. Epub 20170117. doi: 10.1016/j.bbalip.2017.01.004. PubMed PMID: 28108356; PubMed Central PMCID: PMCPMC5513793.

96. Korba AA, Lounici H, Kainiu M, Vincent AT, Mariet JF, Veyrier FJ, et al. Leptospira ainlahdjerensis sp. nov., Leptospira ainazelensis sp. nov., Leptospira abararensis sp. nov. and Leptospira chreensis sp. nov., four new species isolated from water sources in Algeria. International journal of systematic and evolutionary microbiology. 2021;71(12). Epub 2021/12/17. doi: 10.1099/ijsem.0.005148. PubMed PMID: 34914572.

97. Vincent AT, Schiettekatte O, Goarant C, Neela VK, Bernet E, Thibeaux R, et al. Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics. PLoS neglected tropical diseases. 2019;13(5):e0007270. Epub 2019/05/24. doi: 10.1371/journal.pntd.0007270. PubMed PMID: 31120895; PubMed Central PMCID: PMCPMC6532842.

98. Beaute J, Innocenti F, Aristodimou A, Spackova M, Eves C, Kerbo N, et al. Epidemiology of reported cases of leptospirosis in the EU/EEA, 2010 to 2021. Euro Surveill. 2024;29(7). doi: 10.2807/1560-7917.ES.2024.29.7.2300266. PubMed PMID: 38362624; PubMed Central PMCID: PMCPMC10986659.

99. Fernandes LGV, Guaman LP, Vasconcellos SA, Heinemann MB, Picardeau M, Nascimento A. Gene silencing based on RNA-guided catalytically inactive Cas9 (dCas9): a new tool for genetic engineering in Leptospira. Sci Rep. 2019;9(1):1839. Epub 20190212. doi: 10.1038/s41598-018-37949-x. PubMed PMID: 30755626; PubMed Central PMCID: PMCPMC6372684.

100. Randall R, Cooper HK. The Golden Hamster (Cricetus Auratus) as a Test Animal for the Diagnosis of Leptospirosis. Science. 1944;100(2589):133-4. doi: 10.1126/science.100.2589.133. PubMed PMID: 17795954.

101. Van den Ingh TS, Hartman EG. Pathology of acute Leptospira interrogans serotype icterohaemorrhagiae infection in the Syrian hamster. Vet Microbiol. 1986;12(4):367-76. doi: 10.1016/0378-1135(86)90086-6. PubMed PMID: 3538643.

102. Adomas AB, Lopez-Giraldez F, Clark TA, Wang Z, Townsend JP. Multi-targeted priming for genome-wide gene expression assays. BMC Genomics. 2010;11:477. Epub 20100817. doi: 10.1186/1471-2164-11-477. PubMed PMID: 20716356; PubMed Central PMCID: PMCPMC3091673.

103. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc. 2016;11(9):1650-67. Epub 20160811. doi: 10.1038/nprot.2016.095. PubMed PMID: 27560171; PubMed Central PMCID: PMCPMC5032908.

104. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550. doi: 10.1186/s13059-014-0550-8. PubMed PMID: 25516281; PubMed Central PMCID: PMCPMC4302049.

105. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8. doi: 10.1038/nprot.2008.73. PubMed PMID: 18546601.
106. Hernandez-Plaza A, Szklarczyk D, Botas J, Cantalapiedra CP, Giner-Lamia J, Mende DR, et al. eggNOG 6.0: enabling comparative genomics across 12 535 organisms. Nucleic Acids Res. 2023;51(D1):D389-D94. doi: 10.1093/nar/gkac1022. PubMed PMID: 36399505; PubMed Central PMCID: PMCPMC9825578.
107. Koizumi N, Watanabe H. Identification of a novel antigen of pathogenic Leptospira spp. that reacted with convalescent mice sera. Journal of medical microbiology. 2003;52(Pt 7):585-9. PubMed PMID: 12808081.
108. Strohalm M, Kavan D, Novak P, Volny M, Havlicek V. mMass 3: a cross-platform software environment for precise analysis of mass spectrometric data. Anal Chem. 2010;82(11):4648-51. doi: 10.1021/ac100818g. PubMed PMID: 20465224.
109. Scott AJ, Ellis SR, Hofstaedter CE, Heeren RMA, Ernst RK. Spatial lipidomics reveals biased phospholipid remodeling in acute Pseudomonas lung infection. iScience. 2023;26(9):107700. Epub 20230821. doi: 10.1016/j.isci.2023.107700. PubMed PMID: 37680478; PubMed Central PMCID: PMCPMC10480615.
110. Palmer A, Phapale P, Chernyavsky I, Lavigne R, Fay D, Tarasov A, et al. FDR-controlled metabolite annotation for high-resolution imaging mass spectrometry. Nat Methods. 2017;14(1):57-60. Epub 20161114. doi: 10.1038/nmeth.4072. PubMed PMID: 27842059.

