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Acetate, a short-chain fatty acid, inhibits *Coxiella burnetii* replication via direct bacteriostatic effect and through vacuolar modulation and host cell perturbations

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Abstract

Background

Coxiella burnetii, the etiologic agent of Q fever, is an obligate intracellular pathogen that replicates within a specialized acidic compartment termed the *Coxiella*-containing vacuole (CCV). Current treatment options for chronic Q fever require prolonged antibiotic therapy and are limited by toxicity and reduced efficacy within the acidic intracellular niche. Short-chain fatty acids (SCFAs) are host- and microbiota-derived metabolites with documented immunomodulatory and antimicrobial properties; however, their effects on *C. burnetii* remain unknown. We investigated whether acetate, the most abundant SCFA in human body, influences *C. burnetii* growth and CCV biology.

27 *Methods*

28 *C. burnetii* axenic and intracellular growth was measured by colony forming unit (CFU) assay. Acetate
29 MIC and MBC were determined using standard broth microdilution coupled with CFU assays. Confocal
30 microscopy followed by quantitative measurements were used to determine the CCV expansion and CCV
31 luminal pH. Uninfected and *C. burnetii* infected HeLa cells were processed and sent for RNAseq to
32 Plasmidsaurus®.

33

34 *Results*

35 Acetate significantly inhibited *C. burnetii* replication in axenic ACCM-2 medium, completely preventing
36 bacterial growth at concentrations of 20 - 80 mM. Broth microdilution assays identified a minimum
37 inhibitory concentration (MIC) of 1.25 mM and a minimum bactericidal concentration (MBC) of 5 mM,
38 demonstrating marked bacterial sensitivity to acetate. In infected HeLa cells, 24 h acetate treatment
39 reduced intracellular bacterial burden by approximately 50% and markedly impaired CCV expansion,
40 decreasing vacuolar size by 61–67% relative to control cells. Further, acetate elevated CCV luminal pH
41 from approximately 5.2 to 5.5–5.7, indicating reduced vacuolar acidity, a condition known to be
42 unfavorable for *C. burnetii* replication. Transcriptomic analysis revealed extensive host-cell
43 reprogramming following acetate treatment, characterized by altered expression of genes involved in lipid
44 metabolism, membrane trafficking, stress responses, apoptosis, complement activation, and
45 inflammatory signaling pathways.

46

47 *Conclusions*

48 Acetate restricts *C. burnetii* through multiple mechanisms, including direct antibacterial activity, disruption
49 of CCV maturation and acidification, and broad modulation of host-cell transcriptional programs. These
50 findings identify acetate as a previously unrecognized inhibitor of *C. burnetii* and highlight metabolic
51 perturbation of the intracellular niche as a potential vulnerability of this pathogen. More broadly, our

52 results demonstrate that host-derived metabolites can profoundly influence obligate intracellular bacterial
53 survival and pathogenesis.

54 **Background**

55 *Coxiella burnetii* is an unusual gram-negative bacterium that causes a lesser-known zoonotic human
56 disease called Q fever. In humans, acute Q fever typically presents as a self-limiting flu-like illness,
57 pneumonia, or hepatitis, whereas a subset of infections progress to chronic disease, most commonly
58 manifesting as endocarditis or vascular infection [1]. The treatment plan for chronic Q fever is an 18-
59 month-long combined antibiotic therapy, and currently, there is no licensed Q fever vaccine in most
60 countries. As a result, Q fever has emerged as a significant public health concern worldwide, with an
61 increasing number of incidents every year [2-10].

62 *C. burnetii* transmits via the respiratory route, and upon entry into the lower respiratory tract, the
63 bacteria infect alveolar macrophages within the lung parenchyma [11, 12], and establishes a unique
64 phagolysosomal-like replication compartment termed *Coxiella*-containing vacuole (CCV) [13]. Unlike
65 many intracellular pathogens that avoid lysosomal maturation, *C. burnetii* actively requires an acidic
66 vacuolar environment to initiate metabolism, activate its Dot/Icm type IVB secretion system, and support
67 intracellular replication. Perturbations of CCV maturation or luminal pH are therefore detrimental to
68 bacterial survival, rendering the intracellular niche itself an attractive therapeutic target. One of the
69 challenges of treating chronic *C. burnetii* infection with antibiotics like doxycycline is that the acidic
70 environment of the CCV reduces drug activity, thereby diminishing efficacy and necessitating prolonged
71 treatment (18-24 months) in combination with hydroxychloroquine [14, 15]. Moreover, doxycycline is
72 contraindicated in pregnant women and children [16], and long-term use of the drug may cause
73 gastrointestinal disturbances and antibiotic resistance [17]. Therefore, although treatment options exist,
74 the prolonged duration, toxicity concerns, and limited bactericidal efficacy of current regimens underscore
75 the need for improved strategies to better understand and disrupt *C. burnetii* intracellular survival.

76 Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are microbial
77 fermentation products abundant in the intestinal tract and detectable systemically at lower concentrations

78 [18, 19]. Beyond their established roles in regulating host immunity and metabolism, SCFAs have been
79 reported to influence a wide range of infectious processes, either through direct antimicrobial activity or
80 through indirect modulation of host cellular responses [18, 20-23]. Several studies suggest that SCFAs
81 can shape immune response and restrict pathogen growth at mucosal and distal sites, including the lung
82 [18]. However, these effects are highly context-dependent and vary according to pathogen, SCFA
83 species, concentration, and cellular environment. Importantly, how SCFAs influence obligate intracellular
84 bacterial pathogens that rely on acidic lysosomal compartments, such as *C. burnetii*, remains largely
85 unexplored.

86 Given *C. burnetii*'s dependence on host cell metabolism and vacuolar physiology, we
87 hypothesized that SCFAs may interfere with the bacterial intracellular replication by perturbing either
88 bacterial physiology directly or the host cellular environment required for CCV maintenance. In this study,
89 we first assessed the impact of acetate, propionate, and butyrate on CCV expansion. We then focused
90 on acetate to define its effects on intracellular replication, axenic growth, CCV luminal pH, and host
91 transcriptional responses. Rather than positioning SCFAs as immediate therapeutic agents, our goal was
92 to evaluate whether metabolite-driven perturbations can reveal vulnerabilities in *C. burnetii* intracellular
93 biology and identify pathways through which host-derived metabolites influence obligate intracellular
94 pathogens.

95

96 **Materials and Methods**

97 **Bacteria, mammalian cells, and acetate stock solution**

98 *Coxiella burnetii* Nine Mile Phase II (NMII) (clone 4, RSA 439) and mCherry-expressing wild-type (WT)
99 *C. burnetii* [24] were grown for 5 days in acidified citrate cysteine medium-2 (ACCM-2; Cat# 4700-300
100 Sunrise Science Products, Knoxville, TN) [25], washed twice with phosphate-buffered saline (PBS), and
101 stored as previously described [26]. The genome equivalents (GE) for the *C. burnetii* stocks were
102 determined by qPCR using primers for the *Coxiella burnetii* *dotA* gene [27]. The multiplicity of infection
103 (MOI) of each bacterial stock was optimized for mammalian cells for each culture vessel to ~1 internalized

104 bacterium per cell at 37°C and 5% CO₂. Human cervical epithelial cells (HeLa, ATCC CCL-2) were
105 maintained in RPMI (Roswell Park Memorial Institute) 1640 medium with glutamine (Cat. 10-040-CV,
106 Corning, Manassas, VA, USA) containing 10% fetal bovine serum (referred to as 10% RPMI hereafter)
107 (FBS; Cat. A5669701, Gibco, Grand Island, NY, USA) at 37°C and 5% CO₂. Sodium acetate (Cat.
108 A13184, Thermo-Fisher Scientific, Waltham, MA) was dissolved in tissue culture-grade PBS to a final
109 concentration of 2 M. Filter-sterilized aliquots were frozen at -80°C until further use.

