nature portfolio

Peer Review File

In Vivo Dual RNA-Seq uncovers key effectors of epithelial barrier disruption by an extracellular pathogen

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study Giraud-Gatineau et al. provide the host-and-pathogen transcriptional responses of the most common pathogenic Leptospira species in vivo in an animal model of severe leptospirosis. They used dual RNA-sequencing from tissues of infected hamsters to compare transcriptomes of infected vs uninfected tissues and did deep-RNA-sequencing from tissues to enrich bacterial reads that enable comparison of Leptospira transcriptional profiles in vivo and in vitro. The deep-dual-RNAseq method is an example on how to overcome RNA-seq technical difficulties with strict extracellular pathogens where selection of infected cells cannot be used. They followed up with a suite of cell-bio, molecular biology and live-cell imaging techniques to describe how two L. interrogans virulence-modifying proteins promote calcium homeostasis and disruption of cell-cell junctions. The study approach is well-designed, comprehensive, technically rigorous and highly impactful for the Leptospirosis field as well as other pathogens that target paracellular permeability (e.g. H. pylori, Bartonella). The mechanistic part of the study is nicely executed. Minor weaknesses include not using blood as a sample in addition to liver and kidney, and not including data on validation of the transcriptional identified host proteins. If experiments are not feasible at this juncture these points can be included in study limitations.

Comments for the authors:

A) On sequencing, Sequana pipeline, built on Snakemake, provides reproducibility and traceability; detailed tools listed are standard and accepted in the field.
1) The authors mention using “custom primer design on rRNA” with the RiboZero Plus kit. As this is a key step in ensuring adequate depletion of Leptospira rRNA, a clarification if these were custom-designed capture probes specific to Leptospira rRNA (e.g., 16S, 23S), and whether hamster rRNA was fully targeted by the standard probe set could be beneficial. Additionally, was the efficiency of rRNA depletion for both species evaluated (e.g., Bioanalyzer, % rRNA reads)?

2)The authors report detecting 4.57% to 79.34% of Leptospira genes, but the criteria for “gene detection” are not specified. Recommend a clarifying state on threshold used (e.g., ≥1 read, CPM > 1) to improve transparency and enable reproducibility of the transcriptome coverage claims. It would also be of interest to include the percentage of total reads mapping to the bacterial genome before and after deep sequencing.

3)Dual RNA-seq introduces the possibility of cross-mapping between host and pathogen reads, particularly in low-abundance pathogen RNA contexts. While the authors mapped reads to both genomes using STAR and Bowtie2, the manuscript would benefit from clarification on whether steps were taken to prevent or quantify cross-mapping artifacts — for example, through combined genome indexing or ambiguity filtering. This would further strengthen confidence in the assignment of reads to Leptospira or hamster origin.

B) On Other Methods:
1. Animal Model: The use of 4-week-old hamsters and a high infectious dose for this species should be explained in the context of disease modeling and its translational relevance.
2. Serovar: Since L. interrogans serovar Manilae was used it would be interesting to know if the authors expect differences in RNAseq if another L. interrogans serovar was used, what about another species? Given the use of Manilae for infection, why was lung not processed for RNA-seq?
3. Transcriptomic Focus: incorporating blood transcriptomics could have added valuable insights

4. In Vitro Infection Conditions: The manuscript lacks details regarding the infection protocol for L. interrogans in vitro. Specifically, was the infection performed in complete media with serum? This could affect bacterial uptake and cellular responses.
5. In vitro analysis using HEC293T cells which is kidney cell line: it would be interesting to see results in a hepatocyte cell line (e.g.HEPG2) since the highest differences in vivo are seen in liver. Alternatively, this could be added to study limitations
6. Calcium Signaling Assessment: The use of Fluo-8 dyes and microscopy to assess calcium signaling is limited in quantitative power. Flow cytometry could have provided a more accurate and robust quantification. Also, control image quality is poor, making interpretation difficult.
7. F-actin Visualization: The antibody-based F-actin staining displays high background and non-specific signals, complicating interpretation of actin dynamics. Phalloidin staining, a widely used method in the field, offers more reliable visualization of filamentous actin. From the confocal images, no clear changes in actin polymerization are evident. A reduced number of cells in infected groups complicates interpretation based on cell-free area quantification. Regarding cofilin analysis, please clarify whether the total and phosphorylated cofilin bands were obtained from the same blot.
8. RAC1 Signaling Interpretation: The study reports changes at the phosphorylated RAC1 level, typically associated with enhanced actin polymerization. However, the manuscript claims actin disorganization, which appears contradictory. Please clarify, possibly by analyzing downstream effectors of RAC1 that influence actin dynamics.
9. Adhesion Molecule Analysis: While discussing calcium signaling and epithelial barrier function, it is important to also examine adhesion molecules such as ICAM, VCAM, cadherins, and catenins. The authors have cited relevant literature supporting their role in Leptospiral pathogenesis, but no data is presented. This could be tested and/or added as a study limitation.
10. Cell Death Assessment: The authors report no evidence of epithelial cell death. However, no direct assays for cell death pathways (e.g., apoptosis, necrosis) were performed. The MTT assay, being a colorimetric method for cell viability, cannot definitively rule out cell death. The authors should consider performing more specific assays (e.g., Annexin V/PI staining, caspase activity, TUNEL assay) to substantiate cell death.

Reviewer #2

(Remarks to the Author)
GIRAUD-GATINEAU et al.
In this work, the authors, using dual RNA-Seq, determined the in vivo host-pathogen transcriptomic landscape upon infection by the extracellular pathogen Leptospira interrogans. They found a novel mechanism of cell-cell junction disruption, where an increase in intracellular calcium triggered tight junction destabilization, by activating the calmodulin and myosin light chain kinase signalization. Additionally, two novel bacterial effectors of the Virulence-Modifying (VM) proteins family, structurally related to toxin-like proteins, were found. These proteins promoted modulation of calcium homeostasis and disruption of cell-cell junctions, thereby allowing Leptospira translocation across epithelium barriers, tissue colonization and pathogenicity. This is a very interesting and innovative study. The high-quality experimental data support the authors' conclusions.

Reviewer #3

(Remarks to the Author)
The authors report host and bacterial transcriptomes during in vivo infection of L. interrogans using a dual RNAseq approach. They find an increase in calcium signalling and associated changes in tight junction destabilization and identified two VM protein family effectors that that are linked to cell junction disruption.

Overall, the paper is well written and data well presented. The finding that the VM effectors are associated with disruption of cell junctions is quite interesting. However, in general the data supporting some of the findings, for example, the mechanisms underlying calcium signalling, are not that convincing. Calcium signalling induced by other pathogens like Pseudomonas leads to disruption of epithelial junctions, so this mechanism is not unique to Leptospira.