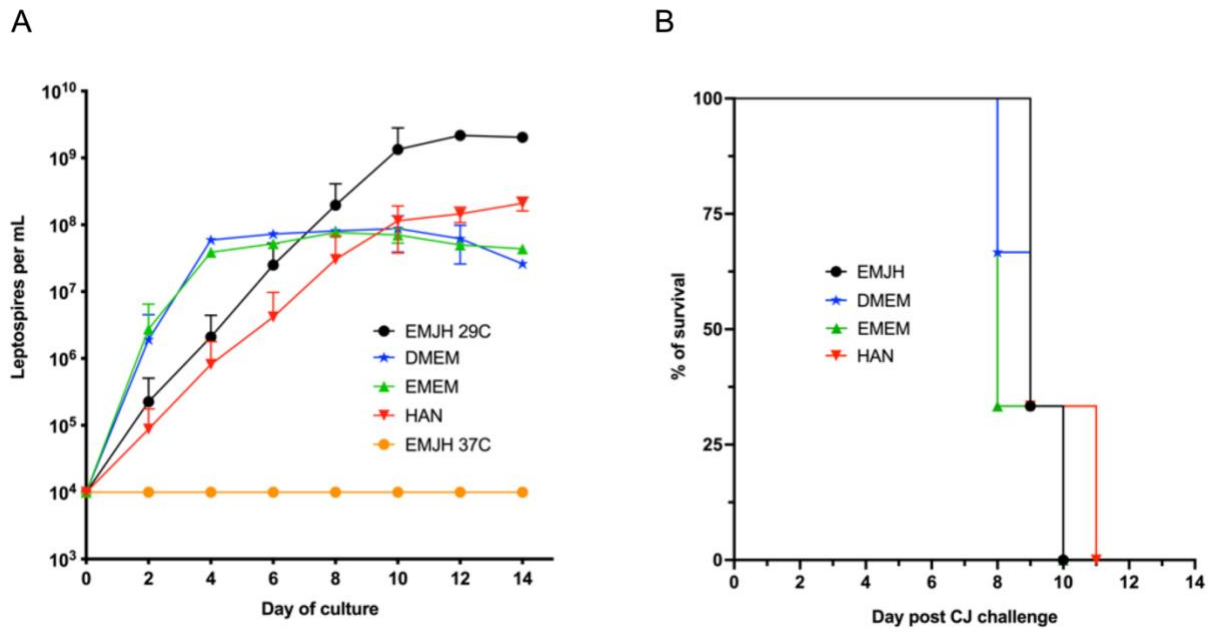
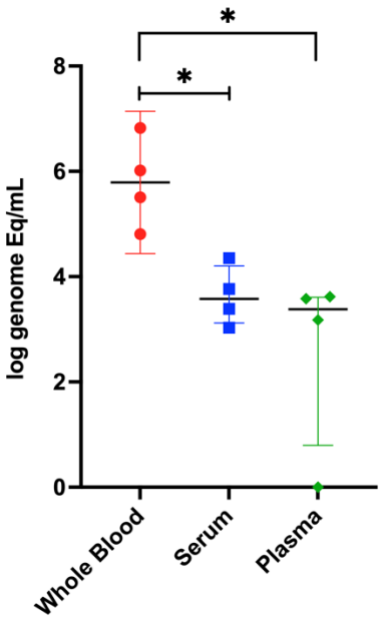


Fig 1. Growth and virulence analysis of pathogenic *Leptospira* cultured on different media and conditions. A. Growth curve of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 in five different growing conditions (medium, temperature, and CO_2 level). Dots and error bars represent the average from two independent experiments and standard deviation, respectively. **B.** Survival curve of virulence experiments. *Leptospira* strain Fiocruz L1-130 was cultured in DMEM, EMEM, or HAN at 37 ^{0}C and 5% CO_2, or EMJH at 30 ºC. Groups of hamsters (n = 3) were challenged with 10^8 leptospires by conjunctival-route (CJ) to demonstrate the ability of pathogenic *Leptospira* to maintain its virulence after culture on different media and conditions. Results presented are representative of one of two independent experiments with similar results.



S1 Fig. Results of qPCR for *Leptospira* using DNA extracted from whole blood, serum, and plasma of hamsters after 7 days post-conjunctival infection with 10^8 leptospires (*L. interrogans* serovar Copenhageni strain Fiocruz L1-130). Numbers are expressed in logarithmic genome equivalent (GEq) with average and standard deviation. *: $P < 0.05$.