110

111 ***C. burnetii* axenic growth measurement**

112 *C. burnetii* axenic growth was measured by culturing in ACCM-2, followed by counting viable colonies in
113 ACCM-2-agarose plates as described previously [28, 29]. Tissue culture flasks containing 5 mL ACCM-2
114 were treated either with PBS (vehicle control) or with 50 or 80 mM acetate. The media pH was measured
115 after adding the treatment conditions to ensure no significant change in the media acidity. The flasks were
116 immediately inoculated with ~ 4×10⁴ bacteria and shaken vigorously. To enumerate the number of viable
117 bacteria at day 0 (the day of inoculation), 100 µL of culture from each flask was serially diluted in ACCM-
118 2, and 10 µL of each dilution was spotted in triplicate on 0.25% ACCM-2 agarose plates. The cultures
119 were processed similarly on day 2 and day 4 to enumerate *C. burnetii* colony-forming units on the
120 indicated time points. The plates were incubated for 6–7 days at 37 °C in 2.5% O₂ and 5% CO₂ in a tri-
121 gas incubator, and the viable colonies were counted to measure bacterial viability. The statistical
122 difference in bacterial growth between PBS, 50 mM, and 80 mM acetate at day 4 was measured using
123 one-way ANOVA with multiple comparisons. Three biologically independent experiments (N=3) were
124 performed, each with technical duplicates for each condition.

125

126 **Determination of Acetate MIC and MBC for *C. burnetii***

127 The minimum inhibitory concentration (MIC) for acetate against *C. burnetii* was determined using the
128 standard broth microdilution assay, as described previously [30], with modifications to adapt it for *C.*
129 *burnetii*. ACCM-2 broth containing 80 mM acetate was diluted 2-fold serially in a 96-well plate (300 µL

per well) to establish a dilution series from 80 mM to 0.078 mM acetate in triplicate. PBS-containing ACCM-2 served as the negative control. Additionally, wells without bacterial inoculum were included at all concentrations of acetate to account for any background signal. To prepare the *C. burnetii* inoculum, 5 μ L of WT *C. burnetii* stock ($GE \approx 1.07 \times 10^{10}$ per mL) was diluted 1:20 in 100 μ L of ACCM-2 media, and 2 μ L of the diluted inoculum was added to each well. The MIC plate was incubated for seven days at 37°C in a tri-gas incubator (5% CO₂, 2.5% O₂). At seven days, the absorbance at 600 nm of each well was measured using a BioTek Cytation 3 Reader (BioTek Instruments, Winooski, VT, USA). A₆₀₀ values for each acetate concentration were compared to the baseline A₆₀₀ of the respective no-acetate controls. The MIC was determined as the acetate concentration that significantly inhibited *C. burnetii* growth compared to the next lower concentration. Three biologically independent experiments (N=3) were performed to ensure reproducibility and consistency. To determine the MBC, 10 μ L of the media from each well of the MIC plate was spotted onto ACCM-2-agarose plates and incubated in the tri-gas incubator for 6-7 days, at which time the plates were read visually and photographed to record bacterial growth. The minimum concentration that killed all bacterial cells was defined as the acetate MBC for *C. burnetii*.

145

146 **Measurement of CCV expansion**

HeLa cells were plated in a 6-well plate (5×10^4 cells per well) and allowed to adhere overnight. The next day, the cells were either mock-infected or infected with mCherry-wild-type *C. burnetii* in 0.5 mL 10% RPMI for 1 h, washed extensively with PBS, and incubated in 10% RPMI. At 2 dpi, the cells were trypsinized, pooled, and resuspended at a final concentration of 1×10^5 cells/mL. 0.5 mL of the cell suspension ($\sim 5 \times 10^4$ cells) was distributed into three microcentrifuge tubes for each of the mock and the *C. burnetii*-infected groups. The tubes were then centrifuged at 1000 $\times$ g for 5 min, and the media was aspirated. The cell pellets were resuspended in 0.5 mL of 10% RPMI containing either PBS or 50 mM or 80 mM acetate and replated onto coverslips placed in a 24-well plate ($\sim 5 \times 10^4$ cells per coverslip). At 3 dpi, cells were fixed in 4% paraformaldehyde (Cat. I28800 Thermo-Fisher Scientific, Rockford, IL, USA)

for 15 min and blocked/permeabilized for 20 min in 1% bovine serum albumin (BSA) and 0.1% saponin in PBS. Cells were then incubated in rabbit anti-LAMP1 (1:1000; Cat. PA1-654A, Thermo-Fisher Scientific) for 1h, followed by Anti-rabbit Alexa Fluor 488 secondary antibody (1:1000, Cat. A11034, Thermo-Fisher Scientific) for 1 h. Following washing with PBS, coverslips were mounted using ProLong Gold with 4',6-diamidino-2-phenylindole (DAPI; Cat. P36983, Life Technologies, Eugene, OR) and visualized on a Keyence BZ-X700 fluorescent microscope using a 63x oil immersion objective. 30-40 CCVs were imaged per condition, and TIFF images were exported along with a microscope-generated scale bar for reference. Images were then imported into ImageJ FIJI [31], where CCVs were detected by LAMP1 localization (green), and the area of each CCV was measured. The statistical difference in CCV areas between PBS, 50 mM, and 80 mM acetate-treated cells was analyzed by one-way ANOVA in GraphPad Prism from three biologically independent experiments (N=3).

***C. burnetii* intracellular growth enumeration**

To quantify *C. burnetii* growth in acetate-treated cells, we also used the CFU assay as described previously [28, 29]. HeLa cells, plated in a 24-well plate, were infected with wild-type (WT) *C. burnetii* in 10% RPMI for 2 h. Upon washing extensively with PBS, cells were scraped into 2 mL fresh 10% RPMI. To determine viable *C. burnetii* at 0 dpi (day of infection), 500 μ L (5×10^4) of infected cells were lysed in sterile water for 5 min. The released bacteria were diluted 1:5 in ACCM-2 and plated in 2-fold serial dilutions onto 0.25% ACCM-2 agarose plates. The remaining cells were split into two groups (labelled Day 2 and Day 3) and replated into a 24-well plate. At 2 dpi, the Day 2 wells were processed for CFU, and Day 3 cells were treated with PBS, 50 mM, or 80 mM acetate. At 3 dpi, after 24 h of acetate treatment, the Day 3 wells were processed for CFU. For the CFU assay, first, the spent media from the culture wells were collected and centrifuged at 20,000xg for 10 min to pellet any previously released bacteria, and supernatants were discarded (tube 1). The cell monolayers were then lysed in sterile water for 5 min, and the released bacteria were diluted 1:5 in ACCM-2 (tube 2). The contents of tubes 1 and 2 were then mixed and spotted in 10-fold serial dilutions onto 0.25% ACCM-2 agarose plates. The plates were

182 incubated for 6–7 days at 37 °C in 2.5% O₂ and 5% CO₂, and the number of colonies was counted to
183 measure bacterial viability. Three biologically independent experiments were performed (N=3), each with
184 technical duplicates for each condition, and the bacteria were spotted in triplicate. Bacterial growth was
185 expressed as fold change in CFU over 0 dpi, and the statistical difference in fold change between PBS,
186 50 mM, and 80 mM acetate conditions was measured using one-way ANOVA with multiple comparisons.

