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RESEARCH ARTICLE

Metabolomic analysis of murine tissues infected with *Brucella melitensis*Bárbara Ponzilacqua-Silva^{1,2}, Alexis S. Dadelahi^{1,2}, Charles R. Moley^{1,2}, Mostafa F. N. Abushahba^{1,2,3}, Jerod A. Skyberg^{1,2,4*}

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Abstract

Brucella is a gram negative, facultative intracellular bacterial pathogen that constitutes a substantial threat to human and animal health. *Brucella* can replicate in a variety of tissues and can induce immune responses that alter host metabolite availability. Here, mice were infected with *B. melitensis* and murine spleens, livers, and female reproductive tracts were analyzed by GC-MS to determine tissue-specific metabolic changes at one-, two- and four-weeks post infection. The most remarkable changes were observed at two-weeks post-infection when relative to uninfected tissues, 42 of 329 detected metabolites in reproductive tracts were significantly altered by *Brucella* infection, while in spleens and livers, 68/205 and 139/330 metabolites were significantly changed, respectively. Several of the altered metabolites in host tissues were linked to the GABA shunt and glutaminolysis. Treatment of macrophages with GABA did not alter control of *B. melitensis* infection, and deletion of the putative GABA transporter BMEI0265 did not alter *B. melitensis* virulence. While glutaminolysis inhibition did not affect control of *B. melitensis* in macrophages, glutaminolysis was required for macrophage IL-1 β production in response to *B. melitensis*. In summary, these results indicate that *Brucella* infection alters host tissue metabolism and that these changes could have effects on inflammation and the outcome of infection.

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Introduction

Brucellosis is a worldwide bacterial zoonosis with a broad host range of animals, spanning from wildlife to agriculturally important livestock [1]. The three main species recognized as substantial health threats and most common human pathogens are *B. melitensis*, *B. abortus*, and *B. suis* [2]. Humans acquire brucellosis through inhalation of infectious aerosols and by consumption of contaminated animal products [3–5]. Human brucellosis can be a severely debilitating disease that requires hospitalization [6]. Human disease is characterized by persistent waves of fever with systemic symptoms that can vary among individuals, including chills, malaise, headaches, and hepato- or splenomegaly [7].

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Brucella replicates intracellularly and has a predilection for organs rich in reticuloendothelial cells such as the spleen and liver [8]. Controlling *Brucella* infection is complicated due to the fact that humoral immunity and some antibiotics are unable to reach the bacteria while inside the cell [9]. Thus, antibiotic therapy is lengthened, increasing the likelihood of treatment-related side effects and leading to non-compliance among those being treated [10, 11]. Aggravating the situation, no vaccine is licensed to prevent human brucellosis.

Metabolic changes in immune cells have an influence on the effectiveness of the immune response and how intracellular bacteria experience stress [12–15]. In the context of brucellosis, macrophages are important for controlling infection but can also be the major niche for *Brucella* survival and replication depending on their metabolic status [1]. Metabolic reprogramming of host cells can be initiated by pathways such as glycolysis, TCA cycle, glutaminolysis, gamma-aminobutyric acid (GABA) shunt, and others, depending on the immune response [16]. For example, M1 polarization of macrophages is marked by increased glycolysis and an impaired TCA cycle, driving the accumulation of several metabolites as well as promoting immune signaling and, in some cases, antimicrobial activities [17–19]. In contrast, M2 polarization of macrophages is marked by mitochondrial oxidative metabolism of fatty acids, leading to an anti-inflammatory and pro-resolution profile which can favor *Brucella* replication [20, 21].

In human and animal tissues, *Brucella* encounter a variety of metabolic conditions in which they must be able to multiply in order to cause disease and spread to new hosts [15]. However, the relative availability of metabolites that *Brucella* encounters across host tissues during infection is largely unknown. Within mice and natural hosts, *Brucella* can target the spleen, liver, and reproductive tracts for replication [22]. Therefore, in this study, we performed global screening of metabolites in spleens, livers, and reproductive tracts at various timepoints after *Brucella* infection. In addition, we investigated the effects of these metabolic changes on inflammatory cytokine production and the requirements for *Brucella* virulence.

Materials and methods

Bacterial strains and growth conditions

Experiments with live *Brucella melitensis* were performed in biosafety level 3 (BSL3) facilities. *B. melitensis* 16M was obtained from Montana State University (Bozeman, Montana) and grown on Brucella agar (Becton, Dickinson) for 3 days at 37°C/5% CO₂. Colonies were then transferred to Brucella broth (Bb; Becton, Dickinson) and grown at 37°C with shaking overnight. The overnight *Brucella* concentration was estimated by measuring the optical density (OD) at 600 nm, and the inoculum was prepared and diluted to the appropriate concentration in sterile phosphate-buffered saline (sPBS). Titer was confirmed by serial dilution of the *B. melitensis* inoculum onto Brucella agar plates.