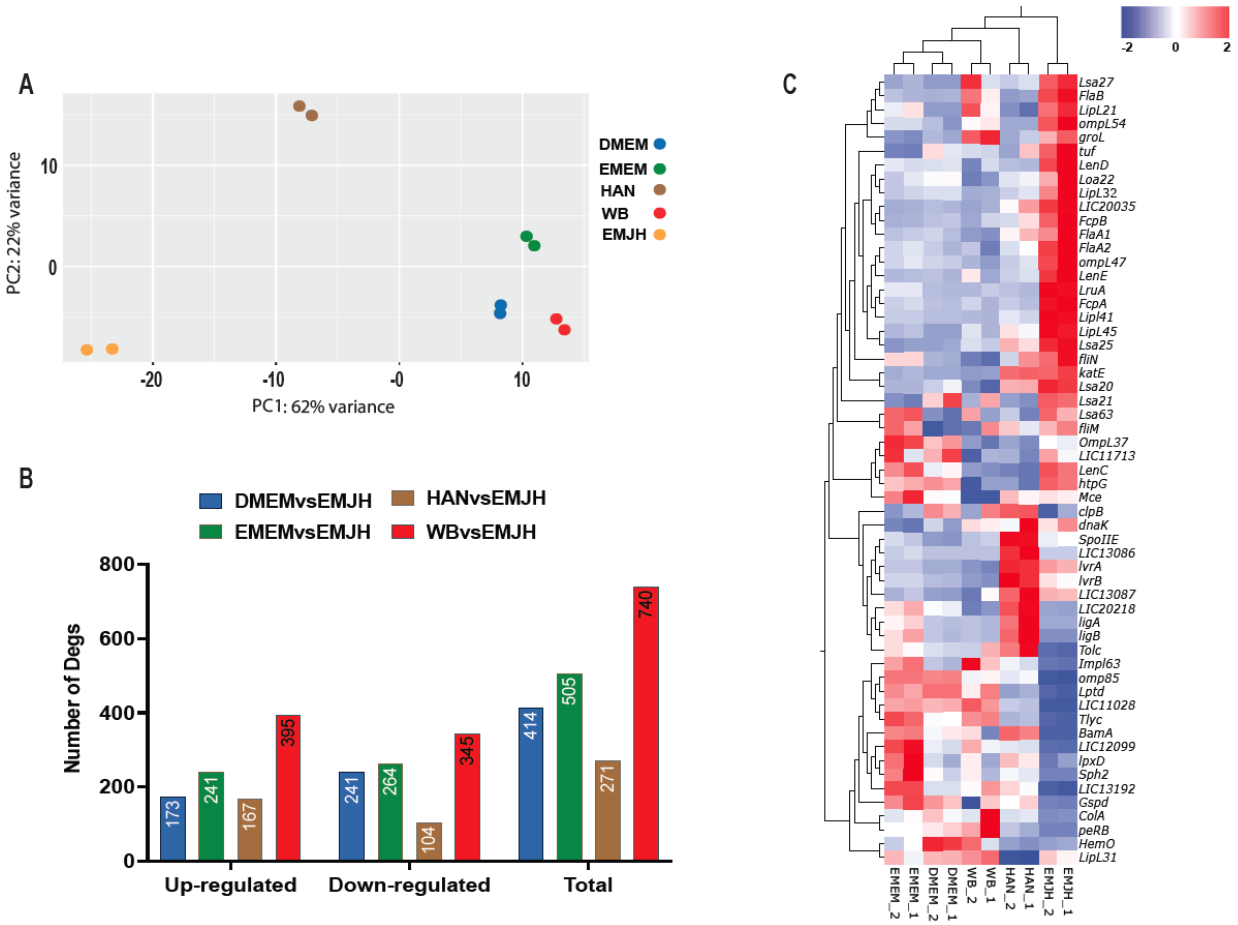
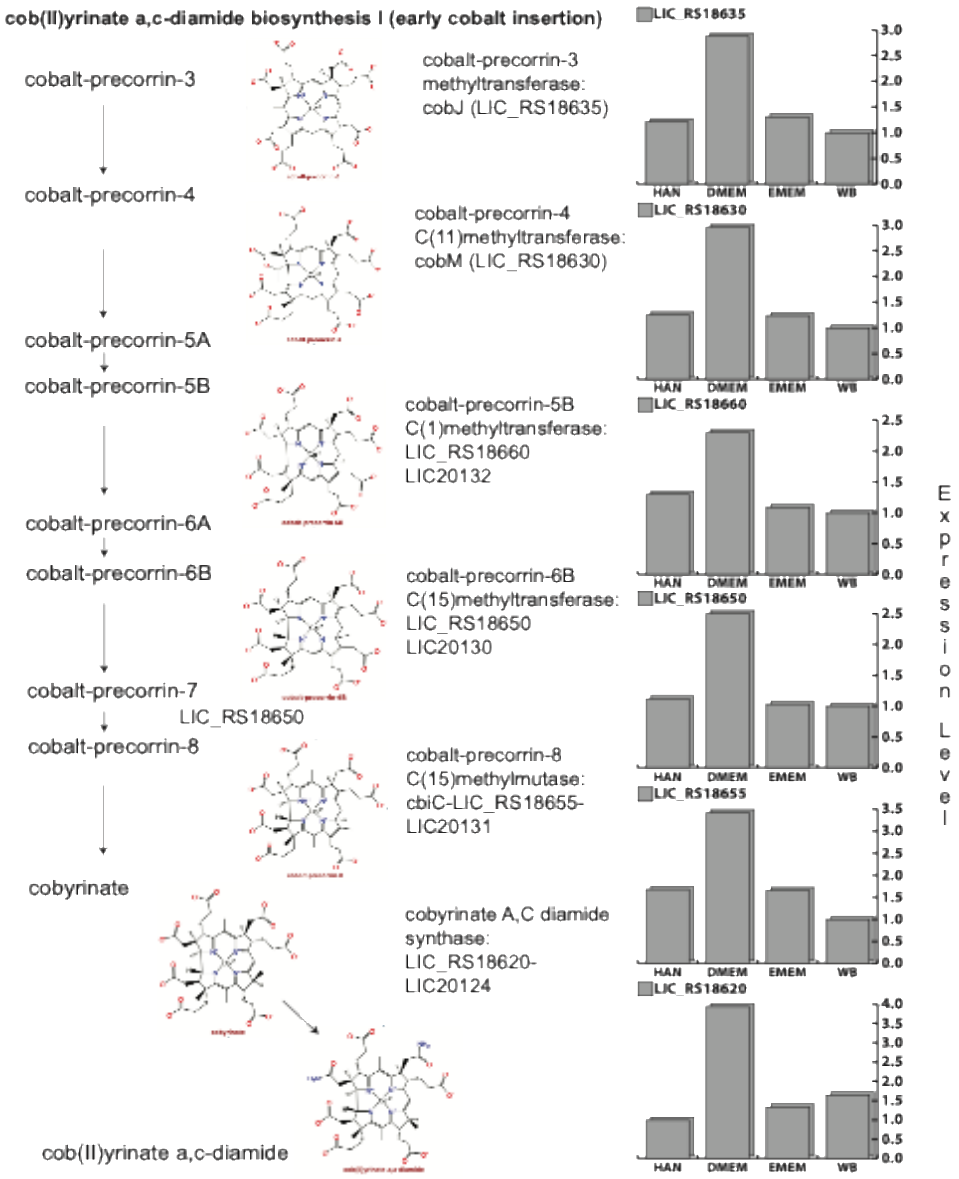