187

188 **CCV pH measurement**

189 The CCV pH from PBS or acetate-treated HeLa cells was measured as described previously [29]. Briefly,
190 HeLa cells were plated in a 6-well plate (5x10⁴ per well) and infected with mCherry-WT *C. burnetii* for 2
191 h. After washing extensively with PBS, cells were incubated in 10% RPMI. At 2 dpi, the cells were
192 trypsinized, pooled, and resuspended at a final concentration of 1x10⁵ cells/mL. 0.5 mL of the cell
193 suspension (~5x10⁴ cells) was distributed into three microcentrifuge tubes. The tubes were then
194 centrifuged at 1000xg for 5 min, and the media were replaced with 10% RPMI containing either PBS or
195 50 or 80 mM acetate. After resuspending the pellets, cells were re-plated onto ibidi-treated channel μ -
196 slide VI0.4 (3x10³ cells per channel; ibidi USA Inc., Verona, WI). The next day (3 dpi), cells were labeled
197 with pH-sensitive Oregon Green 488 dextran (MW, 10,000; Invitrogen, Carlsbad, CA) and pH-stable
198 Alexa Fluor 647 dextran (MW, 10,000; Invitrogen) at a final concentration of 0.5 mg/mL in 10% RPMI for
199 4 h, followed by a 1 h chase to allow for endosomal maturation [29, 32]. After washing with PBS, cells
200 were incubated in 10% RPMI, and individual CCVs were imaged live with a Leica Stellaris (60x oil
201 immersion objective) and with a live-cell setup. Images were captured and processed identically,
202 fluorescence intensity was measured for Oregon Green 488 and Alexa Fluor 647 using ImageJ (Fiji; [31]),
203 and the 488/647 ratio was calculated. To generate a pH standard curve, wild type *C. burnetii*-infected
204 HeLa cells (3 dpi) were incubated in equilibration buffer (143 mM KCl, 5 mM glucose, 1 mM MgCl₂, 1 mM
205 CaCl₂, and 20 mM HEPES) containing ionophores nigericin (10 μ M) and monensin (10 μ M) for 5 min
206 followed by incubation in standard buffers of pH ranging from 4.0 to 7.0 containing ionophores for 5 min
207 before imaging. At least 20 CCVs were measured at each pH, and the 488/647 ratio was plotted against

208 the pH of the respective buffer to obtain a sigmoidal standard curve. The experimental samples were
209 then interpolated to the standard curve to determine the pH; a standard curve was generated for each
210 individual experiment. At least 20 CCVs/cells were measured for each experimental time point per
211 condition for three biologically independent experiments.

212

213 **RNA-seq analysis**

214 The global gene expression changes in acetate-treatment conditions were analyzed by RNA-seq assay.
215 HeLa cells were plated in a 6-well plate (5×10^4 per well) and either mock-infected or infected with WT *C.*
216 *burnetii* for 2 h. After washing extensively with PBS, cells were incubated in 10% RPMI. At 2 dpi, cells
217 were treated with either PBS or 50 mM acetate and incubated in the treatment conditions for 24 h. At 3
218 dpi, cells were harvested, counted, and diluted to 2×10^5 cells/mL. Cells were then centrifuged at 400xg
219 for 5 min, and the pellet was washed once with PBS and centrifuged again. Pellets were then
220 resuspended in 50 μ L Zymo DNA/RNA shield™ (Zymo Research, Irvine, CA, USA) and vortexed
221 thoroughly to lyse the cells and shear the chromosomal DNA. A total of four samples, named Mock-PBS,
222 Mock-acetate, WT-PBS, and WT-acetate, were then sent for RNAseq and analysis to Plasmidsaurus
223 (South San Francisco, CA, USA). Quality of the fastq files was assessed using FastQC v0.12.1. Reads
224 were quality filtered using fastp v0.24.0 with poly-X tail trimming, 3' quality-based tail trimming, a minimum
225 Phred quality score of 15, and a minimum length requirement of 50 bp. Quality-filtered reads were aligned
226 to the reference genome using STAR aligner v2.7.11 with non-canonical splice junction removal and
227 output of unmapped reads, followed by coordinate sorting using samtools v1.22.1. PCR and optical
228 duplicates were removed using UMI-based deduplication with UMIsCollapse v1.1.0. Alignment quality
229 metrics, strand specificity, and read distribution across genomic features were assessed using RSeQC
230 v5.0.4 and Qualimap v2.3, with results aggregated into a comprehensive quality control report using
231 MultiQC v1.32. Gene-level expression quantification was performed using featureCounts (subread
232 package v2.1.1) with strand-specific counting, multi-mapping read fractional assignment, exons, and 3' -
233 UTR as the feature identifiers, and grouped by gene_id. Final gene counts were annotated with gene

234 biotype and other metadata extracted from the reference GTF file. Sample-sample correlations for the
235 sample-sample heatmap and PCA were calculated on normalized counts (TMM, trimmed mean of M-
236 values) using Pearson correlation. Differential expression was done with edgeR v4.0.16 using standard
237 practice, including filtering for low-expressed genes with `edgeR::filterByExpr` with default values.
238 Functional enrichment, when available, is performed using gene set enrichment analysis with gseapy
239 v0.12 using the MSigDB Hallmark gene set.

240

241 **Microscopic imaging and data analyses**

242 Fluorescent and confocal images were processed and analyzed in the ImageJ FIJI software [33]. All
243 statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA). A p-value < 0.05
244 was considered significant. Data are plotted as mean $\pm$ standard error of mean (SEM).

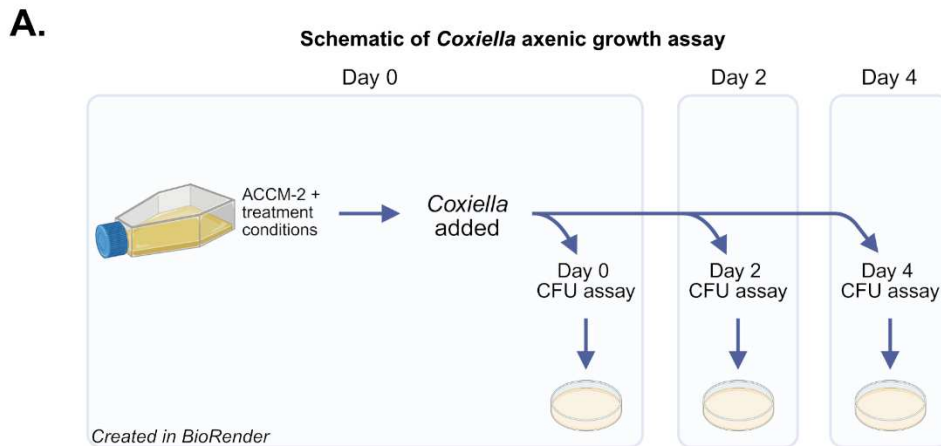
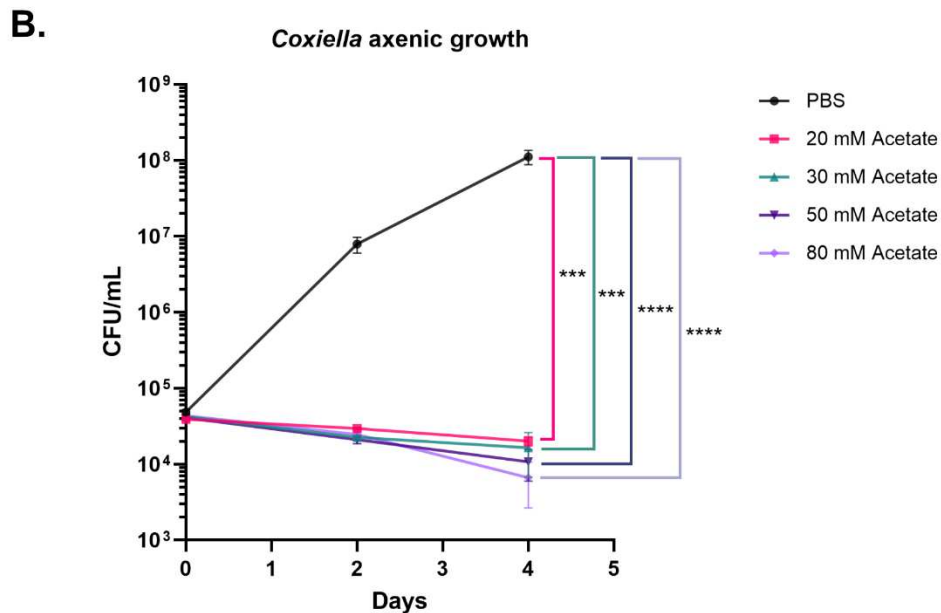
245