Generation of *B. melitensis* AbmeI0265

The BMEI0265 gene in *B. melitensis* 16M was replaced in frame with a chloramphenicol resistance gene (*catR*) from plasmid pKD3 [23] using the suicide plasmid pNTPS139 [24]. Approximately 1,000-bp fragments upstream and downstream of BMEI0265 were amplified by PCR using primers shown in S1 Table. We also generated a 1,044-bp fragment containing the *catR* gene from pKD3 using primers listed in S1 Table. The 5' end of the forward primer used to amplify the upstream fragment of BMEI0265 contained homology to 30 bp upstream of the BamHI site in pNTPS139. The 5' end of the forward primer used to amplify the *catR* gene from pKD3 contained 30 bp homologous to the 3' end of the upstream BMEI0265 fragment. The downstream fragment of BMEI0265 was amplified using a forward primer whose 5' end

contained 30 bp homologous to the 3' end of the *catR* fragment, while the 5' end of the downstream BMEI0265 fragment reverse primer contained 30 bp homologous to the 30bp downstream of the *Sall* site of pNTPS139. pNTPS139 was digested with *Bam*HI/*Sall*. The upstream and downstream BMEI0265 fragments, along with *Bam*HI/*Sall*- digested pNTPS139, were all ligated together using the NEB Hi-Fi DNA assembly kit according to the manufacturer's instructions (New England Biolabs, Ipswich, MA). These plasmids were introduced into *B. melitensis* 16M, and merodiploid transformants were obtained by selection on Brucella agar plus 25 µg/ml kanamycin. A single kanamycin-resistant clone was grown overnight in Brucella broth and then plated onto brucella agar supplemented with 10% sucrose. Genomic DNA from sucrose-resistant, kanamycin-sensitive colonies was isolated and screened by PCR for replacement of the gene of interest.

Mice

All animal procedures were reviewed and approved by the Animal Care and Use Committee (ACUC) of the University of Missouri and followed the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals under Office of Laboratory Animal Welfare assurance number is D16-00249. All animals were checked daily by trained personnel. No overt symptoms of disease were observed in these animals and all efforts were made to minimize suffering. Mice were euthanized via CO₂ inhalation according to American Veterinary Medical Association guidelines at pre-determined endpoints as described in the figure legends. We utilized 6- to 12-week-old C57BL/6J mice that were age and sex-matched for experiments. The number of mice used in each experiment is described in the figure legends. Mice were infected with 1×10^5 CFUs of *B. melitensis* in 200 µL sterile PBS (sPBS) intraperitoneally (i.p.). For coinfections, a 1:1 ratio of WT *B. melitensis* 16M and a chloramphenicol resistant strain (*B. melitensis*Δ*bmeI0265*) was prepared. Following infection, animals were maintained in individually ventilated caging under high-efficiency particulate air-filtered barrier conditions with 12-hour light and dark cycles within animal biosafety level 3 (ABSL-3) facilities at the University of Missouri. Mice were provided food and water ad libitum.

Calculation of tissue bacterial burdens

Animals were euthanized, and spleens, livers, and reproductive tracts (uterus, fallopian tubes, and ovaries) were harvested. Tissues were homogenized mechanically in sPBS [25]. Serial dilutions were performed in triplicate in sPBS and plated onto brucella agar. Plates were incubated for three days at 37°C/5% CO₂, colonies were counted, and the number of CFU/tissue or CFU/mL were calculated. For co-infection experiments, bacterial burdens were determined by plating homogenized spleens or cells on Brucella agar with or without 5 µg/mL chloramphenicol to select for *B. melitensis*Δ*bmeI0265*.

Flow cytometry

Spleens were homogenized and cell suspensions filtered through sterile 40 µm mesh following red blood cell lysis. Fc receptors were blocked in fluorescence-activated cell-sorting (FACS) buffer (2% heat inactivated fetal bovine serum in PBS) before extracellular staining with fluorochrome-conjugated mAbs from eBioscience or Biolegend (San Diego, CA) F4/80 (BM8), Ly-6G (1A8), CD11b (M1/70), and Ly-6C (HK1.4). Cells were then fixed in 4% paraformaldehyde at 4°C overnight before washing and resuspension in FACS buffer. Fluorescence was acquired on a CyAn ADP analyzer (Beckman Coulter, Brea, CA) and FlowJo (Tree Star, Ashland, OR) software was used for analysis. Cells were gated as Macrophages (F4/80⁺), monocytes (CD11b⁺Ly6C^{high}), and neutrophils (CD11b⁺Ly6C^{mid}).

Metabolite quantification by gas chromatography-tandem mass spectrometry (GC-MS)

Nontargeted metabolomics was performed at the University of Missouri Metabolomics Center [26]. ~40 mg of spleen, liver, and reproductive tract (uterus, fallopian tubes, and ovaries) was collected in a final concentration of 80% methanol. Tissues were homogenized and transferred to glass vials and incubated at room temperature with shaking (~140 rpm) for 2 hours. Next, 1.5 mL of CHCl₃ containing 10 µg/mL of docosanol (nonpolar internal standard) was added. The material was then sonicated, vortexed, and incubated at 50°C for 1 h. Then, 1 mL of HPLC grade H₂O containing 25 µg/mL of ribitol (polar phase internal standard) was added, vortexed, and samples were incubated for 1 h at 50°C. Samples were centrifuged at 10°C at 3000x g for 40 minutes to pellet cell debris and separate phases. Upper phase (polar) and lower phase (nonpolar) were individually transferred to new glass tubes and dried in a speed vacuum. Material was stored at -20°C until ready to derivatize. For polar derivatization, samples were resuspended in a solution containing 50 µl of pyridine containing fresh 15 mg/ml methoxyamine-HCl, sonicated, vortexed, and placed in a 50°C oven for 1 h. Samples were allowed to equilibrate to room temperature and then 50 µl of N-methyltrimethylsilyltrifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) (Fisher Scientific) was added. Samples were vortexed, incubated for 1 h at 50°C, centrifuged, and transferred to glass inserts for injection. For nonpolar derivatization, samples were resuspended in 0.8 mL of CHCl₃, 0.5 mL of 1.25 M HCl in MeOH, vortexed and incubated for 4 h at 50°C. Post incubation, 2 mL of hexane was added to the samples, which were vortexed, and the upper layer was transferred to a new autosampler vial to dry. Material was then resuspended by adding 70 µl of pyridine, vortexed, and then 30 µl of MSTFA + 1% TMCS was added prior to incubation for 1 h at 50°C, centrifugation, and transfer to glass inserts for injection. Samples were analyzed using GC-MS on an Agilent 6890 GC coupled to a 5973N MSD mass spectrometer with a scan range from m/z 50 to 650. Separations were performed using a 60 m DB-5MS column (0.25-mm inner diameter, 0.25-mm film thickness; J&W Scientific) and a constant flow of 1.0 mL/min helium gas. Results were interpreted using MetaboAnalyst 5.0 software (<https://www.metaboanalyst.ca/>) [27] with a P value threshold of 0.05.