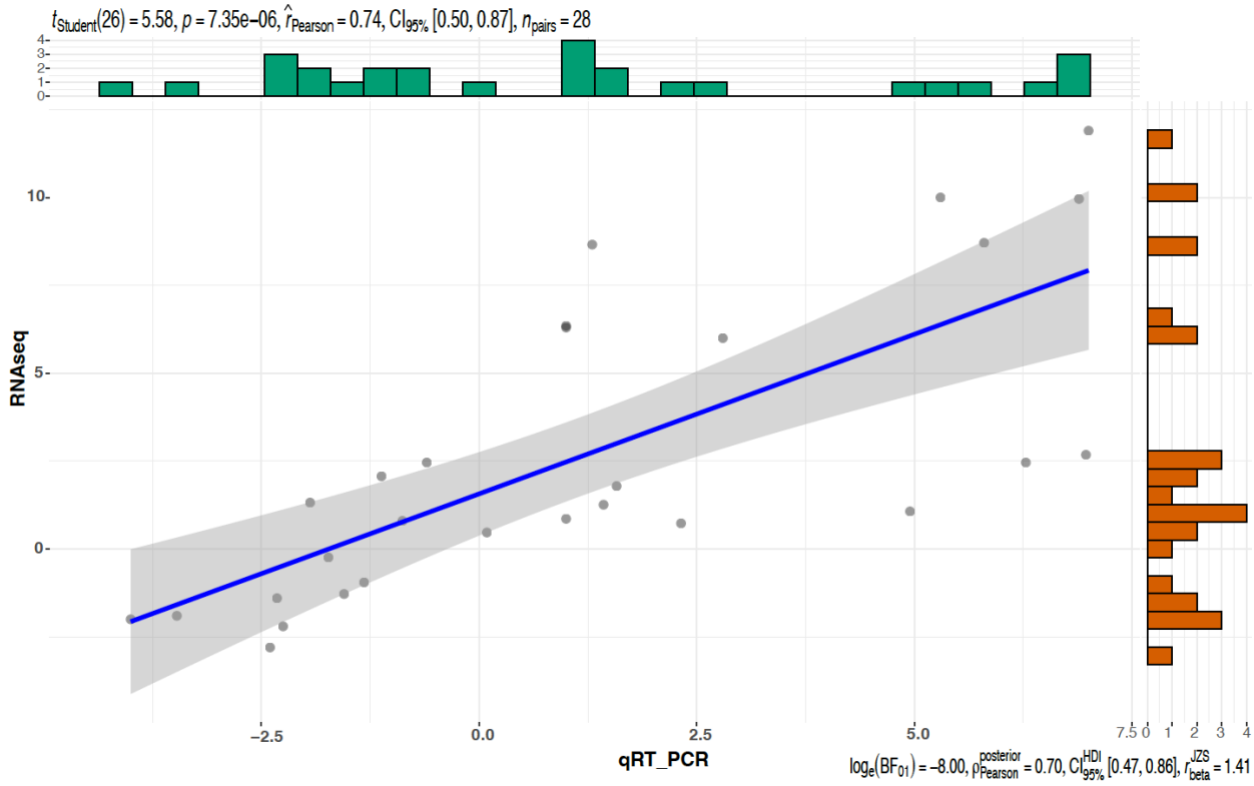