246 **Results**

247 **Acetate treatment inhibits *C. burnetii* growth in axenic medium**

248 We first aimed to determine whether acetate treatment directly affects *C. burnetii* growth in a cell-free
249 environment by inoculating acetate-containing ACCM-2 media, followed by our colony-forming unit (CFU)
250 assay described previously [28]. Fig. 1A shows the schematic diagram of the experimental design. Five
251 independent flasks, containing ACCM-2 media, were treated with either PBS or 20, 30, 50, and 80 mM
252 acetate and inoculated with 4×10^4 CFU/mL of *C. burnetii* at day 0 and incubated for 4 days followed by a
253 CFU assay. Our CFU data from each condition revealed that, while in the PBS control condition, *C.*
254 *burnetii* grew to $\sim 7.8 \times 10^6$ CFU/mL at day 2, and to 1.1×10^8 CFU/mL at day 4 post-inoculation (Fig. 1B),
255 in all of the acetate-containing conditions, *C. burnetii* growth remained unchanged or even slightly reduced
256 (Fig. 1B), suggesting a complete attenuation of bacterial growth in the presence of acetate in cell-free
257 medium. One-way ANOVA between PBS and treatment conditions revealed P values < 0.001 for all
258 conditions, suggesting significant attenuation of *C. burnetii* growth. To further analyze the dose-
259 dependent effects of acetate on *C. burnetii*, we next aimed to determine the minimum inhibitory and

260 minimum bactericidal concentrations (MIC and MBC) of acetate for *C. burnetii*. To this end, we performed
261 a standard broth microdilution test with a 2-fold serial dilution of acetate in a 96-well format as described
262 previously [30]. Our data show that although 0.625 mM acetate exerts a significant inhibitory effect, 1.25
263 mM acetate showed a complete inhibition of *C. burnetii* growth, which was also maintained in further
264 higher concentrations (Fig. 2A). Therefore, we determined that the acetate MIC for *C. burnetii* in ACCM-
265 2 medium is 1.25 mM. Bacterial viability data from each of the wells of the broth microdilution plate on
266 ACCM-2 agar revealed a complete absence of *C. burnetii* growth in 5 mM acetate, and therefore this was
267 determined to be the acetate MBC for *C. burnetii*. Therefore, although the acetate MIC is 1.25 mM, the
268 MBC is 5 mM. Taken together, these data strongly suggest a direct negative effect of acetate on *C.*
269 *burnetii* growth in cell-free medium.



270

271 **Figure 1. Acetate directly inhibits *C. burnetii* growth in cell-free axenic medium. (A)** Schematic
 272 diagram of *C. burnetii* axenic growth measurement by CFU assay. T25 flasks with 5 mL ACCM-2 were
 273 treated either with PBS or with 50 or 80 mM acetate and were immediately inoculated with the bacteria.
 274 To enumerate the number of viable bacteria at day 0, 100 μ L of culture from each flask was serially diluted
 275 in ACCM-2, and spotted in triplicate on ACCM-2 agarose plates. The cultures were processed similarly
 276 on day 2 and day 4 to enumerate viable bacteria. The plates were incubated for 6–7 days at 37 °C in
 277 2.5% O₂ and 5% CO₂ in a tri-gas incubator. **(B)** Enumeration of viable *C. burnetii* over time showed
 278 significant attenuation in bacterial growth in acetate-treated conditions compared to PBS. Visually, almost

no turbidity was observed in the flasks with acetate over time. Data shown as mean±SEM from three independent experiments (N=3), each with technical duplicates for each condition, as analyzed by one-way ANOVA; ***, P<0.001; ****, P<0.0001.

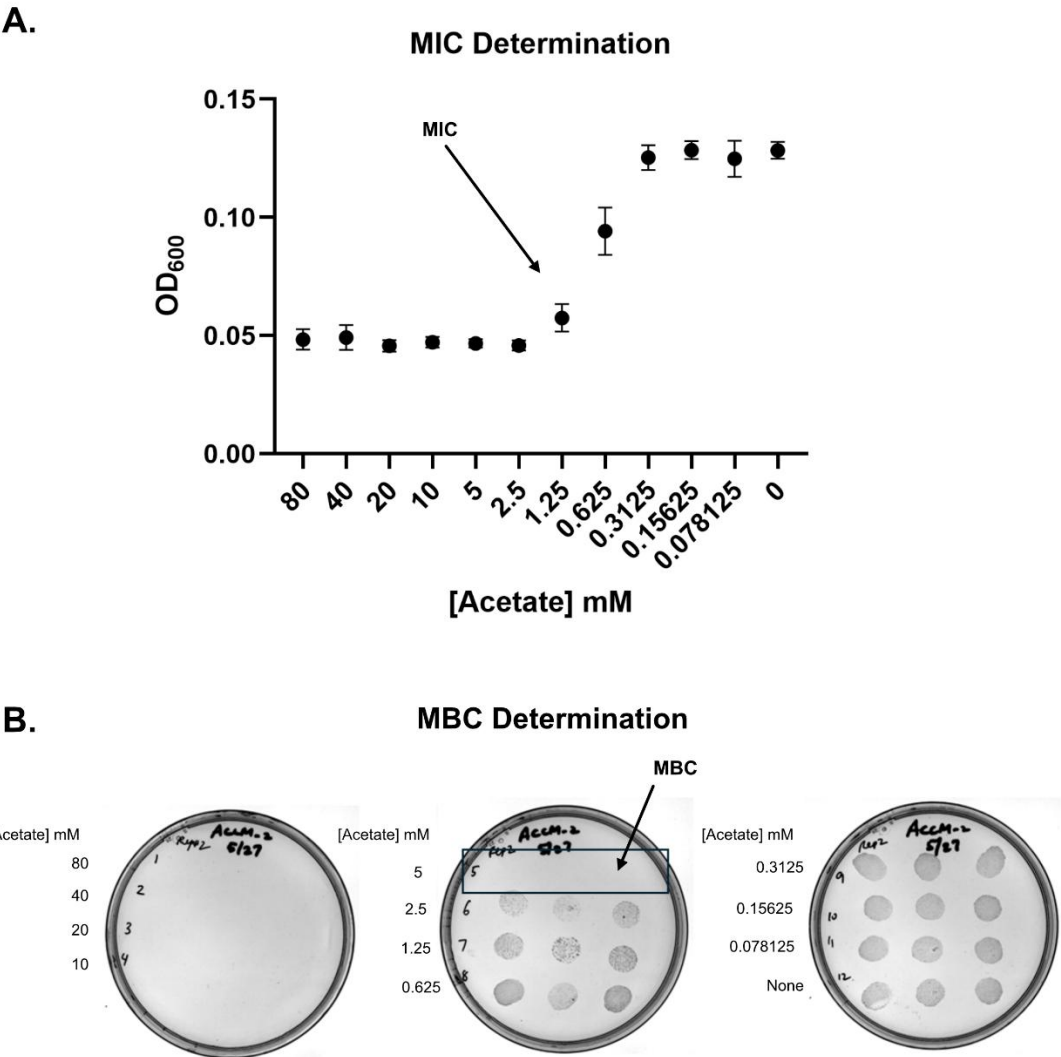


Figure 2. The minimum inhibitory concentration (MIC) of acetate for *C. burnetii* is 1.25 mM and the minimum bactericidal concentration (MBC) is 5 mM. ACCM-2 broth containing 80 mM acetate was diluted 2-fold serially in a 96-well plate (300 μ L per well) to establish a dilution series. PBS-containing ACCM-2 served as the negative control. 5 μ L of WT *C. burnetii* stock was diluted in ACCM-2 media, and