Macrophage generation, treatments, and infections

Cells were flushed from the femurs and tibias of C57BL/6J mice with sPBS supplemented with 5 µg/mL of gentamicin. Bone marrow-derived macrophages (BMDMs) were generated by cultured in complete medium with glutamine (CM; RPMI 1640, 10% fetal bovine serum [FBS], 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate) containing 30 ng/ml recombinant murine macrophage colony-stimulating factor (M-CSF; Shenandoah Biotechnology, Warwick, PA). After 3 days of culture, cells were washed with 30 mL of pre-warmed sPBS and fresh CM containing 30 ng/ml M-CSF was added to the culture flasks. After 3 days, adherent cells were collected by adding 0.05% trypsin (MilliporeSigma). Cells were plated at 1x10⁶ cells/ml in fresh CM (with or without glutamine) and allowed to adhere. Cells were infected at a multiplicity of infection (MOI) of 100 *B. melitensis* 16M or coinfecting with 1:1 ratio of *B. melitensis* 16M and *B. melitensis* Δ*bmeI0265* each at a MOI of 100. Cells were infected for 4 h, washed with sPBS, and then cultured in CM containing 50 µg/ml gentamicin for 30 minutes. Cells were then washed with sPBS and left to incubate in CM containing 2.5 µg/ml gentamicin for the remainder of the experiment. For GLS inhibition, 10 µM of Telaglenastat (MedChemExpress LLC, Monmouth Junction, NJ) was added to cells 12 h prior to infection [28–30]. For GABAergic modulation, BMDMs were treated with GABA (100 µM) or bicuculline (BIC; 100 µM; GABA receptor antagonist) (Sigma-Aldrich, St. Louis, MO) at the

same time that gentamicin containing media was added to the cells. At 24 h, 48 h, and 72 h post infection, supernatants were collected, and macrophages were washed and then lysed. These lysates were plated on brucella agar or brucella agar supplemented with chloramphenicol as explained above to determine the amount of intracellular *Brucella*. Supernatants were used for quantification of cytokines as described below.

Cytokine quantification

Cell culture supernatants were filtered prior to measurement of cytokines. IL-1 β levels were measured with a mouse IL-1 β ELISA Ready Set Go kit (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions.

Statistical analysis

For CFU and cytokines, data are expressed as mean $\pm$ standard deviation (SD). Student's unpaired T-test were used to assess differences in means between two groups with significance at $p < 0.05$, while ANOVA followed by Tukey's test with significance at $p < 0.05$ was used for comparisons between ≥ 3 groups. N values and the number of experimental repeats are provided in the figure legends. All statistical analyses were performed with Prism software (version 10.1.1, GraphPad).

Multivariate statistical analysis for metabolite data was performed with Metaboanalyst (v5.0) (<https://www.metaboanalyst.ca>). The normalized values were used for statistical analyses such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), heatmaps, and volcano plots after log transformation and autoscaling with Metaboanalyst software. Pathway analysis was also performed in Metaboanalyst (V5.0) using KEGG database to identify the biological significance of metabolic pathways associated with infection. Pathways with $P < 0.05$ were plotted indicating potential biological relevance.

Results

Brucellosis progression in mice and tissue metabolism variations during different phases of infection

We investigated changes in tissue metabolite levels during progression of intraperitoneal *Brucella* infection in the spleens, livers, and female reproductive tracts of C57BL/6J mice. Bacterial loads peaked at day seven post-infection in all three tissues (Fig 1A–1C). At 14- and 28-days post-infection (dpi) CFU levels in spleens had a ~ 5 -10-fold reduction compared to 7 dpi (Fig 1A). Similarly, in livers, there was a ~ 100 -fold reduction in CFUs between the 14 and 28 day timepoints relative to day 7 post-infection (Fig 1B). In reproductive tracts, CFU counts were ~ 10 -fold lower at 14 dpi and ~ 100 -fold lower at 28 dpi compared to 7 dpi (Fig 1C).

Next, we analyzed the levels of innate immune cells in the spleen during infection. At 7 and 14 dpi the proportions of macrophages, monocytes, and neutrophils were significantly higher compared to naïve mice (Fig 1D). At 14 dpi, neutrophil proportions were significantly increased compared to all other time points indicating that two weeks post-infection may be the peak of splenic inflammation in this model.

Based on these findings, we chose four time points to investigate metabolic changes during experimental brucellosis, pre-infection (naïve), 7 dpi representing the peak of bacterial loads, 14 dpi as the peak of inflammation, and 28 dpi as a starting point of chronic infection with some resolution of inflammation.