Fig 2. Whole transcriptome and hierarchical cluster between different treatments. A. First two Principal Component Analysis (PCA) between replicates of all the treatments evaluated: EMJH, EMEM, DMEM, HAN, and Whole-blood (WB). **B.** Total number of differentially expressed genes (DEGs) between all treatments versus EMJH. **C.** Hierarchical cluster of different treatments based on the expression of selected genes associated with the pathogenic mechanism of leptospirosis infection. (*P* value < 0.01, and log2FC ± 2;n = 2).



S2 Fig. Relative expression of all the genes involved in Cobalamin production in different *in vitro* growth conditions and in whole blood (WB). Bars represent expression levels of each gene on leptospires cultured in different media.



S3 Fig. Pearson correlation coefficient results for validation of RNA-Seq analysis using RT-qPCR. Dots represent comparison results of four genes tested on all four media conditions analyzed: EMJH, HAN, EMEM, and EMEM.

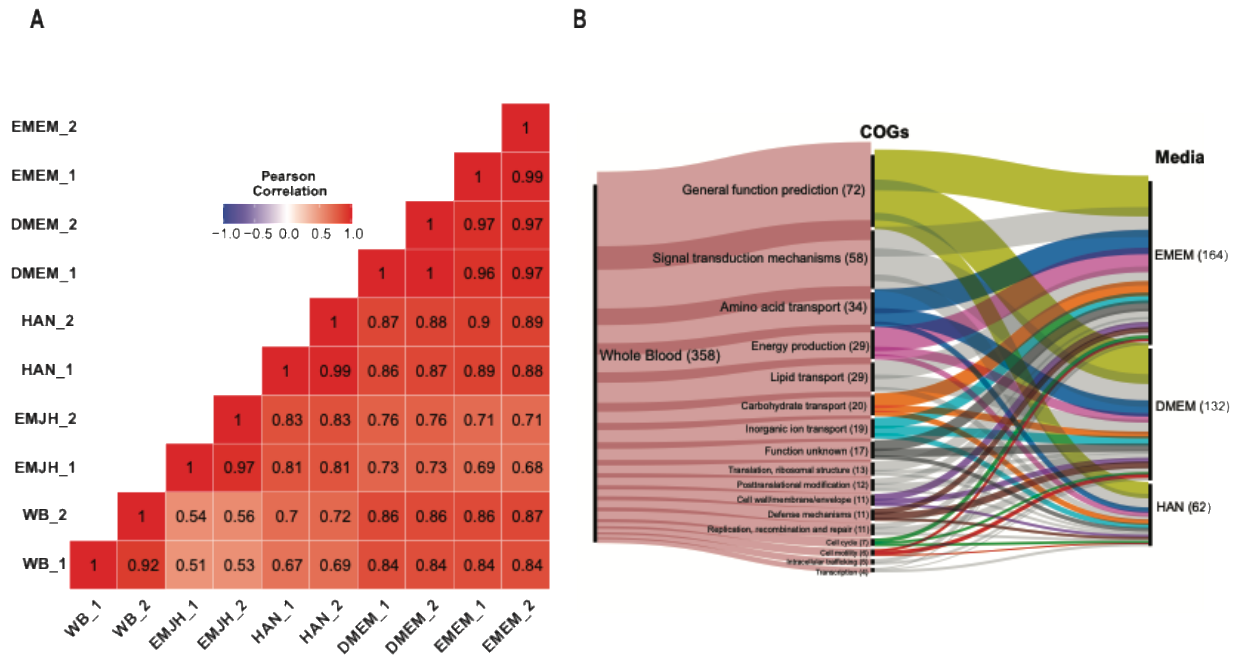


Fig 3. Correlation and cluster of orthologous genes analysis. A. Pearson correlation of normalized log raw counts of all differentially expressed genes (DEGs) between different growth conditions (*P* value <0.01). **B.** Sankey plot of orthologous gene clusters (COG) identified in *Leptospira* isolated from whole blood (WB) samples and shared with *Leptospira* grown in different *in vitro* media.

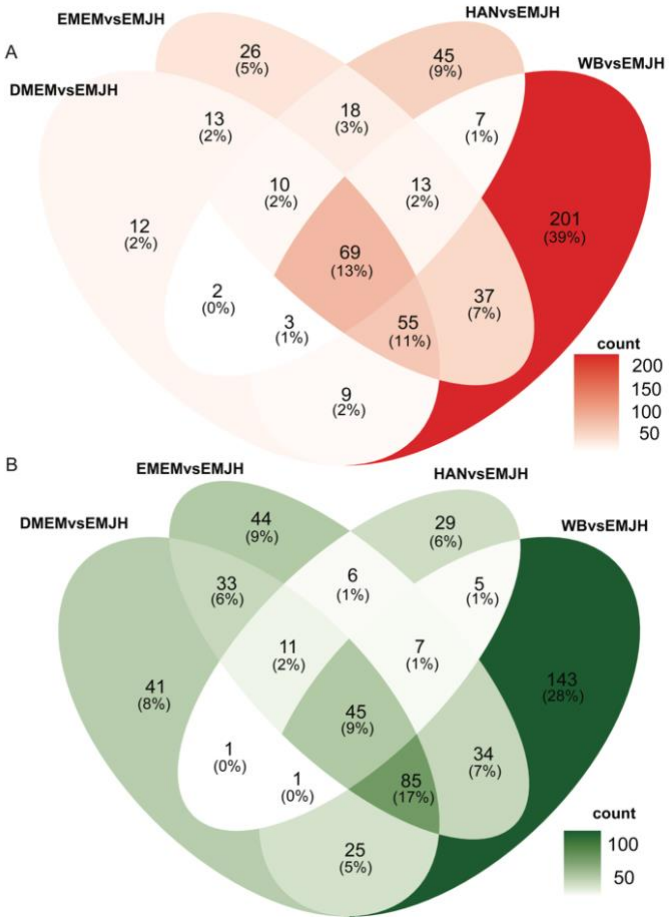


Fig 4. Venn diagram of differentially expressed genes (DEGs) between all treatments versus EMJH. **A.** Venn diagram of up-regulated DEGs. **B.** Venn diagram of down-regulated DEGs. *P* value < 0.01, and log2FC ± 2 (n = 2).