My specific concerns with the study are
• Introduction: The authors claim in line 34 ‘the mechanisms employed by extracellular pathogens remain comparatively underexplored’ and in line 44 that molecular mechanisms of cell-cell junction disruption are not understood. This is not true as there are numerous examples of virulence mechanisms at host-pathogen interfaces of extracellular pathogens like staphylococcus, pseudomonas, clostridium etc. There are several bacterial toxins including staphylococcal, clostridial toxins that have been shown to disrupt epithelial barriers.
• Would be good to know the numbers of sequencing reads obtained from bacterial and the hamster host
• Figure 4 essentially demonstrates that actin reorganisation and epithelial cell junction disruption is seen in human HEK293T cells. This section essentially is reporting what has already been previously reported in the literature.
• Figure 5- Could the authors quantify fluorescence over time with the Fluo-8 AM staining assay? This would provide a non-microscopy-based quantification of intracellular calcium.
• Please include quantification of band intensities from blots showing the phosphorylation of MCL2 blots/ normalised to MCL2 and the loading control. Data should typically be from at least three blots.
• Most of the conclusions made on inhibitor effects depend on the ZO-1 staining and cell-free area quantification, which are both microscopy-based. Were these microscopy quantifications performed blinded? Immunoblotting ZO-1 or quantifying another marker would help strengthen these data.
• The differences in the %leptospires recovered after translocation is lower, however the difference is quite nominal, and it is

not clear if the differences are statistically significant.
• Figure 6. It is nice to see that the LIMLP_11660 mutant supernatants are affecting translocation, and ZO-1 staining. Do whole WT and mutant bacteria incubated with the cells also have similar effects? And also, is there an increased calcium signalling seen with whole bacteria.
• Line 256, states 'infection' with mutants, although the figure legend for 6G says supernatants were added to cells- please clarify.
• I think it is possible that the proteins are bound to receptors on the cell surface- I don't think the data shown support the conclusion that the FLAG tagged protein is internalised. Have the authors tried to do immunofluorescence with anti-FLAG to study protein localisation in the host cell?
• Methodology: No details were provided regarding the quantification of images- how many cells, fields were counted? Icy software was mentioned, no version and no details on what was quantified- for e.g., cell free area, how was this defined etc.
• Statistics- unpaired t tests have been used in many figures, e.g. figure 1 B where there are multiple data sets being compared-I believe an ANOVA or similar should be used here.

Reviewer #4

(Remarks to the Author)
This is a beautiful paper that uses dual RNAseq to identify bacterial and host pathways altered during in vivo infection (VM proteins and calcium signalling/actin cytoskeleton) and culminates with demonstrating a mechanistic link between the two. Whilst it does not yet show exactly what the molecular basis is – what is the cellular target of the two VM proteins and how do they affect calcium signalling/cytoskeleton? – it is my opinion that these first findings presented here are substantial enough in scope to warrant publication in Nature Communications.

Major(ish) concerns:
1. I am not convinced by the transwell assays used in Fig. 4L, 4J and 6B. As performed, it cannot definitely say that the increase in bacteria in the lower chamber is due to migrated bacteria, rather than an increase in bacterial growth through replication occurring over this time period. A better assay would be to infect the cell layers with the bacteria as done here, but then test for passage of an unrelated, inert particle such as FITC-dextran, a protein, or an inactivated bacterium. This will separate out permeability from cell growth. The version used in 6C is OK because this measures transfer of L. biflexa in monolayers infected with L. interrrogans.
2. There is not enough information about the in vitro sample to asses whether this is an appropriate control, and this is important as it forms the basis of all the comparative dualRNAseq analysis on bacterial transcripts. Was this isolated at exponential growth or stationary growth? Also, is it known (from the literature) how many of these genes differ at different stages of in vitro growth? i.e. which of these genes are generally variable to growth conditions, compared with those specifically altered during in vivo infection?

Minor comments:
1. Line 86, at a first read it sounds like these percentages refer to the percentage of total in vivo reads that are bacterial. After reading more carefully it became apparent that this refers to the percentage of total genes encoded in the bacterial genome that are expressed. It would be good to rephrase this to make it clearer to understand. Also it should be 4.57 not 4,57.
2. Fig 2 – confusing to use red/blue for different organs in Fig 2C, as everywhere else it refers to up/down regulated.
3. Fig 3C, it is not clear why these particular "poorly characterised" genes were selected for display here. Highest fold change? Most interesting?
4. Line 153 and Fig 4A, talk about actin rearrangement but this cannot be seen (at least to my eye) in the panel it refers to.
5. Fig 4H – is there a reason why JAM-1 not included here?
6. Lines 176-191 – this substantial section does not refer to any images in the main paper, but only to supplementary figures. I think if the authors want to dicuss this data they should put some in the main figures.
7. Fig 5G, write on these panels that the red refers to ZO-1, as written on Fig 5C. Makes it clearer to reader.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this revised manuscript the authors addressed my previous comments and concerns to the original submission.

Reviewer #3

(Remarks to the Author)
The authors have addressed most of my comments satisfactorily. I however noticed that the statistical information provided is not in line with the journal requirements (eg exact p values, test details).

page 1 line 42 -change to occludin

Reviewer #4

(Remarks to the Author)
Authors have addressed all my concerns

"In Vivo Dual RNA-Seq uncovers key toxin-like effectors of epithelial barrier disruption and tissue colonization by an extracellular bacterial pathogen"
Giraud-Gatineau et al.

Point-by-point response to the Reviewers

Reviewer #1 (Remarks to the Author)

In this study Giraud-Gatineau et al. provide the host-and-pathogen transcriptional responses of the most common pathogenic Leptospira species in vivo in an animal model of severe leptospirosis. They used dual RNA-sequencing from tissues of infected hamsters to compare transcriptomes of infected vs uninfected tissues and did deep-RNA-sequencing from tissues to enrich bacterial reads that enable comparison of Leptospira transcriptional profiles in vivo and in vitro. The deep-dual-RNAseq method is an example on how to overcome RNA-seq technical difficulties with strict extracellular pathogens where selection of infected cells cannot be used. They followed up with a suite of cell-bio, molecular biology and live-cell imaging techniques to describe how two L. interrogans virulence-modifying proteins promote calcium homeostasis and disruption of cell-cell junctions. The study approach is well-designed, comprehensive, technically rigorous and highly impactful for the Leptospirosis field as well as other pathogens that target paracellular permeability (e.g. H. pylori, Bartonella). The mechanistic part of the study is nicely executed. Minor weaknesses include not using blood as a sample in addition to liver and kidney, and not including data on validation of the transcriptional identified host proteins. If experiments are not feasible at this juncture these points can be included in study limitations.

We genuinely thank Reviewer #1 for the thoughtful and encouraging evaluation of our study. We appreciate his.her positive feedback on our deep Dual RNA-Seq approach, as well as the relevance of our findings for the field of leptospirosis and beyond.

Minor weaknesses:

1. *"Minor weaknesses include not using blood as a sample in addition to liver and kidney, …"*

Response. We fully agree with Reviewer #1 that including blood in the Dual-RNASeq analysis in addition to liver and kidney would have been interesting and relevant for leptospiral infection. However, the aim of this study was to focus on liver and kidney tissues to decipher paracellular transmigration mechanisms for bacterial dissemination and tissue colonization. We acknowledge though that blood analysis would be instrumental to determine the host immune and inflammatory responses upon leptospirosis at the early phase of the infection and this will be investigated in future studies. This point is now discussed as a limitation of the study (lines 455-459) (see point B-3).

2. *"…and not including data on validation of the transcriptional identified host proteins"*

Response. We have now included RT-qPCR analyses of selected host DEGs related to inflammation, calcium signaling and actin/cell junction pathways of liver and kidneys (**Supplementary Fig. 2E-F**). This analysis is described in the Results section (lines 145-148, and 248) and the legend of **Supplementary Fig. 2** was modified accordingly (Supplementary Information, lines 41-46).