To investigate metabolic diversity within spleens, livers, and female reproductive tracts we extracted polar metabolites, which were then derivatized and analyzed via GC-MS. The

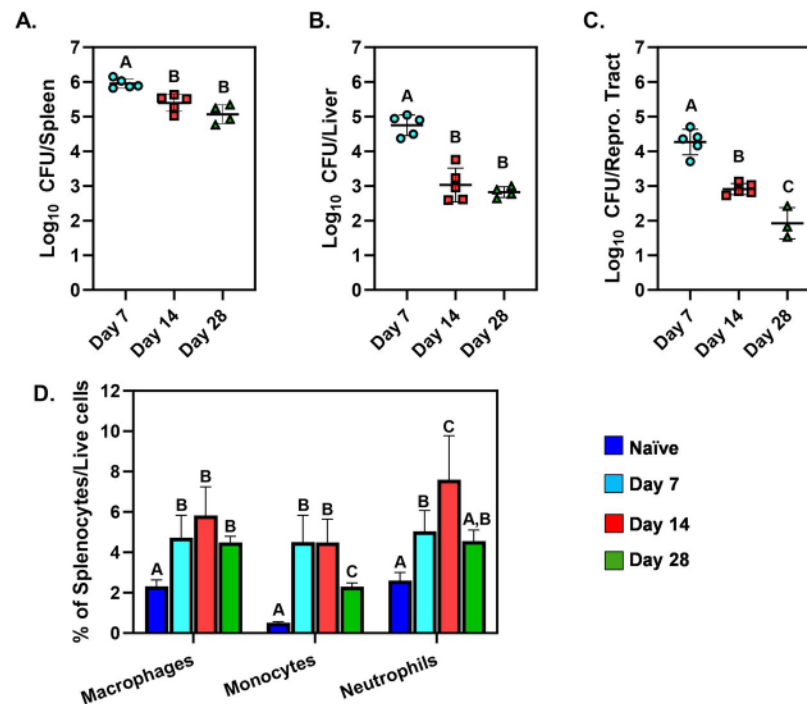


Fig 1. Brucellosis progression in mice. Spleens, livers, and reproductive tracts were harvested from naïve C57BL/6J mice or from C57BL/6J mice at 7-, 14-, and 28-days after i.p. infection with 10^5 CFUs of *B. melitensis* ($n = 4-6$ /group). Bacterial levels were determined in spleens (A), livers (B), and reproductive tracts (C). Flow cytometry (D) was also performed to determine the percentages of macrophages (F4/80⁺), monocytes (CD11b⁺Ly6C^{high}) and neutrophils (CD11b⁺Ly6C^{mid}) amongst splenocytes. Error bars depict S.D. of the mean. Means with the same letter are not statistically different from each other ($P < 0.05$ by ANOVA). Data are from one experiment.

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clusters were plotted by Partial Least-Squares Discriminant Analysis (PLS-DA) (Fig 2A–2C). PLS-DA is a supervised data reduction method influencing the dimension in a graph representation that incorporates group (timepoint) information [27]. This approach was selected to investigate how infection alters metabolic shifts associated with specific time-related changes, emphasizing the variation between groups relative to intra-group differences. All three tissues demonstrated altered metabolic profiles when comparing infected animals with naïve mice. The total number of compounds showing significant changes in spleens, livers, and reproductive tracts were 117, 30, and 32, respectively (S2–S4 Tables).

Heat map visualization showed a distinct splenic metabolite profile separating uninfected from infected animals (Fig 2D), though one sample from the day 28 timepoint displayed variable metabolite levels relative to the other samples within its group. The most remarkable changes in relative levels of compounds appeared to be between naïve and 14 dpi mice. In particular, we found elevated levels of aspartate, succinate, and lactate at 14 dpi in spleens (Fig 2E). These findings were of interest as lactate is the end product of glycolysis and therefore used as a marker for this pathway [18, 31]. Lactate can also serve carbon source for, and promote the growth of, *B. abortus* within macrophages [32]. Aspartate is associated with the urea cycle [33], and can contribute to the growth of some *Brucella* strains [34], while succinate was recently shown to be linked with HIF-1 α stabilization and activation, which facilitates the metabolic shift from mitochondrial phosphorylation (OXPHOS) to glycolysis in several settings including *Brucella* infection [19, 35, 36]. Collectively, these findings from metabolite screening suggest a *Brucella*-driven change in tissue metabolism.