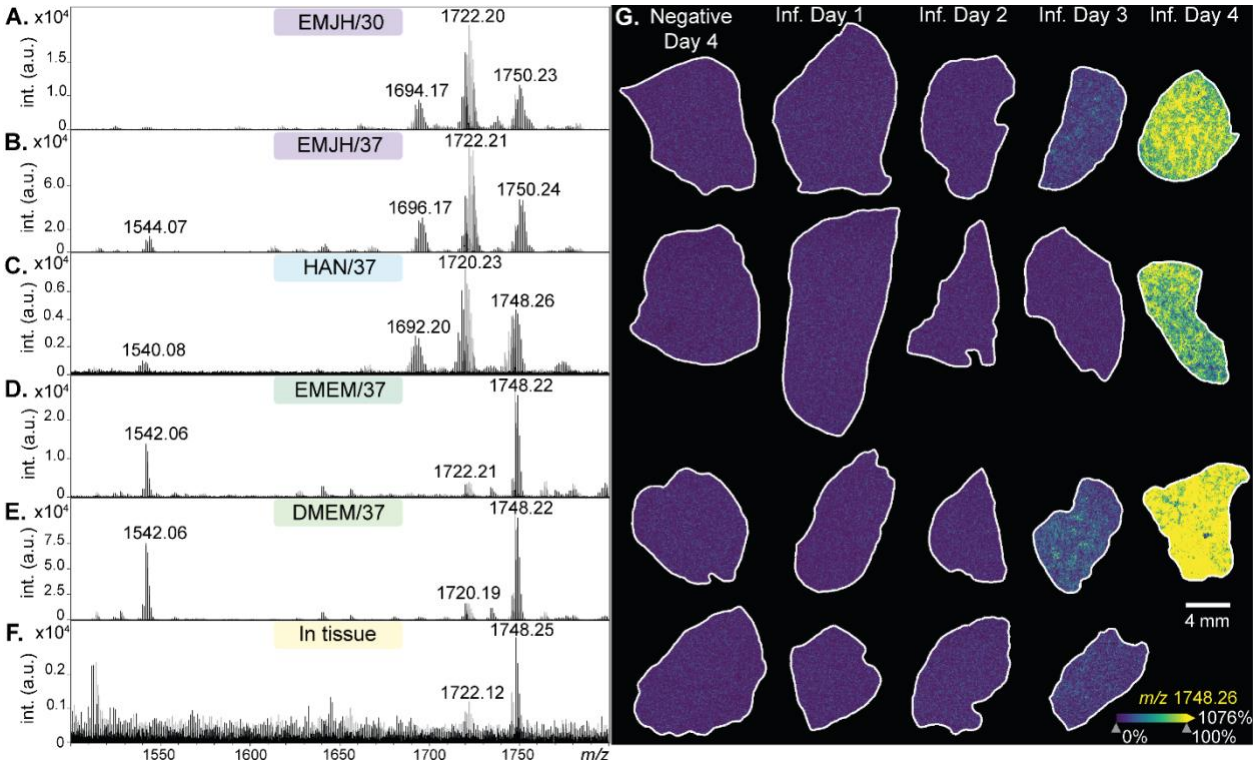
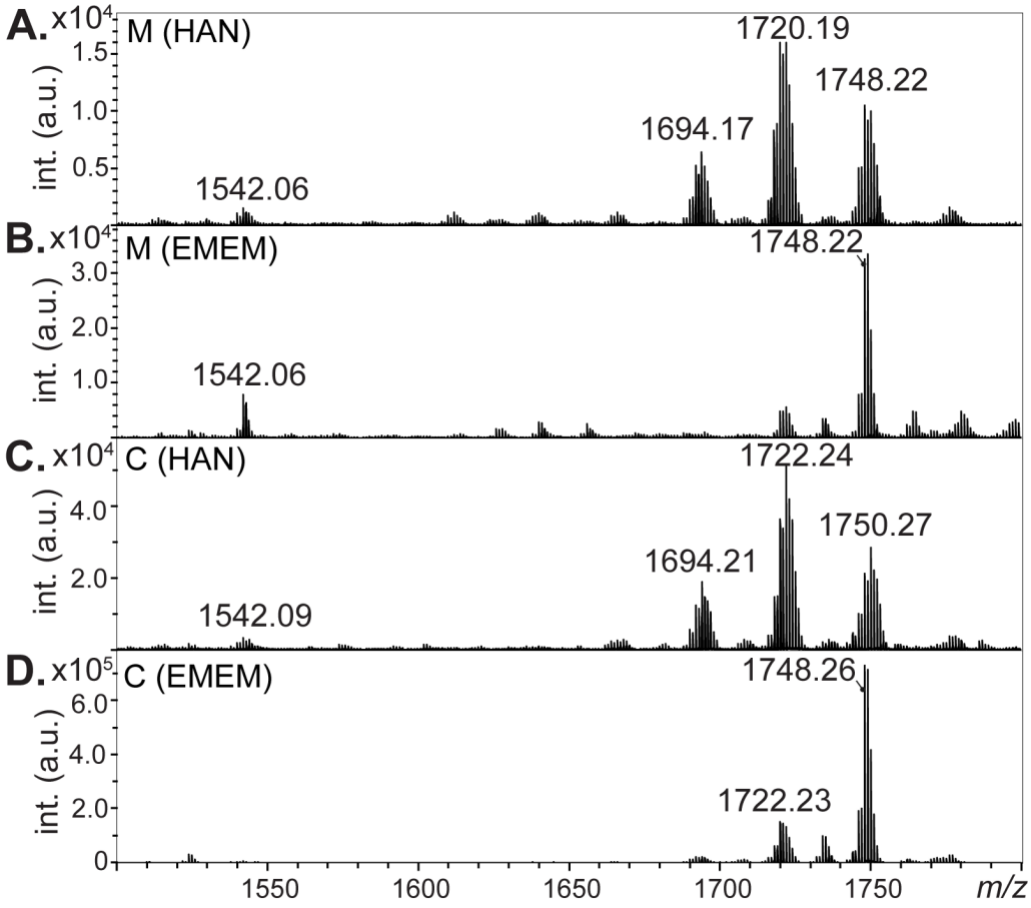