Comments for the authors:

A) On sequencing, Sequana pipeline, built on Snakemake, provides reproducibility and traceability; detailed tools listed are standard and accepted in the field.

1) The authors mention using "custom primer design on rRNA" with the RiboZero Plus kit. As this is a key step in ensuring adequate depletion of Leptospira rRNA, a clarification if these were custom-designed capture probes specific to Leptospira rRNA (e.g., 16S, 23S), and whether hamster rRNA was

fully targeted by the standard probe set could be beneficial. Additionally, was the efficiency of rRNA depletion for both species evaluated (e.g., Bioanalyzer, % rRNA reads)?

Response. We used custom-designed capture probes specific to *Leptospira* 16S and 23S rRNA for rRNA depletion. These were generated using Sequana 0.180 Ribodesigner, an open-source tool developed in-house (https://github.com/sequana/sequana; Cokelaer et al. (2017) DOI: 10.21105/joss.00352). The probes were designed using the default parameters and based on the rRNA sequences in the *Leptospira* reference genome (NZ_CP011931.1). Briefly, Sequana Ribodesigner determines the length of the probes and the spacing between them according to the input rRNA sequences and it returns a probe set optimized for ribosomal depletion. For the host, the standard probes included in the Illumina Stranded Total RNA Prep with Ribo-Zero kit are a pool of oligos targeting mitochondrial rRNA (human, mouse, and rat); no details are provided by the manufacturer about the sequences.

We assessed rRNA depletion efficiency for both *Leptospira* and hamster samples using preliminary sequencing (Shallow Sequencing). Based on the preliminary sequencing results, we found that around 5% of rRNA remained among the *Leptospira* samples, and between 1 and 5% of rRNA among the hamster samples, confirming efficient depletion of rRNA from both species. As suggested, this information is now included in the Methods section (lines 575-578).

2) The authors report detecting 4.57% to 79.34% of Leptospira genes, but the criteria for "gene detection" are not specified. Recommend a clarifying state on threshold used (e.g., ≥1 read, CPM > 1) to improve transparency and enable reproducibility of the transcriptome coverage claims. It would also be of interest to include the percentage of total reads mapping to the bacterial genome before and after deep sequencing.

Response. We thank the Reviewer for raising this important point. In our study, a gene was considered as "detected" only if it had at least one read mapped in each biological replicate. Meaning that genes with zero reads in any of condition were excluded from the list of detected genes. We have now clarified this criterion in the legend of **Fig. 1** (line 959) to improve transparency.

As recommended, we have modified **Supplementary Fig. 1** to include the percentage of reads mapping to the *Leptospira* genome before and after deep sequencing. To provide a more comprehensive view of the sequencing output and underscore the relevance of the dataset of *Leptospira* transcriptome analysis, we have also added the absolute number of reads mapped to the *Leptospira* genome before and after deep sequencing. In addition, the number of reads mapped to the hamster genome is also reported. The legend of **Supplementary Fig. 1** was modified accordingly (Supplementary Information, lines 17-25).

3) Dual RNA-seq introduces the possibility of cross-mapping between host and pathogen reads, particularly in low-abundance pathogen RNA contexts. While the authors mapped reads to both genomes using STAR and Bowtie2, the manuscript would benefit from clarification on whether steps were taken to prevent or quantify cross-mapping artifacts — for example, through combined genome indexing or ambiguity filtering. This would further strengthen confidence in the assignment of reads to Leptospira or hamster origin.

Response. We thank the reviewer for this important methodology point. To assess potential cross-mapping between *Leptospira* and hamster reads, we had aligned the uninfected hamster RNA-Seq libraries to the *Leptospira* genome (see Tables "D1-D3_Kidney_raw_counts.txt" and "D1-D3_Liver_raw_counts.txt" in the GEO deposit). Despite tens of millions of reads per sample, only a very small number of hits were observed, and these mapped exclusively to rRNA loci. No reads aligned to *Leptospira* protein-coding genes, confirming that cross-mapping artifacts were negligible. This control analysis therefore supports the reliability of read assignment in the infected samples. The absence of cross mapping has been mentioned in the Methods section (lines 588-591).

B) On Other Methods:

1. Animal Model: The use of 4-week-old hamsters and a high infectious dose for this species should be explained in the context of disease modelling and its translational relevance.

Response. The use of weanling hamsters (3-4 weeks old) is standard in leptospirosis research because they are highly susceptible to *L. interrogans*. This allows for robust disease modelling with consistent outcomes (Wunder et al. (2016) https://doi.org/10.1128/iai.00094-16; Gomes-Solecki et al. (2017) https://doi.org/10.3389/fimmu.2017.00058). Intraperitoneal injection of 10^6 to 10^8 of *L. interrogans* is routinely used in this model to reliably induce acute and severe leptospirosis, that reproduces the pathological and clinical features of human disease, including vascular damages, renal and hepatic injuries. This approach has been validated across numerous studies to ensure translational relevance, supporting vaccine efficacy assessments and mechanistic investigations (Wunder et al. (2021) DOI: https://doi.org/10.7554/eLife.64166). In this study, our rational to use the higher relevant dose (10^8 leptospires) was to obtain the highest *Leptospira* burden in liver and kidneys (Wunder, et al. (2016) https://doi.org/10.1128/iai.00094-16) and maximize the recovery of *Leptospira* RNA for the dual RNA-Seq analysis. We have modified the manuscript to include the relevance of the experimental animal model used in this study for mimicking clinical features of acute human leptospirosis (lines 101-103).

2. Serovar: Since L. interrogans serovar Manilae was used it would be interesting to know if the authors expect differences in RNAseq if another L. interrogans serovar was used, what about another species? Given the use of Manilae for infection, why was lung not processed for RNA-seq?

Response. We thank the Reviewer for raising an insightful question related to two important aspects in leptospirosis research: the potential variability between *Leptospira* serovars and species, and the rationale for organ selection in the RNA-seq design performed in this study.

The *Leptospira* serovar is determined by the *rfb* locus, which encodes the O-antigen of the lipopolysaccharide (LPS). This locus varies across serovars and likely contributes to host specificity, particularly in reservoir hosts where infection is asymptomatic. In our study, we used *L. interrogans* serovar Manilae, which is well established to induce acute leptospirosis in hamsters, a susceptible model that reproduces human-like disease manifestations. If a different *L. interrogans* serovar had been used, provided that (i) the hamster remains a susceptible host, and (ii) the new strain encodes at least the two VM proteins studied here, we would expect a broadly similar pattern for dissemination and bacterial gene expression. Thus, we hypothesize that if hamsters are still susceptible to the selected strain, the differences will be minimal. However, certain differences could arise, particularly in the host's innate immune response, as this is known to be modulated by the LPS structure. Importantly, the impact may be more pronounced when comparing different species (e.g., *L. borgpetersenii* or *L. kirschneri*), as there may vary in terms of their tropism, virulence and host adaptation mechanisms. We fully agree that the use of other *Leptospira* species and serovars represents a valuable direction for future studies. This point is now addressed in the Discussion section (lines 497-505).