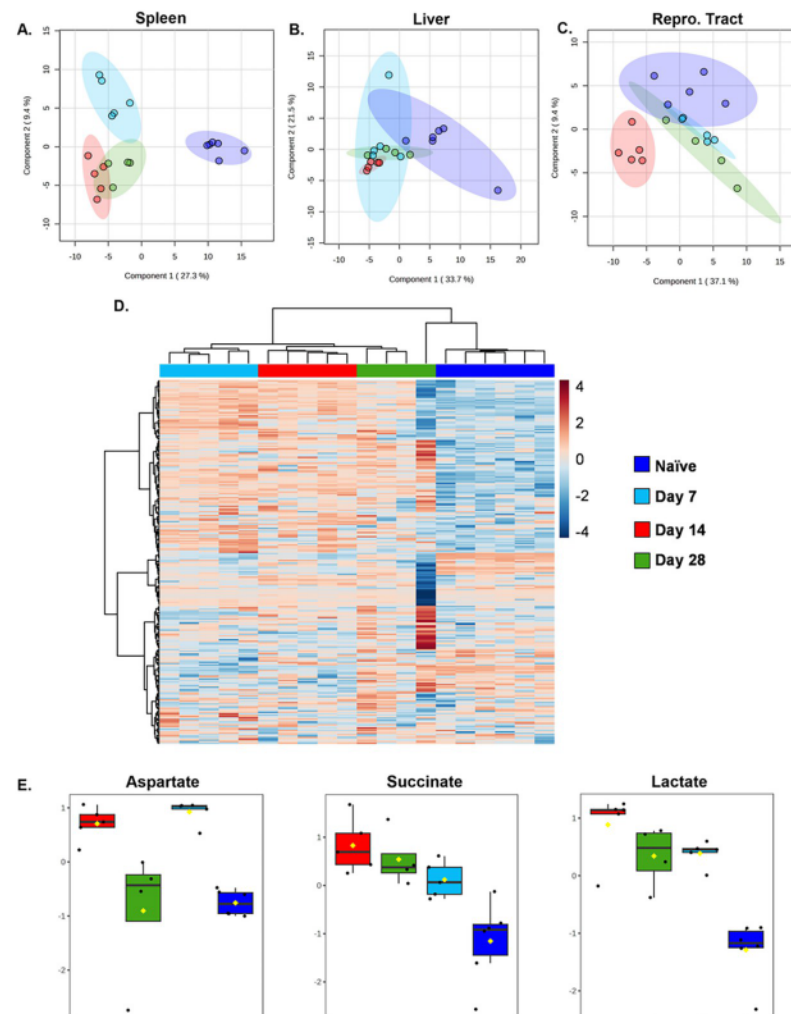


Fig 2. Tissue metabolism variations during different phases of *Brucella melitensis* infection using GC-MS analysis. C57BL/6J mice (WT, $n = 4-6$ mice/group) were infected i.p. with 10^5 CFUs of *B. melitensis*. Naïve mice (dark blue) were included as controls. Spleens, livers, and reproductive tracts were harvested at 7- (light blue), 14- (red), and 28-days (green) post-infection (dpi). Tissues were derivatized and analyzed via GC-MS. Score plot of Partial Least Square-Discriminant Analysis (PLS-DA) relative to time post-infection in spleens (A), livers (B), and female reproductive tracts (C). In A-C component 1 (X-axis) and component 2 (Y-axis) refer to the first and second latent variables generated during the analysis. Higher percentages mean that these components capture more of the data's relevant information. Component 1 captures the largest, and component 2 captures the second-largest difference in the data, respectively. Metabolite heatmap representation in spleen from tissues of naïve animals and from mice infected with *Brucella* for 7, 14 or 28 days (D). Box plots for select metabolites elevated at 14 dpi vs. naïve mice determined by one-way ANOVA with Fisher's LSD post hoc. The Y axis is Log_{10} values of normalized instrument response for the select metabolites (aspartate, succinate, and lactate). (E). Data are from one experiment.

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Changes in tissue metabolism at the peak of *Brucella*-induced inflammation

To confirm that the metabolic profile of spleens, livers, and reproductive tracts are altered by *Brucella* infection at the peak of inflammation, we performed another metabolomic experiment on tissues from additional naïve and 14 dpi animals. In this experiment, we employed a Principal Component Analysis (PCA) which is an unsupervised technique that analyzes each

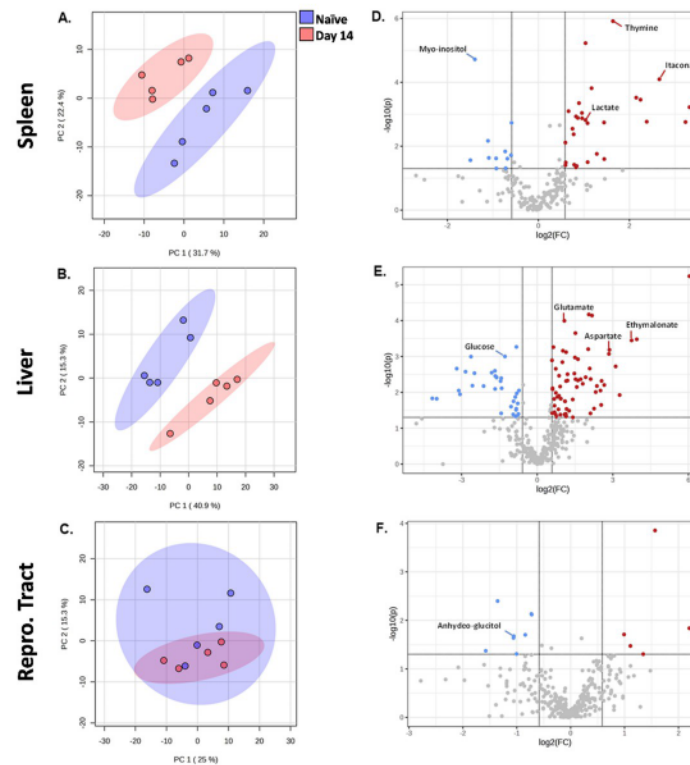


Fig 3. Global analysis of tissue metabolic profiles at the peak of inflammation after *Brucella melitensis* challenge. C57BL/6J mice (WT, $n = 5$ mice/group) were infected i.p. with 10^5 CFUs of *B. melitensis*. Principal Component Analysis (PCA) plot showing metabolic profiles at 14 dpi and in naïve mice, in spleens (A) livers (B), and female reproductive tracts (C). In (A–C) component 1 (X-axis) and component 2 (Y-axis) refer to the first and second latent variables generated during the analysis. Higher percentages mean that these components capture more of the data's relevant information. Component 1 captures the largest, and component 2 captures the second-largest difference in the data, respectively. Volcano plot demonstrating down- (blue) and up-regulated (red) metabolites at 14 dpi compared with uninfected samples in spleens (D), livers (E), and female reproductive tract (F). For each metabolite, the $\log_2$ fold change (threshold 1.5) is displayed on the x-axis and the $-\log_{10}$ (P-value) is displayed on the y-axis. $P < 0.05$. Data are from one experiment.