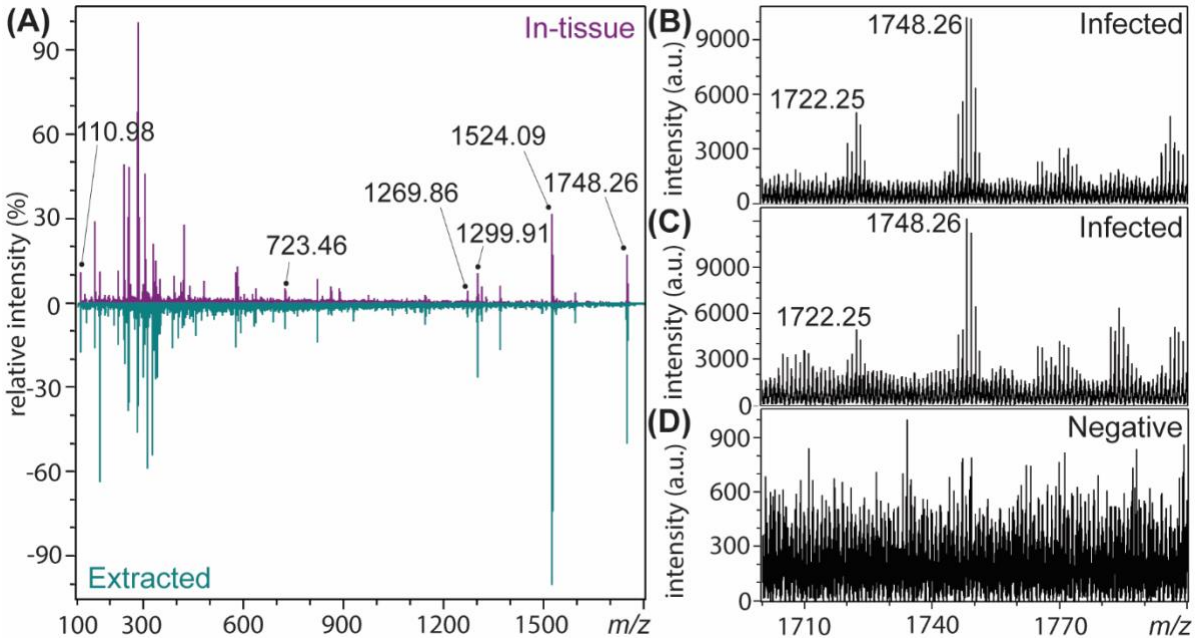