Regarding the organ selection in our study, we focused on liver and kidney, as they are the primary sites of *Leptospira* colonization in the hamster model of acute infection. Both organs typically harbor the highest bacterial loads and exhibit clear histopathological lesions during acute disease (Haake (2006) https://doi.org/10.1002/9780471729259.mc12e02s02; Wunder et al. (2016) https://doi.org/10.1128/iai.00094-16). While we acknowledge the lung as a relevant colonization site in leptospirosis associated with severe pulmonary haemorrhagic syndrome, given the technical constraints of Dual RNA-seq for pathogen transcript recovery, we prioritized organs with the highest bacterial burden. Nonetheless, we fully agree that transcriptomic analysis of infected lung tissue remains of high interest and the use of other *Leptospira* species and serovars represent a valuable direction for future studies. This points is now addressed in the Discussion section (lines 459-462).

3. Transcriptomic Focus: incorporating blood transcriptomics could have added valuable insights.

Response. We fully acknowledge the importance of studying blood transcriptional response upon infection by *Leptospira* and we agree that incorporating blood transcriptomics would offer valuable insights into the early host-pathogen interactions during *Leptospira* infection. In fact, we recently

contributed to a study describing the transcriptomic profiling of *L. interrogans* exposed to whole blood from hamster (Garcia et al. (2025) https://www.biorxiv.org/content/10.1101/2025.01.22.634353v1). A comprehensive Dual RNA-Seq analysis of blood upon infection by different *Leptospira* strains (e.g., different serovars) would be a powerful way to uncover both bacterial adaptation mechanisms and host-specific immune responses during the critical early phase of infection. This point is now addressed in the Discussion section (lines 455-459) (see minor comments, point #1).

4. In Vitro Infection Conditions: The manuscript lacks details regarding the infection protocol for L. interrogans in vitro. Specifically, was the infection performed in complete media with serum? This could affect bacterial uptake and cellular responses.

Response. As described in the Methods section, infections were performed using complete cell culture medium (Minimum Essential Medium Eagle, MEME) supplemented with 10% heat-inactivated foetal bovine serum and 2mM L-glutamine. *L. interrogans* strains were diluted directly in this medium prior to infection of HEK-293T cells. As verified in previous studies (Giraud-Gatineau et al. (2024) PLOS Pathogen DOI: 10.1371/journal.ppat.1012161), the use of heat-inactivated serum does not impair *L. interrogans* viability and, importantly, allow avoiding any serum effect on the host-*Leptospira* response. We believe that our infection setup reflects standard conditions that preserve both host cell physiology and bacterial viability, allowing us to investigate interactions at the host-pathogen interface. This point is now clarified in the Methods section (line 615).

5. In vitro analysis using HEK293T cells which is kidney cell line: it would be interesting to see results in a hepatocyte cell line (e.g.HEPG2) since the highest differences in vivo are seen in liver. Alternatively, this could be added to study limitations

Response. We sincerely appreciate the Reviewer's insightful suggestion. We have performed infection with the human hepatocyte cell line HepG2 and we could show disruption of cell junction (ZO-1 and pan-cadherin) upon infection with *L. interrogans*. These results are now presented in the **Supplementary Fig. 6** and we have modified the manuscript (lines 228-231) and the Supplementary Information file (lines 84-103) accordingly.

We have also included experimental evidence of disruption of cell-cell junctions in the hepatocyte HepG2 cells when incubated with the *L. interrogans* strains WT and mutants (*LIMLP_11660*, *dcas9-LIMLP_11655* and *LIMLP_11660+dcas9-LIMLP*_11655) (lived leptospires or supernatants). These new results are presented in **Supplementary Fig. 13**. The manuscript (lines 354-355) and Supplementary Information file (lines 222-251) were modified accordingly.

6. Calcium Signaling Assessment: The use of Fluo-8 dyes and microscopy to assess calcium signalling is limited in quantitative power. Flow cytometry could have provided a more accurate and robust quantification. Also, control image quality is poor, making interpretation difficult.

Response. To provide a more accurate and robust quantification, as recommended, we have analyzed calcium content by flow cytometry with the Fluo-8 AM dye. We could confirm the increased calcium content upon infection by *L. interrogans*, observed previously by microscopy. These data are now presented in **Fig. 5C**. In addition, a similar quantification is also provided for the infection of human epithelial cells with the different VM *Leptospira* mutant strains (lived bacteria or supernatants) in **Supplementary Fig. 15**. The manuscript (lines 278 and 377-382) and the legends of **Fig. 5** (lines 1080-1083) and **Supplementary Fig. 15** (Supplementary Information file, lines 265-273) were modified accordingly (see Reviewer #3, point 4).

7. F-actin Visualization: The antibody-based F-actin staining displays high background and non-specific signals, complicating interpretation of actin dynamics. Phalloidin staining, a widely used method in the field, offers more reliable visualization of filamentous actin. From the confocal images, no clear changes in actin polymerization are evident. A reduced number of cells in infected groups complicates interpretation based on cell-free area quantification. Regarding cofilin analysis, please clarify whether the total and phosphorylated cofilin bands were obtained from the same blot.

Response. We apologize for having misled Reviewer #1. The antibody used for filamentous actin (F-actin) visualization in our study (Actistain 555; PHDH1, from Tebu) is in fact a phalloidin conjugate, and therefore, the staining method employed is indeed Phalloidin-based. We agree with the Reviewer that this information was not clearly stated in the manuscript, and we have therefore modified the Methods section to explicitly indicate that Phalloidin staining was used (line 631-632).
We understand Reviewer #1's concern regarding the apparent lower number of cells in the image of infected sample (**Fig. 4A-B**). We wish to stress out that *L. interrogans* does not impair HEK-293T cell proliferation or cell death under the condition used in our study, as verified by cell viability assay (MTT assay for cell proliferation and Annexin V-Propidium iodide markers for cell death, see point B-10). The observed lower cell density in the images of infected samples is due to increased cell dispersion and loss of cell-cell contacts during infection, but not to a reduction in total cell number. Importantly, the microscope field of view and magnification were kept constant across all conditions, which may contribute to the visual impression of fewer cells in infected samples. The cell-free area quantification is a metric used here not to infer cell death but to assess the disruption of cell-cell junctions and monolayer integrity, which are hallmarks of barrier dysfunction. Since the number of cells remains constant in both conditions and since the staining clearly delineates cell borders, we believe this quantification remains valid and informative.
Regarding cofilin analysis, the total and phosphorylated cofilins were not detected on the same immunoblot membrane, due to their very similar molecular weights (~19 kDa). As such, separate blots with identical protein loads and processed under the same experimental conditions were used for these detections. This point is now mentioned in the legend of **Supplementary Fig. 5** (Supplementary Information, lines 79-80) to clarify the experimental setup.

8. RAC1 Signaling Interpretation: The study reports changes at the phosphorylated RAC1 level, typically associated with enhanced actin polymerization. However, the manuscript claims actin disorganization, which appears contradictory. Please clarify, possibly by analyzing downstream effectors of RAC1 that influence actin dynamics.