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sample independently, emphasizing the largest variation between individuals [27]. This methodology was selected to detect general metabolic patterns and visualize the global variation in the dataset of each tissue. Via PCA, metabolite profiles in spleens and livers were again distinct in naïve and infected animals, clustering separately in the Principal Component Analysis (PCA) (Fig 3A and 3B and S5 and S6 Tables). However, reproductive tracts did not show defined separation (Fig 3C and S7 Table) which is in contrast to our findings in Fig 2. This difference could be because we employed an unsupervised approach here (PCA) while in Fig 2 we utilized a PLS-DA which incorporates group level information into the analysis.

We performed volcano tests combining fold change analysis (1.5 threshold) and T-test ($P < 0.05$) to determine metabolites modified by infection (Fig 3D–3F). In spleens 11 compounds were lower in infected compared to naïve tissues, including myo-inositol (Fig 3D). Twenty-nine compounds were significantly higher in infected spleens, including lactate, aspartate, itaconate, malate, and glutamate, suggesting an increase in the level of the TCA cycle intermediates in infected spleens (Fig 4A). Livers from infected mice were found to have 34 compounds with lower relative levels and 67 compounds with significantly higher levels (Fig

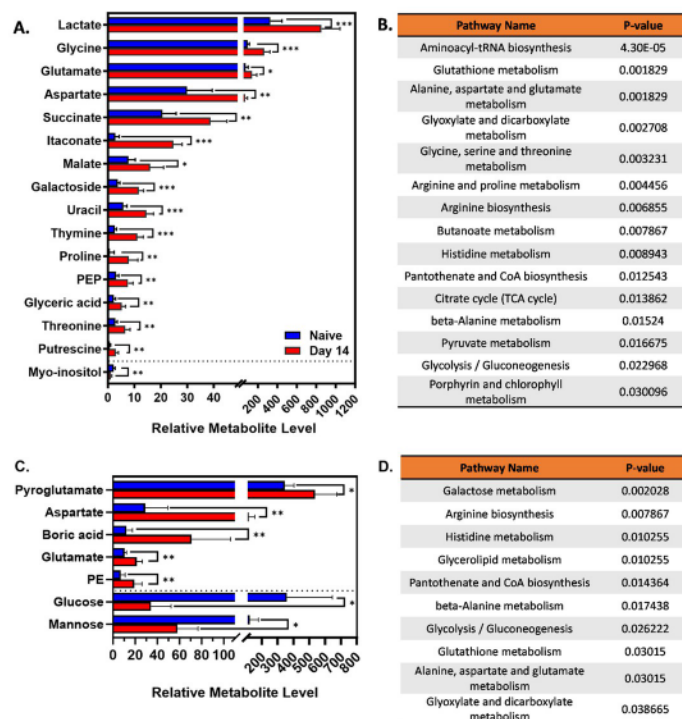


Fig 4. Peak of inflammation is associated with remarkable changes in tissue metabolic pathways. C57BL/6J mice (WT, n = 5 mice/group) were infected i.p. with 10^5 CFUs of *B. melitensis*. Relative metabolite levels and metabolic pathway analyses between 14 dpi and naïve mice in spleens (A, B) and livers (C, D) by GC-MS. Error bars stand for S.D. of the mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ via T test. Data are from one experiment, n = 5 mice/group.

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3B and 3E). Infected livers showed elevated pyroglutamate, aspartate, boric acid, and glutamate, among other metabolites (Fig 4C). Five compounds were elevated, and 8 compounds were reduced in reproductive tracts from infected mice. Of these compounds, only 1,5-anhydroglucitol was identifiable (Fig 3F).

In addition, we performed a metabolic pathway analysis in spleens and livers targeting potential cellular signaling and metabolic networks that could play a role in *Brucella* infection. Several identified pathways modified by infection were linked to the TCA cycle, including glutaminolysis, the arginosuccinate shunt, glycolysis, and the GABA shunt (Fig 4B and 4D). These data demonstrate a change in host metabolism concurrent with the peak of inflammation during infection. Of the identified pathways, there were some that might be involved with the cellular immune response against *Brucella*, including glutaminolysis and glycolysis.

Inhibition of the glutaminolysis pathway dampens IL-1 β production in response to *Brucella*

Our metabolomics data indicated alteration of the glutaminolysis pathway in response to *Brucella* infection. Glutamine is a vital compound in cellular metabolism and is involved in many functions, ranging from protein biosynthesis to mitochondrial respiration [37]. Glutaminolysis is one of the means responsible for replenishing the TCA cycle via the breakdown of glutamine into glutamate. The key enzyme in that process is glutaminase (GLS). These reactions replenish the TCA cycle leading to the production of itaconate and succinate, which subsequently promotes a similar effect as the Warburg effect (aerobic glycolysis) in cancer cells [36].