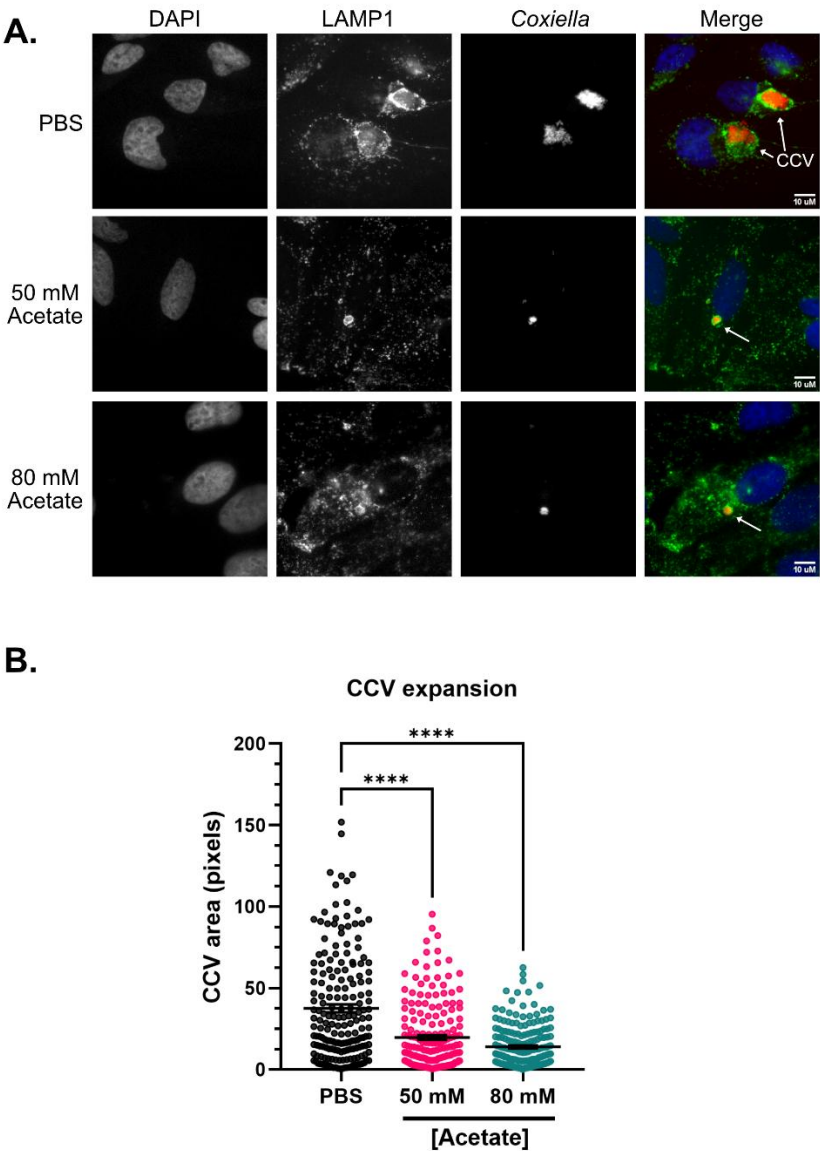
288 2 μ L of the diluted inoculum was added to each well. The MIC plate was incubated for seven days at
289 37°C in a tri-gas incubator (5% CO₂, 2.5% O₂). At seven days, the absorbance at 600 nm of each well
290 was measured using a BioTek Cytation 3 plate Reader. A₆₀₀ values for each acetate concentration were
291 compared to the baseline A₆₀₀ of the respective no-acetate controls. The MIC was determined as the
292 acetate concentration that significantly inhibited *C. burnetii* growth compared to the next lower
293 concentration. Data shown as mean $\pm$ SEM from three independent experiments (N=3), each with
294 technical triplicates for each condition, and analyzed by unpaired t-test; ****, P<0.0001.

295

296 **Acetate treatment negatively affects CCV expansion**

297 The direct effect of acetate on *C. burnetii* axenic growth prompted us to assess its effects on the
298 intracellular *C. burnetii*. Being an obligate intracellular bacterium, *C. burnetii* relies on the CCV for
299 multiplication, metabolism, and protection from the host defense [34]. The CCV expands markedly from
300 2 to 4 days post-infection (dpi), and large, mature CCVs may indicate successful *C. burnetii* colony
301 establishment within host cells. Therefore, to assess the effects of acetate on CCV maturation, we
302 compared the CCV size in the PBS and acetate-treated HeLa cells at 3 dpi. Our quantitative microscopy
303 data revealed that at the 24-h time point, 50 and 80 mM acetate treatments on *C. burnetii*-infected HeLa
304 cells decreased the CCV areas by ~ 61% (P < 0.0001) and ~ 67% (P < 0.0001), respectively, compared
305 to the PBS-treated cells (Fig 1A, B). However, upon qualitative observation, overall cell size, shape, and
306 nuclei size remained unchanged in the treatment conditions at this time point (Fig 1A). Therefore, these
307 data suggest that acetate negatively affects CCV expansion and maturation. These acetate
308 concentrations were chosen for the treatment conditions as an average of the 'high' and 'low' level SCFA
309 mixtures described previously [35] and to reflect the mean acetate concentration in the human body [36].
310 We further attempted to measure the CCV areas in acetate-treated cells at later time points upon
311 prolonged treatment. However, treatments longer than 24 hours appeared to negatively affect overall cell

312 number under our experimental conditions. Therefore, we used a 24-h treatment across all downstream
313 experiments in this study.



314

315 **Figure 3. Acetate inhibits *C. burnetii*-containing vacuole expansion in HeLa cells.** (A)

316 Representative images of *C. burnetii*-infected HeLa cells, treated with either PBS or 50 and 80 mM

317 acetate. HeLa cells were infected with mCherry-*C. burnetii* for 2 h. At 2 dpi, cells were replated on

318 coverslips and treated with the respective conditions. At 24 h post-treatment (3 dpi), cells were fixed with

319 4% PFA and subjected to an immunofluorescent assay with anti-LAMP1 antibody to label the CCVs. Cells
320 were visualized with a Leica Stellaris confocal microscope, and the images were analyzed in ImageJ FIJI.
321 **(B)** Measurement of the CCV area revealed significantly smaller CCVs at 3 dpi in both 50 and 80 mM
322 acetate-treated HeLa cells compared to the PBS control. Data shown as mean $\pm$ SEM of at least 25 cells
323 per condition in each of three independent experiments (N=3) as analyzed by one-way ANOVA; ****,
324 P<0.0001.

325

326 **Acetate treatment reduces the intracellular *C. burnetii* burden**

327 Previous studies have indicated that CCV expansion does not directly correlate with *C. burnetii*
328 multiplication [37]. Therefore, to examine the degree to which acetate affects the bacterial intracellular
329 life cycle, we quantified *C. burnetii* intracellular multiplication in acetate-treated HeLa cells using our
330 colony-forming unit (CFU) assay described previously [38]. Fig. 4A shows a schematic diagram of the
331 experiment. We first analyzed the absolute CFU values in PBS, 50 mM, and 80 mM acetate treatment
332 conditions at each time point, which revealed that starting at $\sim 2 \times 10^5$ CFU/mL at 0 dpi, *C. burnetii* grew to
333 $\sim 1.2 \times 10^6$ CFU/mL at 2 dpi, at which point the host cells were treated with PBS or acetate (Fig. 4B). At 3
334 dpi, although *C. burnetii* continued to grow in PBS-treated cells to $\sim 2.3 \times 10^6$ CFU/mL, both acetate
335 treatment conditions significantly inhibited *C. burnetii* growth to only $\sim 1.4 \times 10^6$ CFU/mL at 3 dpi (P < 0.01
336 for both 50 and 80 mM acetate conditions compared to PBS control; Fig. 4B). Next, to normalize any
337 effect of the starting CFUs on the *C. burnetii* growth rate, we analyzed the mean fold change in growth
338 over 0 dpi in each treatment condition (Fig. 4C). These data show that *C. burnetii* multiplied by ~ 4.4 -fold
339 from 0 to 2 dpi, and continued to grow in PBS-treated cells by ~ 10 -fold at 3 dpi. However, under both 50
340 and 80 mM acetate, *C. burnetii* grew by only ~ 5 -fold at 3 dpi, suggesting a $\sim 50\%$ reduction in *C. burnetii*
341 intracellular growth in acetate-treatment conditions. Taken together, these data suggest that 24-h of
342 acetate treatment attenuates *C. burnetii* intracellular growth (P < 0.01 for both 50 and 80 mM acetate

conditions compared to PBS control; Fig. 4C). However, whether acetate directly affects *C. burnetii* or inhibits *C. burnetii* through modulating the host cell environment was unclear at this point.