Response. We thank Reviewer #1 for this insightful comment on the apparent discrepancy between RAC1 activation and the observed actin disorganization during infection by *L. interrogans*. Activated phosphorylated RAC1 is indeed well-characterized Rho-family GTPase that typically activates downstream effectors such as WAVE complex, PAK kinases or cofilin, thereby promoting branched actin polymerization in cells (Spence & Soderling (2015) DOI: 10.1074/jbc.R115.655118; Sit & Manser (2011) https://doi.org/10.1242/jcs.064964.). However, RAC1 was also shown to regulate membrane ruffling, lamellipodia turnover, and dynamic cytoskeletal reorganization (Marston et al. (2019) https://doi.org/10.1073/pnas.1808830116), which depending on the context can manifest as actin rearrangement.
In our infection model, the elevated Rac1 phosphorylation likely reflects activation of Rac1 signaling pathways in response to bacterial infection. However, this activation does not necessarily lead to organized actin structures, as Rac-1 mediated signaling can promote dynamic turnover of actin filaments rather than stable actin structures (Marston et al. (2019) https://doi.org/10.1073/pnas.1808830116; Sun et al. (2007) DOI: 10.1083/jcb.200705122).
We also observed increased activation of the downstream effectors cofilin and WAVE-2 (**Supplementary Fig. 5B**), confirming Rac1 pathway involvement and enhanced actin dynamics. However, despite this activation, cell-cell junctions remained disrupted, indicating that Rac1 alone is insufficient to maintain cytoskeletal integrity.
Therefore, *L. interrogans* may perturb additional regulatory pathways or express effectors that directly interfere with actin-binding proteins, leading to actin reorganization and junctional disorganization.

9. Adhesion Molecule Analysis: While discussing calcium signaling and epithelial barrier function, it is important to also examine adhesion molecules such as ICAM, VCAM, cadherins, and catenins. The authors have cited relevant literature supporting their role in Leptospiral pathogenesis, but no data is presented. This could be tested and/or added as a study limitation.

Response. We agree that adhesion molecules such as ICAM, VCAM, or cadherins play critical roles in maintaining epithelial integrity and, indeed, several of these factors have already been shown to participate in disruption of cell-cell junction associated with leptospirosis (H. Sato & J. Coburn (2017) https://doi.org/10.1371/journal.pntd.0005830; Sebastian et al. (2021) DOI: 10.1111/cmi.13343). Our aim was to further characterize *L. interrogans* dissemination, focusing on unexplored molecular pathways such as disruption of tight junction. Nevertheless, as suggested, we have assessed the effect of *Leptospira* on the integrity of cadherins. Using a pan-cadherin antibody, we could show a decrease of pan-cadherin content in the plasma membrane upon infection by *Leptospira* (presented now in **Fig. 4E**). we could confirm that infection by *L. interrogans* compromises cadherin junction, as previously shown (H. Sato & J. Coburn (2017) https://doi.org/10.1371/journal.pntd.0005830). We also confirmed that the activation of calmodulin-MLCK pathway through Ca^{2+} signaling partially restored pan-cadherin localization at cell-cell junctions (presented in **Supplementary Fig. 9**). The manuscript (lines 241-242, 245-248, 303, 313, 671, and 962), the legend of **Fig. 4** (lines 1064-1069) and the Supplementary Information file (lines 146-160) were modified accordingly. In addition, we included the experimental evidence of the decrease of pan-cadherin in plasma membrane of cell-cell junctions in epithelial cells when incubated with the supernatants of or infected with different *L. interrogans* strains (WT and VM mutants) (presented in now **Supplementary Fig. 12A**). The manuscript (lines 370, 373-378), and the Supplementary Information files (lines 200-208) were modified accordingly.

10. Cell Death Assessment: The authors report no evidence of epithelial cell death. However, no direct assays for cell death pathways (e.g., apoptosis, necrosis) were performed. The MTT assay, being a colorimetric method for cell viability, cannot definitively rule out cell death. The authors should consider performing more specific assays (e.g., Annexin V/PI staining, caspase activity, TUNEL assay) to substantiate cell death.

Response. We thank Reviewer #1 for this thoughtful comment on the limitation of MTT assay. While the MTT assay suggests preserved cellular viability, we agree that this method does not distinguish between different modes of cell death. As suggested, we have performed Annexin V/PI staining to directly assess apoptosis and necrosis, thereby providing a more definitive evaluation of the survival of infected epithelial cells. We did not detect any apoptosis nor necrosis upon infection of HEK-293T cells (presented now in **Fig. 4D**) or HepG2 cells (presented now in **Supplementary Fig. 6B**). The Methods section (lines 666-670), the legends of **Fig. 4** (lines 1011-1015) and **Supplementary Fig. 6B** (Supplementary Information, lines 86-92) have been modified.

Reviewer #2 (Remarks to the Author)

GIRAUD-GATINEAU et al.

In this work, the authors, using dual RNA-Seq, determined the in vivo host-pathogen transcriptomic landscape upon infection by the extracellular pathogen Leptospira interrogans. They found a novel mechanism of cell-cell junction disruption, where an increase in intracellular calcium triggered tight junction destabilization, by activating the calmodulin and myosin light chain kinase signalization. Additionally, two novel bacterial effectors of the Virulence-Modifying (VM) proteins family, structurally related to toxin-like proteins, were found. These proteins promoted modulation of calcium homeostasis and disruption of cell-cell junctions, thereby allowing Leptospira translocation across epithelium barriers, tissue colonization and pathogenicity. This is a very interesting and innovative study. The high-quality experimental data support the authors' conclusions.

We would like to thank Reviewer #2 for his/her very positive evaluation of our study. We really appreciate that our findings were acknowledged for their novelty, innovation, significance, and quality.

Reviewer #3 (Remarks to the Author)

The authors report host and bacterial transcriptomes during in vivo infection of L. interrogans using a dual RNAseq approach. They find an increase in calcium signalling and associated changes in tight junction destabilization and identified two VM protein family effectors that that are linked to cell junction disruption. Overall, the paper is well written and data well presented. The finding that the VM effectors are associated with disruption of cell junctions is quite interesting. However, in general the data supporting some of the findings, for example, the mechanisms underlying calcium signalling, are not that convincing. Calcium signalling induced by other pathogens like Pseudomonas leads to disruption of epithelial junctions, so this mechanism is not unique to Leptospira.
My specific concerns with the study are

We would like to thank Reviewer #3 for his.her thoughtful and pertinent suggestions and reviews.

1. Introduction: The authors claim in line 34 'the mechanisms employed by extracellular pathogens remain comparatively underexplored' and in line 44 that molecular mechanisms of cell-cell junction disruption are not understood. This is not true as there are numerous examples of virulence mechanisms at host-pathogen interfaces of extracellular pathogens like staphylococcus, pseudomonas, clostridium etc. There are several bacterial toxins including staphylococcal, clostridial toxins that have been shown to disrupt epithelial barriers.
Response. We agree with the reviewer that some mechanisms of infection by extracellular pathogens, including mechanisms of epithelial barriers disruption, have already been characterized. We realized that the aforementioned statement could be interpretated as overstated, and we have modified consequently the introduction to acknowledge previous studies on extracellular pathogen-induced junction disruption, particularly by specific toxins (lines 39-40 and 49-52).
We would like to emphasize though that while mechanisms of cell-cell junction disruption are established for some pathogens, mechanisms for understudied bacteria, which do not encode known virulence factors are less understood. By demonstrating a role of *Leptospira* VM protein family effectors, structurally distinct from previously characterized toxins, in promoting cell-cell junction disruption and modulating host calcium signaling, the present study will significantly advance the field of infection by extracellular pathogens.