Fig 5. **Lipid A profiles of *L. interrogans* serovar Manilae strain L495 in different growth conditions. A**. EMJH at 30 °C, regular incubator without a CO_2 level control. **B**. EMJH at 37 °C and 5% CO_2. **C** HAN at 37 °C and 5% CO_2. **D**. EMEM at 37 °C and 5% CO_2. **E** DMEM at 37 °C and 5% CO_2. **(A–E)** Lipid A profiles determined by FLAT. **F.** Liver of an infected hamster. **G.** Ion images of a lipid A ion (*m/z* 1748.26 ± 10 ppm),as acquired in hamster liver tissue. Hamsters were infected with 10^8 *Leptospira*, and sacrificed at days 1, 2, 3 and 4 (n = 4 per group; one hamster did not survive the challenge). Negative controls were infected with EMJH medium only and sacrificed at day four post-injection (n = 4). Data normalized to the total ion count, 100% on the color scale corresponds to 99% quantile. **(F–G)** Data collected by mass spectrometry imaging at 50 µm spatial resolution. All data acquired in the negative ion mode on a timsTOF flex MALDI-2 instrument (Bruker).



S4 Fig. Comparison of Lipid A profiles of *L. interrogans* serovar Manilae strain L495 and serovar Copenhageni strain Fiocruz L1-130 in different growth conditions. Strains L495 (M) and Fiocruz L1-130 (C) were cultured in HAN and EMEM and lipid A profile was analyzed by mass spectrometry.



S5 Fig. Mass spectrometry analysis of *Leptospira* lipid A from infected hamster liver. **A.** Tandem mass spectrometry analysis of the *m/z* 1748.26 lipid A ion. Top panel (purple): lipid A in tissue by mass spectrometry imaging. Bottom panel (green): lipid A extracted from liver homogenates. **B–C.** Lipid A signal in liver extracts from hamster four days post-challenged with *L. interrogans* serovar Manilae strain L495. Tissue used for extraction correspond to Figure 4G in the main manuscript (Day 4, tissue section on the bottom and top of the panel; lipid A extraction from the tissue depicted in the middle was not successful). **D.** Representative spectrum of liver extracts from uninfected controls; all negative for presence of the lipid A signal (n = 4). Tissues used for extraction correspond to Figure 5G in the main manuscript (Negative, Day 4).