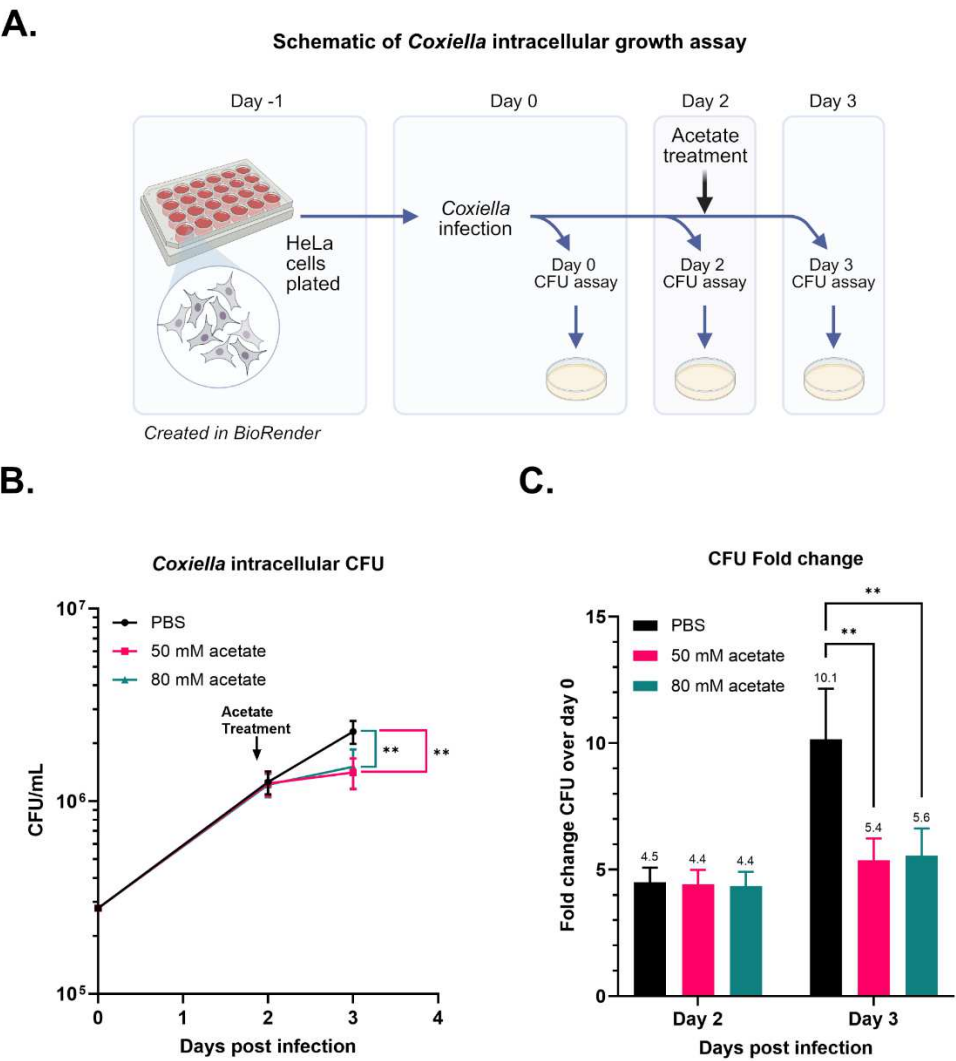


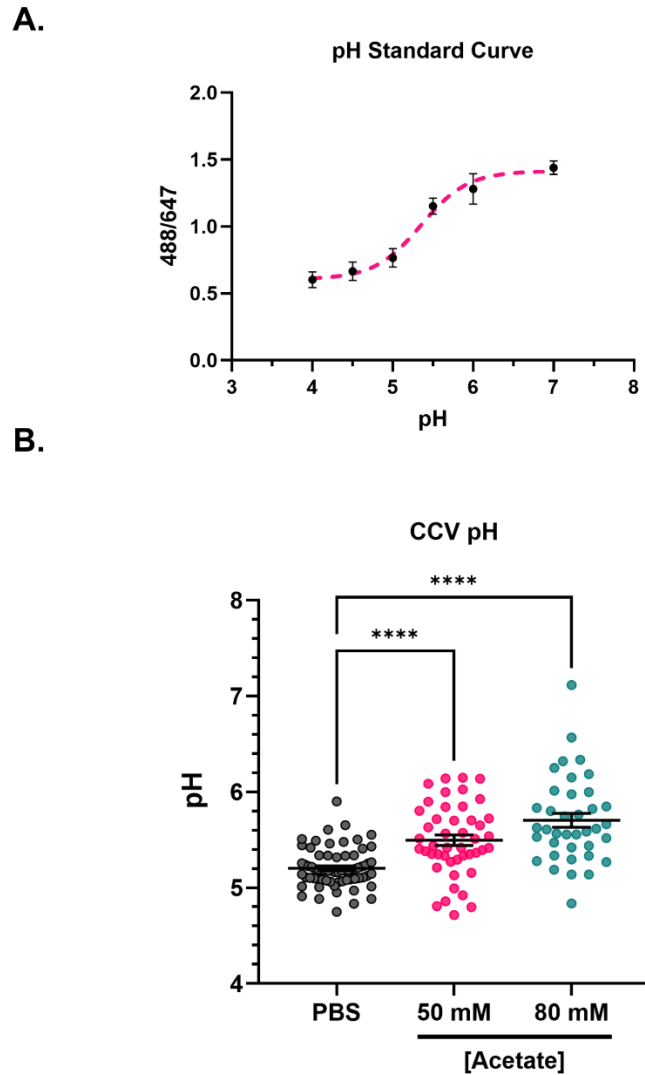
Figure 4. Acetate treatment reduces intracellular *C. burnetii* replication. (A) Schematic diagram of *C. burnetii* intracellular growth measurement by CFU assay. HeLa cells were infected with *C. burnetii* in a 24-well plate for 2 h and incubated. To determine viable *C. burnetii* at 0 dpi (day of infection) infected cells were lysed in sterile water, diluted 1:5 in ACCM-2 broth and plated on ACCM-2 agarose plates. The remaining cells were split into two groups (labelled Day 2 and Day 3), and replated into a 24-well plate. At 2 dpi, the Day 2 wells were processed for CFU, and Day 3 cells were treated with PBS, 50

352 mM, or 80 mM acetate. At 24 h post-treatment (3 dpi), the Day 3 wells were processed for CFU assay.
353 **(B)** Enumeration of viable *C. burnetii* by absolute CFU count. All three treatment conditions showed
354 similar growth from 0 to 2 dpi, whereas the acetate-treated conditions showed significantly reduced
355 growth at 3 dpi. **(C)** The fold change of CFU over 0 dpi showed ~ 50% reduction in *C. burnetii* growth in
356 acetate-treated conditions compared to that in PBS-treated condition. Data shown as mean±SEM from
357 three independent experiments (N=3) as analyzed by one-way ANOVA; **, P<0.01.