2. Would be good to know the numbers of sequencing reads obtained from bacterial and the hamster host
Response. A recommended, we have indicated the number of reads mapping in *Leptospira* genome before and after deep sequencing, and the number of reads mapped to the hamster genome (see **Supplementary Fig. 1**). The legend of **Supplementary Fig. 1** was modified accordingly (Supplementary Information, lines 17-25) (see also Reviewer #1, point A-2).

3. Figure 4 essentially demonstrates that actin reorganisation and epithelial cell junction disruption is seen in human HEK293T cells. This section essentially is reporting what has already been previously reported in the literature.
Response. We fully agree with the Reviewer that the disruption of epithelial and endothelial cell junctions and actin reorganization upon *L. interrogans* infection have been previously reported in the literature. In fact, we have acknowledged these previous studies (H. Sato & J. Coburn (2017) https://doi.org/10.1371/journal.pntd.0005830; Sebastian et al. (2021) DOI: 10.1111/cmi.13343). Our purpose was not to re-establish these known phenomena, but rather to use them as cellular readout for validating the dual RNA-Seq data and the functional investigation of the two newly identified VM proteins. Indeed, dual RNA-Seq with an extracellular pathogen *in vivo* is an approach which is still rare in the field. Data presented in **Fig. 4** essentially serve to demonstrate that the experimental set up used in the study recapitulates key aspects of cell-cell junction disruption associated with leptospiral infection.

4. Figure 5- Could the authors quantify fluorescence over time with the Fluo-8 AM staining assay? This would provide a non-microscopy-based quantification of intracellular calcium.

Response. As recommended, we have now included the quantification of Fluo-8 AM fluorescence intensity by flow cytometry to provide a non-microscopy-based quantification of intracellular calcium. We could confirm the increase of calcium content upon infection by *L. interrogans* previously observed by microscopy. These data are now presented in **Fig. 5C**. In addition, a similar quantification is also provided for the infection experiments of human epithelial cells with the different VM *Leptospira* mutant strains (lived bacteria or supernatants) in **Supplementary Fig. 15**. The manuscript (lines 278 and 377-382) and the legends of **Fig. 5** (lines 1080-1083) and **Supplementary Fig. 15** (Supplementary Information file, lines 265-273) were modified accordingly (see also Reviewer#1, point B-6).

5. Please include quantification of band intensities from blots showing the phosphorylation of MCL2 blots/ normalised to MCL2 and the loading control. Data should typically be from at least three blots.

Response. As suggested, we have provided now the quantification of the band intensities corresponding to total and phosphorylated MCL2 performed in triplicate (see **Fig. 5G**). The legend of **Fig. 5** has been modified accordingly (lines 1106-1109).

6. Most of the conclusions made on inhibitor effects depend on the ZO-1 staining and cell-free area quantification, which are both microscopy-based. Were these microscopy quantifications performed blinded? Immunoblotting ZO-1 or quantifying another marker would help strengthen these data.

Response. We thank the reviewer for this important and challenging remark. We acknowledge that microscopy-based quantifications of ZO-1 and cell-free areas were not conducted in a blinded manner. This limitation is now clearly disclosed in the Methods section (lines 635-636) to maintain full transparency.

To strengthen the reliability of the data obtained with ZO protein staining and inhibitors, and upon suggestions of Reviewer #1 (see points B-5 and B-9), we obtained the following additional results. We obtained the evidence that (i) infection by *L. interrogans* leads to the disruption of cadherin in human epithelial cells and treatment with inhibitors (BAPTA-AM, KN-83 and ML-7) reduces this disruption (**Fig. 4E**, **Supplementary Fig. 9**), (ii) cadherin amounts at the cell-cell junction are reduced when epithelial cells are incubated with supernatant of WT *L. interrogans* culture (**Supplementary Fig. 12)**, (iii) reduction of both ZO-1 and cadherin upon infection by *L. interrogans* is also observed in infected human HepG2 hepatocytes (**Supplementary Fig. 6**), and (iv) VM proteins participate in the reduction of ZO-1 and cadherin amounts at the cell-cell junction, as demonstrated using supernatants from VM mutants cultures (*LIMLP_11660*, *dcas9-LIMLP-11655* and *LIMLP-11660+dcas9-LIMLP_11655*) in both epithelial cells and human HepG2 hepatocytes (**Supplementary Fig. 12 and 13**). In conclusion, the significant role of VM effectors in cell-cell junction disruption and modulation of calcium signaling can be observed with different markers (ZO-1 and cadherin) and cell lines. Together with our finding that a defect of translocation across human epithelial cells can be observed using supernatant of *L. interrogans* mutant (**Fig. 6C**), these results provide robust and convergent evidence supporting a mechanistic link between VM effectors and cell junction integrity.

7. The differences in the %leptospires recovered after translocation is lower, however the difference is quite nominal, and it is not clear if the differences are statistically significant.

Response. We fully agree with the Reviewer's observation on the relatively low percentage of *L. interrogans* translocation observed in transwell assay experiments. It is important to note that considerable variations in the translocation rates of *L. interrogans* are reported in the literature, depending on the experimental model used, ranging from approximately 4-8% (Vieira *et al.* (2013) DOI: 10.1128/IAI.00020-13; Zhu et al (2023) https://doi.org/10.1371/journal.ppat.1011313) up to 60% (Martinez-Lopez et al. (2010) https://doi.org/10.1371/journal.pntd.0000918). This variability likely reflects complex factors, such as fluid dynamics, host cell types and microenvironmental conditions, influencing *L. interrogans* dissemination. The transwell assay, while useful, exhibits low efficiency and has inherent limitations. The use of negative controls is essential in this assay. In the present study, we

have used the saprophytic species *L. biflexa* that consistently displayed a significantly lower translocation rate at different time points compared to *L. interrogans* (see **Fig. 4L**) as negative control. In addition, VM mutant strains exhibited a lower translocation ability (**Fig. 6B**) and VM proteins in the supernatant of *L interrogans* culture increases the ability of *L. biflexa* to translocate epithelial barriers as demonstrated in **Fig. 6C** using WT and VM mutant strains. This validates the specificity of *L. interrogans* translocation capacity and supports the presence of dedicated mechanisms employed by this pathogenic species to cross cellular barriers. Although the absolute translocation rates remain low, altogether these findings strengthen the biological relevance of the transwell assays presented in the present study (see also Reviewer #4, Major Concern point#1).

8. Figure 6. It is nice to see that the LIMLP_11660 mutant supernatants are affecting translocation, and ZO-1 staining. Do whole WT and mutant bacteria incubated with the cells also have similar effects? And also, is there an increased calcium signalling seen with whole bacteria.

Response. We thank the reviewer for this important question. Analyses of cell junction integrity (as assessed by ZO-1 and cadherin stainings) upon direct infection of epithelial HEK293T and hepatocyte HepG2 cells with whole *L. interrogans* WT and VM mutant strains are now included in the revised manuscript (**Supplementary Fig. 12B-C, 13A and 13C**). Junction disruption is equally observed with whole *L. interrogans* WT and most mutant strains, but cell-cell junction disruption is partially reduced only when both LIMLP_11660 and LIMLP_11655 are concomitantly inactivated or silenced (mutant *LIMLP_11660*+dcas9-*LIMLP_11655*). This demonstrates that both VM proteins act cooperatively to promote efficient junction disruption during infection. The manuscript (lines 351-355), the legend of **Supplementary Fig. 12** (Supplementary Information file, lines 209-220) and **Supplementary Fig. 13** (Supplementary Information file, lines 224-231 & 240-245) have been modified.