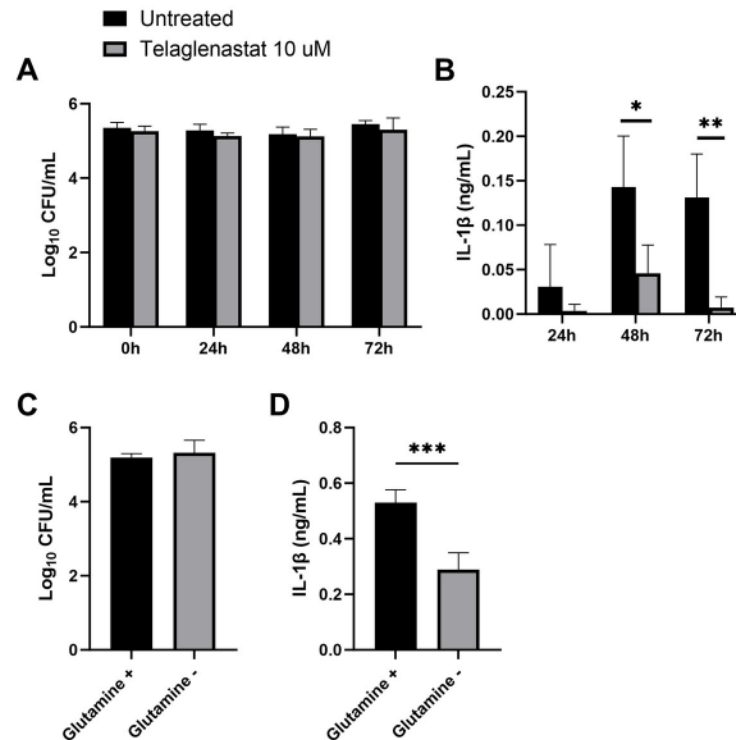


Fig 5. GLS inhibition decreases IL-1 β secretion *in vitro*. Macrophages from C57BL/6J mice were infected with *B. melitensis* 16M at an MOI of 100, and at 0, 24, 48 and 72 hours post infection, intracellular CFU levels were determined (A) and IL-1 β was measured in supernatants via ELISA (B). Macrophages were infected with *B. melitensis* 16M at an MOI of 100 and cultured in media with or without glutamine supplementation, and intracellular bacteria were quantified (C) and IL-1 β was measured in supernatants (D) 72 hours after infection. Error bars depict SD of the mean, n = 4 wells/group. Data are representative of 2 independent experiments. * P<0.05; ** P<0.01; *** P<0.001 compared to WT macrophages via T test.

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Therefore, we investigated the role of glutamine catabolism in BMDMs infected with *B. melitensis*.

To chemically inhibit the glutaminolysis pathway, we treated BMDMs with the GLS inhibitor CB-839, commercially known as Telaglenastat. GLS inhibition did not significantly affect the ability of macrophages to control intracellular *Brucella* infection at 24, 48, and 72 hours (Fig 5A). However, Telaglenastat treatment dampened IL-1 β secretion at 48 and 72 hours after infection (Fig 5B). Glutamine-dependent anaplerosis is the largest source for succinate as a metabolite which in turn enhances IL-1 β production [35]. As glutamine is required for glutaminolysis and subsequent anaplerosis of the TCA cycle, we infected BMDMs with *B. melitensis* in complete media with and without glutamine. Despite bacterial clearance not being affected by glutamine (Fig 5C), IL-1 β secretion was dampened in the absence of glutamine 72 hours after infection (Fig 5D). Collectively, these data demonstrate that glutaminolysis plays a role in IL-1 β production but does not contribute to control of *Brucella* infection in BMDMs.

GABA supplementation does not alter control of *Brucella* by macrophages

Glutamate is also the precursor of GABA, an inhibitory neurotransmitter with the potential to enhance antimicrobial defenses against intracellular bacteria [38, 39]. To investigate if the host GABAergic system controls intracellular *B. melitensis* we treated infected BMDMs with GABA

(100 μ M) or bicuculline (BIC; 100 μ M; GABA receptor antagonist) (Fig 6A). No difference was observed between treatment groups at 48 hours.

Our *in vivo* metabolomics data indicated that the GABA shunt was altered in mouse spleens at two-weeks post-infection (Fig 4A and 4B). *B. abortus* has been previously shown to encode two GABA transporters, *bab1_1794* and *bab2_0879*, with moderate and high GABA import rates, respectively, but these GABA transporters were not required for virulence [40]. There are other genes within the *Brucella* genome that are annotated to encode putative GABA transporters including *B. melitensis* BMEI0265. We therefore generated an isogenic BMEI0265 mutant (*B. melitensis* Δ bmei0265). However, we did not find *B. melitensis* Δ bmei0265 to be attenuated in mouse spleens two weeks post-infection (Fig 6B). These findings suggest that *bmei0265* does not likely play a role in *B. melitensis* virulence.

Discussion

Over the past decade, immunometabolism studies have become key to understanding how metabolism of host cells impacts the outcome of infection [41]. In the context of *Brucella* infection, studies have indicated that modulation of glycolysis can affect intracellular replication and inflammation [32, 42], while enhanced glucose availability within M2 macrophages can promote *Brucella* replication [20].

Here, we investigated the interface of host-*Brucella* interactions by first screening tissue metabolic fluctuations during the course of infection. We found that infection caused more consistent separation of metabolite profiles from spleen and liver relative to reproductive tracts from non-pregnant mice (Figs 2 and 3). This could be due to the absence of estrus synchronization before the experiment. In addition, while *Brucella* can infect the reproductive tract of both pregnant, and non-pregnant mice [43, 44], pregnancy can alter the immune response [45] which could in turn affect metabolite availability. Therefore, investigating the metabolite profiles of reproductive tracts from pregnant mice infected with *Brucella* should be studied in the future.

Untargeted GC-MS showed a *Brucella*-driven change in tissue metabolism at 14 dpi, particularly in metabolites correlated with the TCA cycle. Mitochondrial changes in metabolites related to the TCA cycle can regulate the function and activation of immune cells [46, 47]. We have