358

359 **Acetate reduces the luminal acidity of *C. burnetii*-containing vacuoles**

360 Our previous studies have established that the luminal pH of the CCV is maintained at ~5.2 [24, 29, 38],
361 and any deflection in the CCV pH caused by external factors is detrimental to *C. burnetii* intracellular
362 growth [24]. Therefore, to better understand the SCFA mechanism of action of *C. burnetii* intracellular
363 growth inhibition, we aimed to determine whether acetate treatment alters the CCV pH. Acetate and other
364 SCFAs can freely move through the biological membranes [39], and CCV membrane is essentially
365 plasma membrane. Therefore, we hypothesized that acetate will directly affect CCV luminal environment.
366 To test this, we measured the CCV pH in PBS and acetate-treated cells. A representative pH standard
367 curve prepared from the control cells shows a sigmoidal curve (Fig. 5A), confirming the validity of the
368 experimental procedure. Upon interpolating the experimental samples to the standard curve, we
369 observed an average CCV pH of ~ 5.204 in PBS-treated cells, as expected (Fig. 5B). However, to our
370 surprise, there was a dose-dependent increase in CCV pH in acetate-treated cells. Specifically, the
371 average CCV pH in 50 mM acetate-treated cells was ~5.496 ($P < 0.0001$ compared to PBS control), and
372 that in 80 mM acetate-treated cells was ~5.703 ($P < 0.0001$ compared to PBS control), both significantly
373 higher than that in the PBS control. These data suggest that, contrary to our hypothesis, acetate treatment
374 significantly reduces CCV acidity. These seemingly modest but significant change in CCV pH may
375 negatively affect the *C. burnetii* intracellular multiplication.



376

377 **Figure 5. Acetate treatment reduces CCV pH.** HeLa cells infected with mCherry-*C. burnetii* and pulsed
 378 with Oregon Green 488 (pH-sensitive; green) and Alexa 647 (pH-stable; magenta) dextran for 4 h
 379 followed by a 1 h chase. Cells were imaged with a confocal microscope, and Oregon Green 488 and Alexa
 380 Fluor 647 intensities were quantified in Fiji, and 488/647 ratios were determined. **(A)** Ratiometric pH
 381 measurement in HeLa cells treated with ionophores and standardized pH buffers revealed a sigmoidal
 382 standard curve as expected. **(B)** The 488/647 ratios determined from each CCV in PBS or acetate-treated
 383 HeLa cells at 3 dpi were interpolated to the standard curve to determine the CCV pH in the respective

384 treatment conditions. These data revealed that CCVs are significantly less acidic in the acetate-treated
385 conditions than in the PBS condition. Data shown as mean $\pm$ standard error of mean (SEM) of at least 20
386 CCVs from three independent experiments as analyzed by one-way ANOVA with Tukey's post hoc test;
387 ****, $P < 0.0001$.

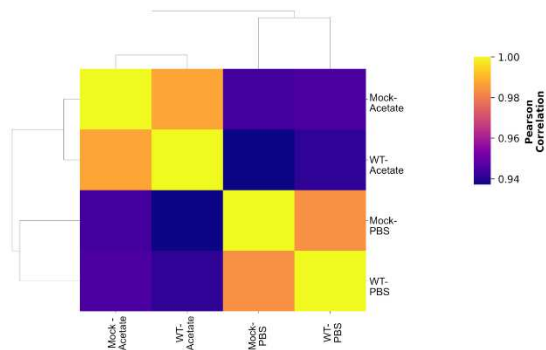
388

389 **Acetate treatment alters lipid metabolism, membrane homeostasis, and stress response** 390 **regulators in *C. burnetii*-infected cells**

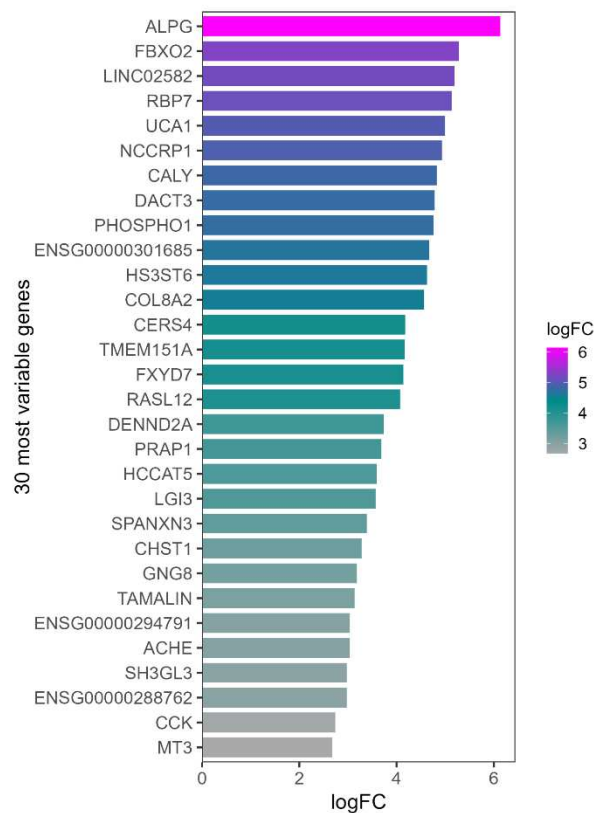
391 To identify host pathways associated with acetate-mediated inhibition of *C. burnetii* growth, we performed
392 RNA-sequencing from mock and *C. burnetii*-infected cells treated with 50 mM acetate or PBS control.
393 The sample–sample correlation heatmap showed strong clustering by treatment conditions rather than
394 *C. burnetii* infection (Fig. 6A). The acetate-treated samples (Mock-acetate and WT-acetate) clustered
395 closely together and exhibited high correlation with each other (Pearson $r \approx 0.98$ – 1.00), indicating highly
396 similar global transcriptomic profiles. Likewise, the control samples (Mock-PBS and WT-PBS) clustered
397 together with a similarly high correlation. In contrast, correlations between the acetate-treated and PBS-
398 treated samples are relatively lower (approximately $r \approx 0.94$ – 0.96), demonstrating a transcriptional
399 separation between the treatment groups, independent of the *C. burnetii* infection. Fold-change analysis
400 revealed widespread transcriptional remodeling following acetate treatment. In mock-infected cells,
401 approximately 10,815 genes were upregulated, and 4,466 genes were downregulated by at least twofold
402 compared with PBS-treated cells (Supplemental file S1). A similar pattern was observed in *C. burnetii*-
403 infected cells, with 11,291 genes upregulated and 4,451 genes downregulated (Supplemental file S2).
404 Notably, 5,530 genes were consistently upregulated, and 1,038 genes were consistently downregulated
405 in both backgrounds. Together, these results indicate that acetate treatment is the dominant factor
406 shaping global gene expression patterns in this dataset. Among the 30 most highly altered transcripts
407 between WT-acetate and WT-PBS were genes involved in lipid metabolism and membrane homeostasis.
408 Most notably, RBP7 (rank 4), CERS4 (rank 13), and PRAP1 (rank 18) are involved in lipid metabolism
409 [40–45], suggesting that acetate treatment alters host lipid regulatory networks. Several genes associated

with vesicular trafficking and membrane remodeling were also differentially expressed, such as TMEM151A (rank 14), DENND2A (rank 17), TAMALIN (rank 24), and SH3GL3 (rank 27). These proteins are implicated in endosomal transport and Rab GTPase-regulated trafficking pathways [46-49], processes that are extensively exploited by *C. burnetii* to establish and maintain its intracellular replicative niche [29, 50]. Altered expression of these genes suggests that acetate treatment may disrupt normal endosomal maturation and recycling pathways required for CCV expansion and bacterial growth. In addition, acetate treatment induced expression of genes linked to cellular signaling and stress responses. Notably, DACT3, a negative regulator of Wnt/ β -catenin signaling [51], was among the most variable transcripts. Similarly, induction of MT3, a metallothionein involved in oxidative stress responses and metal homeostasis [52], suggests activation of host stress pathways that could further limit bacterial survival. A number of long non-coding RNAs, including UCA1 [53], LINC02582 [54], and HCCAT5 [55], were also among the most highly upregulated transcripts, indicating that acetate treatment may trigger broader transcriptional and epigenetic reprogramming of infected host cells. A functional enrichment analysis of the dysregulated genes in WT-acetate compared to WT-PBS cells further identified several genes associated with immune and stress response pathways, including coagulation (51 genes), complement activation (63 genes), estrogen-response signaling (83 genes), apoptosis (58 genes), KRAS signaling (77 genes), IL-2/STAT5 signaling (77 genes), and broad inflammatory response pathways (66 genes) (Fig. 7A-H). These pathway-level changes suggest a transition toward a more stress-activated and immune-responsive cellular state. Collectively, these findings suggest that acetate globally modulates of host lipid metabolism, endosomal trafficking, and cellular stress-response pathways. Further studies will be needed to validate these data and identify specific molecular mechanisms of action of acetate in *C. burnetii*-infected cells.