Regarding intracellular calcium concentrations during infection, we observe a similar increase in calcium content when infecting epithelial cells with whole *L. interrogans* or when incubating cells with supernatant of *L. interrogans* culture. These data are now presented in **Supplementary Fig. 15**. The manuscript (line 382) and the legend of **Supplementary Fig. 15** have been updated (Supplementary Information, lines 265-273).

Interestingly, the impact of inactivating VM genes on cell-cell disruption (as assessed by ZO-1 and cadherin staining) and calcium signaling is more pronounced with supernatants than with whole bacteria. This indicates that *L. interrogans* possesses additional factors, beyond secreted VM proteins, such as adhesins or other surface molecules, that contribute to the disruption of cell junctions and modulate intracellular calcium levels directly or indirectly.

9. Line 256, states 'infection' with mutants, although the figure legend for 6G says supernatants were added to cells- please clarify.

Response. We thank the reviewer for pointing out this mistake. We have corrected it accordingly (lines 361, 371, 378-379, and 382).

10.I think it is possible that the proteins are bound to receptors on the cell surface- I don't think the data shown support the conclusion that the FLAG tagged protein is internalised. Have the authors tried to do immunofluorescence with anti-FLAG to study protein localisation in the host cell?

Response. We do appreciate the reviewer's comment which helps improving the clarity and accuracy of our conclusion. Reviewer #3 is totally right to consider that our data do not conclusively demonstrate internalization of the VM proteins inside host cells. In fact, we have attempted to detect the flagged VM protein inside the host cells using anti-3xFLAG antibodies. Unfortunately, these experiments did not yield detectable fluorescence of VM despite using the same antibody that successfully detects VM in immunoblots. The lack of immunofluorescence signal could be explained by a limited accessibility of the FLAG epitope into the cellular context, an insufficient antibody penetration in fixed cells, and/or a too low abundance of the internalized protein. We have accordingly revised some of the conclusions in the manuscript to reflect these considerations and limitations. We have stated that VM (i) is secreted by *L. interrogans*, and (ii) interacts with and modulates the host cell

environment (lines 28 (abstract) and lines 372-373), rather than being definitively internalized. The title of **Fig. 6** was also modified (lines 1154-1155).

11.Methodology: No details were provided regarding the quantification of images- how many cells, fields were counted? Icy software was mentioned, no version and no details on what was quantified- for e.g., cell free area, how was this defined etc

Response. We thank the reviewer for raising this point. For each experiment, a minimum of 3 random fields per condition and at least 100 cells were analyzed to ensure representative sampling. A more detailed description of the quantification and imaging protocol (number of fields and cells analyzed, defining regions of interest, the version of Icy software) has now been included in the Methods section (lines 647-659).

12.Statistics- unpaired t tests have been used in many figures, e.g. figure 1 B where there are multiple data sets being compared-I believe an ANOVA or similar should be used here.

Response. We thank the reviewer for this important comment regarding the statistical analyses. We provide now analyses by One-way ANOVA test when the unpaired *t* test was not relevant. The legends of Fig. 1B, Fig. 5B, Fig. 5E-F, Fig. 5I-J, Fig. 6B-D, and Fig. 6G have been modified accordingly.

Reviewer #4 (Remarks to the Author)

This is a beautiful paper that uses dual RNAseq to identify bacterial and host pathways altered during in vivo infection (VM proteins and calcium signalling/actin cytoskeleton) and culminates with demonstrating a mechanistic link between the two. Whilst it does not yet show exactly what the molecular basis is – what is the cellular target of the two VM proteins and how do they affect calcium signalling/cytoskeleton? – it is my opinion that these first findings presented here are substantial enough in scope to warrant publication in Nature Communications.

We are grateful to Reviewer #4 for his.her appreciative and positive assessment of our study and recognising the importance of our findings.

Major(ish) concerns:

1. I am not convinced by the transwell assays used in Fig. 4L, 4J and 6B. As performed, it cannot definitely say that the increase in bacteria in the lower chamber is due to migrated bacteria, rather than an increase in bacterial growth through replication occurring over this time period. A better assay would be to infect the cell layers with the bacteria as done here, but then test for passage of an unrelated, inert particle such as FITC-dextran, a protein, or an inactivated bacterium. This will separate out permeability from cell growth. The version used in 6C is OK because this measures transfer of L. biflexa in monolayers infected with L. interrogans.

Response. We thank the reviewer for raising this important point regarding the relevance and limitations of the transwell assays presented in the present study. We fully agree with the importance of distinguishing active migration across epithelial barriers from bacterial replication. In our experimental system, however, it is very unlikely that bacterial multiplication explains the observed increase in bacteria number in the lower chamber as *L. interrogans* do not replicate in EMEM medium during the timeframe of the transwell assays, although they remain viable. To further address this concern, we performed an experiment with a non-motile *L. interrogans flaA2* mutant, shown to grow at a rate comparable to that of the WT strain. As shown in the Reviewer only Figure below, under identical conditions, the *flaA2* mutant did not cross epithelial monolayers, in contrast to the WT strain (see panel B below). This strongly supports that the increased number of bacteria recovered in the lower chamber is mainly due to active transmigration rather than to bacterial growth.

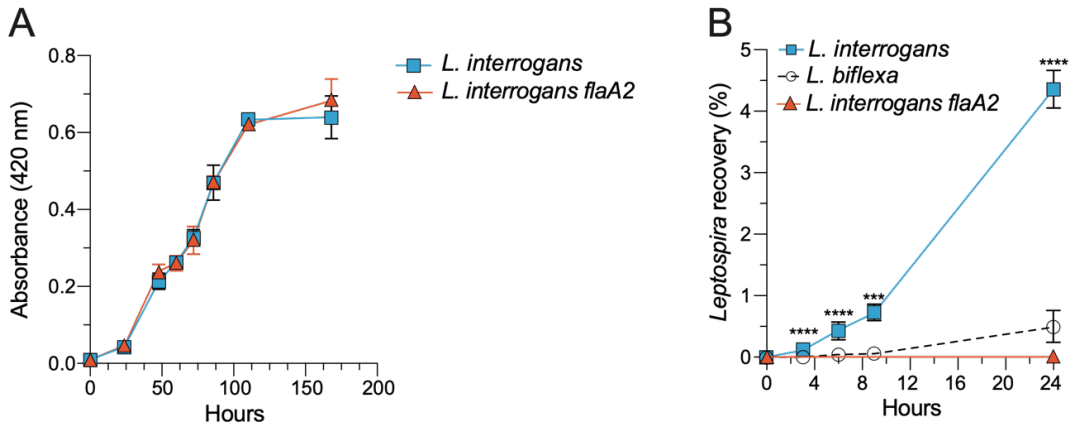

Figure Reviewer Only. Transmigration of the non-motile *L. interrogans flaA2* mutant
(**A**) Growth of *L. interrogans* WT (blue squares) and *L. interrogans flaA2* mutant (orange triangles). Both strains were cultivated in EMJH medium. Bacterial growth was assessed by measuring the absorbance at 420 nm. Data are the mean ± SD from 3 independent experiments.
(**B**) Ability of *L. interrogans* WT (blue squares), *L. interrogans flaA2* mutant (orange triangles) and *L. biflexa* WT (empty circles) to translocate through human epithelial cells. Bacteria were inoculated in the upper chamber of a cell culture insert containing human epithelial cell monolayer. The percentage of *Leptospira* recovered in the lower chamber was determined by qPCR analysis. Values obtained at the indicated times were normalized by the inoculum and expressed as percentage. Data are mean ± SD from 3 independent experiments. ***, $p < 0.001$; ****, $p < 0.0001$ (unpaired, two-tailed t test).