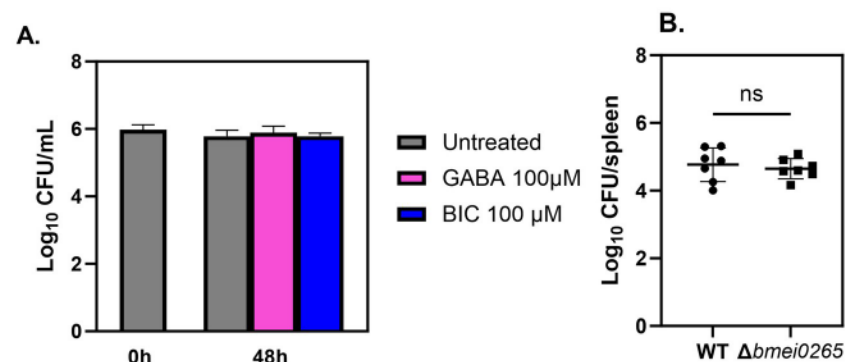


Fig 6. GABAergic system does not play a role in controlling *B. melitensis* 16M in BMDMs. (A) Macrophages from C57BL/6J mice ($n = 4$ wells/group) were infected with *B. melitensis* 16M at an MOI of 100. After infection, cells were treated with GABA (100 μ M) or bicuculline (BIC; 100 μ M; GABA_AR antagonist) and intracellular CFUs were determined at 0, 48 and 72 hours post-infection. (B) C57BL/6J mice ($n = 7$) were challenged i.p. with a 1:1 ratio of 10^5 *B. melitensis* 16M and *B. melitensis* Δ bmei0265, and relative splenic CFU levels were determined fourteen days post-infection. Error bars depict SD of the mean. Data in (A) are representative of 2 independent experiments while data in (B) are combined from two experiments.

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previously demonstrated the impact of itaconate, an intermediate metabolite from the TCA cycle, on mouse susceptibility to *Brucella* [18]. In the present study, we found itaconate levels to be higher in spleens from infected mice (Fig 4A). In line with these results, we suggest that TCA cycle-correlated metabolites have an impact on efficient immune responses against *B. melitensis*.

Metabolic pathway analysis displayed a link between infection and several pathways such as glutaminolysis, the arginosuccinate shunt, glycolysis, and the GABA shunt. Among the metabolites showing key changes, glutamate levels were significantly increased in infected livers compared to uninfected controls. Glutamate plays a role in macrophage polarization, replenishment of the TCA cycle via glutaminolysis, and in the GABA shunt at the mitochondria level [17, 37, 46, 48]. Glutaminolysis has been found to be critical in the metabolic reprogramming of M1-like human macrophages infected with *M. tuberculosis*, demonstrating its importance in the proinflammatory response [28]. Similarly, we found that glutaminolysis inhibition with telaglenastat impairs secretion of the pro-inflammatory IL-1 β cytokine (Fig 5B). It is established that IL-1 is crucial to control *Brucella* infection in mice [49, 50]. However, while GLS inhibition dampened IL-1 β production, we found that GLS inhibition did not affect the ability of BMDMs to control intracellular *B. melitensis* (Fig 5A). Conversely, a study using a telaglenastat analog (BPTES) reported increased CFUs in a macrophage cell line infected with *B. abortus* [51]. The divergent results could be due to differences between the two inhibitors, the types of macrophages, and the strains of *Brucella*.

Furthermore, glutamate is also involved in the GABA shunt, a process responsible for producing and conserving the supply of gamma-aminobutyric acid. GABA is an inhibitory neurotransmitter recently found to impact the host immune system [38, 39, 52]. Studies performed with *M. tuberculosis*, *Salmonella* Typhimurium, and *Listeria monocytogenes* demonstrated that GABAergic system activation by GABA or its receptor agonist enhances macrophage antimicrobial defense against these intracellular bacteria [53]. However, our results suggested that the GABAergic system does not play a role in controlling *B. melitensis* intracellularly.

The conversion of glutamate into GABA is catalyzed by the enzyme glutamate decarboxylase (GAD), which provides a pH homeostasis mechanism in some pathogenic bacteria, including *L. monocytogenes* [54]. Several *Brucella* species have a functional GAD system; however, the system is lost in host-adapted pathogens such as *B. melitensis*, *B. abortus*, and *B. suis* [40]. Nonetheless, *Brucella* has a GABA transporter with an undefined role in the metabolic utilization of GABA which may play a role in the pathogenesis of *Brucella* infection [37]. According to our results, the potential transport of GABA transport by BMEI0265 does not play a role in *B. melitensis* virulence under the conditions tested here. However, because *Brucella* encodes multiple GABA transporters, the effect of deleting a single transporter could be masked by the presence of transporters with redundant function. Therefore, more studies are needed to understand the mechanisms of GABA signaling on host immune defense against *Brucella*.

In conclusion, we show here that metabolite screening of spleens, livers, and reproductive tracts suggested a *Brucella*-driven change in tissue metabolism, with the most remarkable changes in host metabolism occurring at the peak of inflammation around two weeks after *B. melitensis* infection. Additionally, metabolite changes were related to intracellular pathways linked to mitochondria mechanisms. Between these candidate pathways, glutaminolysis was demonstrated to play a role in IL-1 β production but did not contribute to macrophage control of *Brucella* infection *in vitro*.

Supporting information

S1 Table. Primers used in this study.
(DOCX)

S2 Table. Metabolite levels in spleens from naïve mice, and from spleens at 7, 14, and 28 days post-infection with *B. melitensis*.
(CSV)

S3 Table. Metabolite levels in livers from naïve mice, and from livers at 7, 14, and 28 days post-infection with *B. melitensis*.
(CSV)

S4 Table. Metabolite levels in reproductive tracts from naïve female mice, and from reproductive tracts at 7, 14, and 28 days post-infection with *B. melitensis*.
(CSV)

S5 Table. Metabolite levels in spleens from naïve mice, and from spleens at 7 days post-infection with *B. melitensis*.
(CSV)

S6 Table. Metabolite levels in livers from naïve mice, and from livers at 7 28 days post-infection with *B. melitensis*.
(CSV)

S7 Table. Metabolite levels in reproductive tracts from naïve female mice, and from reproductive tracts at 7 days post-infection with *B. melitensis*.
(CSV)

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