2. There is not enough information about the in vitro sample to assess whether this is an appropriate control, and this is important as it forms the basis of all the comparative dualRNAseq analysis on bacterial transcripts. Was this isolated at exponential growth or stationary growth? Also, is it known (from the literature) how many of these genes differ at different stages of in vitro growth? i.e. which of these genes are generally variable to growth conditions, compared with those specifically altered during in vivo infection?

Response. We thank the reviewer for raising this important point about the relevance of *in vitro*-cultivated *Leptospira* as control for the transcriptional response *in vivo*. We agree that in transcriptomic studies under *in vivo* conditions, one key question is the suitability of the baseline comparison. For the control, *L. interrogans* were cultivated at 30°C in the classical EMJH medium for *Leptospira* spp. and harvested at exponential phase ($OD_{420} \approx 0.3$; 10^9 leptospires/mL) and RNAs were extracted from 10^{10} leptospires. This information has been indicated in the Methods section (line 569). The shift from the EMJH culture medium to the host tissues corresponds to dramatic, complex and pleiotropic environmental changes, having a strong and significant influences on gene expression in bacteria. Transcriptomes of *Leptospira* upon exposure to the host temperature (37°C) (Lo et al. (2006) https://doi.org/10.1128/iai.00755-06), the host osmolarity (Matsunaga et al. (2007) https://doi.org/10.1128/iai.01619-06), or to serum (Xue et al. (2010) DOI: 10.1371/journal.pntd.0000857) allow inferring to some extent what are the specific host-related signal that influence the expression of a particular gene. Unfortunately, no transcriptome of *Leptospira* at different stages of the *in vitro* growth was published to date.
Nevertheless, we believe that the comparison with in vitro-cultivated and exponentially growing *Leptospira* remains relevant and useful in the context of transcriptomic studies on *Leptospira* field. Thus, differential gene expression *in vivo* reflects adaptation to the complex and pleiotropic infectious environment, highlighting processes linked to pathogenicity.

Minor comments:

1. Line 86, at a first read it sounds like these percentages refer to the percentage of total in vivo reads that are bacterial. After reading more carefully it became apparent that this refers to the percentage of total genes encoded in the bacterial genome that are expressed. It would be good to rephrase this to make it clearer to understand. Also it should be 4.57 not 4,57.

Response. As recommended, we have changed the sentence and stated that the percentages referred here correspond to the fraction of all *Leptospira* genes encoded in the genome that were detected as expressed, not to the proportion of bacterial reads in the total sample. We have also corrected 4,57 into 4.57 (lines 115-116).

2. Fig 2 – confusing to use red/blue for different organs in Fig 2C, as everywhere else it refers to up/down regulated.

Response. We have modified colors of **Fig. 2C** to avoid confusing with red and blue of up- and down-regulated genes, respectively. Kidney and liver samples correspond now to brown and green, respectively. The legend of **Fig. 2** was modified accordingly (line 978).

3. Fig 3C, it is not clear why these particular "poorly characterised" genes were selected for display here. Highest fold change? Most interesting?

Response. Around 30% of *L. interrogans* genome encodes for hypothetical proteins. We have chosen to present the most up- and down-regulated poorly characterized and hypothetical genes as this information might be important for future studies. In fact, the two VM proteins characterized in the present study are annotated as hypothetical proteins as well.

4. Line 153 and Fig 4A, talk about actin rearrangement but this cannot be seen (at least to my eye) in the panel it refers to.

Response. We agree with the reviewer that the effect visible in **Fig. 4A** is more related to changes in overall cell morphology than to a clear actin rearrangement. However, in infected cells, we observe that the cytoskeleton appears less expanded compared to uninfected cells. While this does not show a classical reorganization of actin filaments, the actin staining seems more condensed around the nucleus with reduced intercellular contacts, in contrast to the more spread morphology of uninfected cells. We have revised the manuscript accordingly to better reflect these observations (lines 208-209).

5. Fig 4H – is there a reason why JAM-1 not included here?

Response. JAM-1 was included in the confocal analysis (**Fig. 4F-G**) to assess junctional organization upon *L. interrogans* infection; however, the mechanistic validation in this study focused on the ZO family of tight junction proteins. ZO proteins are key scaffolding components that link transmembrane proteins to the actin cytoskeleton and play a central role in maintaining barrier integrity. In our study, these proteins showed the most pronounced alterations upon *L. interrogans* infection and were therefore prioritized for protein validation by western blot. While JAM-1 provided complementary localization data by confocal microscopy regarding cell-cell junction disruption, our mechanistic investigations were focused on ZO proteins and cadherins (in the revisited manuscript).

6. Lines 176-191 – this substantial section does not refer to any images in the main paper, but only to supplementary figures. I think if the authors want to dicuss this data they should put some in the main figures.

Response. Findings presented in this study indicate that infection by *L. interrogans* modulates the ER activity, but we could not establish its participation in the cell-cell junction disruption mechanism. Therefore, we presented these data as the Supplementary Figures. However, we agree that the section describing ER activity on infected cells might be seen as lengthy compared to its importance in the manuscript. We have now shortened this section (lines 242-255).

7. Fig 5G, write on these panels that the red refers to ZO-1, as written on Fig 5C. Makes it clearer to reader.

Response. We thank the reviewer for this thoughtful suggestion. We have now added colors legend in panel of Fig. 4B, Fig. 4I, Fig. 5A, Fig. 5H (Fig. 5G in the previous version of the manuscript), Fig. 6D and Fig. 6G.

NCOMMS-25-28572A

"*In vivo* Dual RNA-Seq uncovers key effectors of epithelial barrier disruption by an extracellular pathogen" Giraud-Gatineau *et al*.

Point-by-point response to the Reviewers

We sincerely thank all the Reviewers for their previous helpful and constructive feedback. We are pleased that they are satisfied with the revised version.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In this revised manuscript the authors addressed my previous comments and concerns to the original submission.
We are pleased that Reviewer #1 finds that her.his concerns have been appropriately addressed.

Reviewer #3 (Remarks to the Author):

The authors have addressed most of my comments satisfactorily.
We are pleased that Reviewer #3 finds that most of her.his concerns have been appropriately addressed.

I however noticed that the statistical information provided is not in line with the journal requirements (eg exact p values, test details).
Response. We thank the Reviewer for this remark. We have now provided all the necessary detailed information about the statistic tests (nature of tests, sample size, nature of replicates, *p*-values, etc.) performed in this study in the Figure legends and in the Source Data file (for Fig. Supplementary 2). This now complied with the editorial requirements of the journal.

page 1 line 42 -change to occludin
Response. We thank the Reviewer for pointing out this mistake. We have corrected "occluding" into "occludin" (Line 44, word highlighted in yellow).

Reviewer #4 (Remarks to the Author):

Authors have addressed all my concerns
Response. We are pleased that Reviewer #4 finds that her.his concerns have been appropriately addressed.