A. Sample correlation heatmap with hierarchical clustering



B. Gene expression analysis in WT-acetate vs. WT-PBS



432

433

434 **Figure 6. Acetate treatment upregulates lipid metabolism and membrane homeostasis genes.**

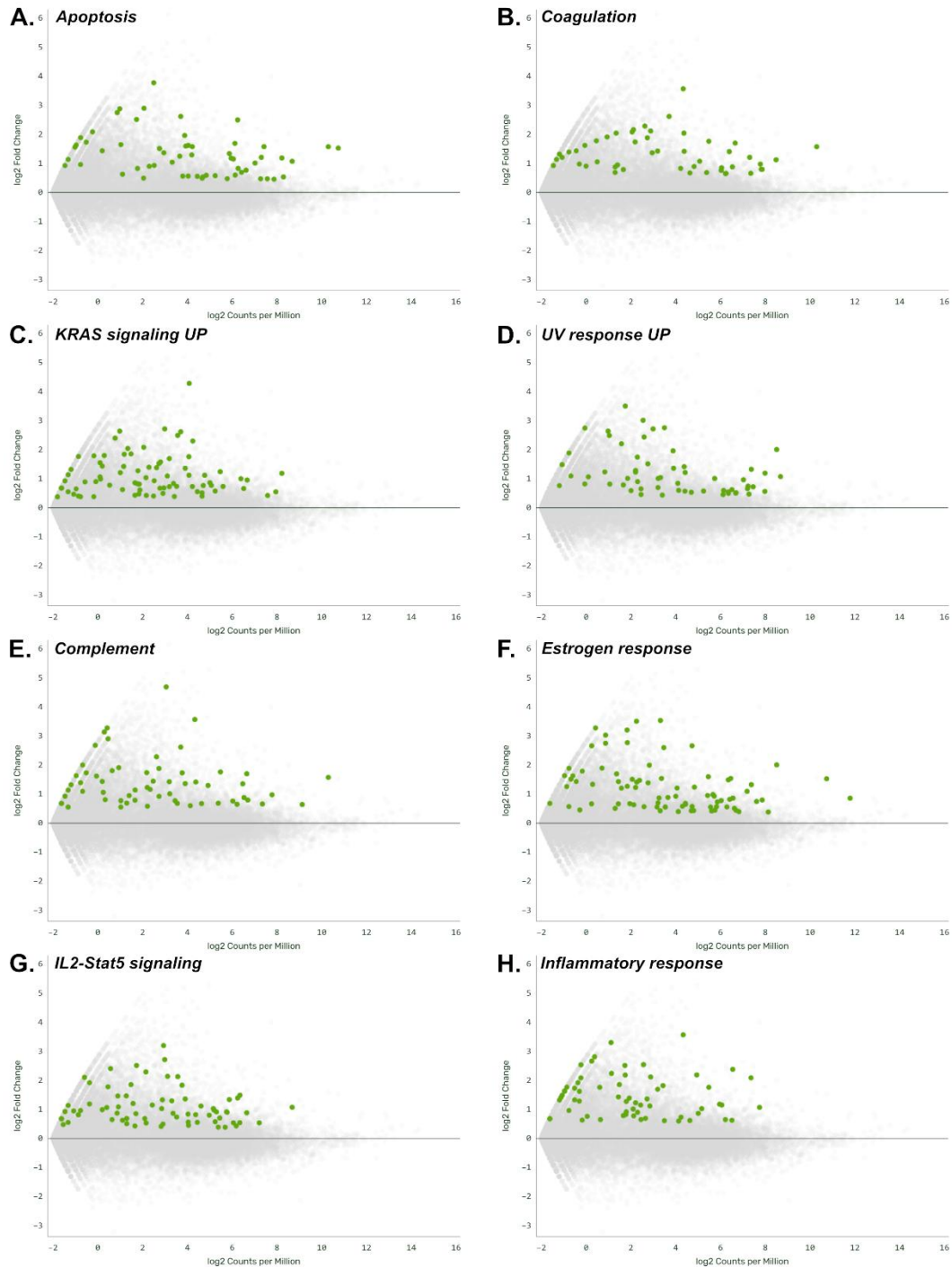
435 HeLa cells were plated and either mock-infected or infected with WT *C. burnetii*. At 2 dpi, cells were

436 treated with either PBS or 50 mM acetate and incubated in the treatment conditions for 24 h. At 3 dpi,

437 cells were harvested, counted, and diluted to 2×10^5 cells/mL. Cells were centrifuged, and pellets were
438 resuspended in Zymo DNA/RNA shield™. Samples were then sent for RNA-seq analysis to
439 Plasmidsaurus®. **(A)** A sample–sample correlation heatmap showed strong clustering by treatment
440 conditions rather than *C. burnetii* infection. The acetate-treated samples (Mock-acetate and WT-acetate)
441 clustered closely together and exhibited high correlation with each other (Pearson $r \approx 0.98$ –1.00), and
442 the control samples (Mock-PBS and WT-PBS) clustered together with a similarly high correlation
443 (Pearson $r \approx 0.98$ –1.00). **(B)** Fold change analysis of the 30 most variable genes between *C. burnetii*-
444 infected, PBS-treated vs. *C. burnetii*-infected, acetate-treated cells revealed genes associated with lipid
445 metabolism and membrane trafficking.

446

447



448

449 **Figure 7. Acetate treatment globally upregulates stress-associated and inflammatory response**
 450 **genes.** HeLa cells were plated and either mock-infected or infected with WT *C. burnetii*. At 2 dpi, cells
 451 were treated with either PBS or with 50 mM acetate and incubated in the treatment conditions for 24 h.

At 3 dpi, cells were harvested, counted, and diluted to 2×10^5 cells/mL. Cells were centrifuged, and pellets were resuspended in Zymo DNA/RNA shield™. Samples were then sent for RNA-seq analysis to Plasmidsaurus. (A-F) Differential gene expression plot, along with functional enrichment analysis, revealed that acetate treatment globally upregulated coagulation (51 genes), complement activation (63 genes), estrogen-response signaling (83 genes), apoptosis (58 genes), KRAS signaling (77 genes), IL-2/STAT5 signaling (77 genes), and broad inflammatory response pathways (66 genes).

Discussion

In this study, we demonstrated that acetate, the most abundant short-chain fatty acid (SCFA) in humans, restricts *Coxiella burnetii* replication through multiple complementary mechanisms. Specifically, acetate directly inhibits bacterial growth in axenic culture, impairs intracellular replication, disrupts maturation of the *Coxiella*-containing vacuole (CCV), and induces broad transcriptional changes in host cells. Together, these findings identify acetate as a previously unrecognized inhibitor of *C. burnetii* and reveal a metabolic vulnerability of an obligate intracellular pathogen that depends on a highly specialized acidic intracellular niche.

Our study is the first report of a potent direct effect of acetate on *C. burnetii* growth. In axenic ACCM-2 medium, acetate completely inhibited *C. burnetii* replication. Consistent with this observation, broth microdilution assays revealed a remarkably low minimum inhibitory concentration (MIC) of 1.25 mM and a minimum bactericidal concentration (MBC) of 5 mM. These findings indicate that *C. burnetii* is highly sensitive to acetate and suggest that the metabolite exerts direct antimicrobial stress independent of host-cell factors. Similar antimicrobial effects of SCFAs have been described against several bacterial pathogens and are often associated with disruption of intracellular pH homeostasis, metabolic imbalance, and membrane dysfunction [18, 56, 57]. Whether acetate similarly perturbs central metabolism or membrane integrity in *C. burnetii* warrants further investigation.

In addition to its direct antibacterial effects, acetate significantly impaired *C. burnetii* intracellular replication. A 24-hour acetate treatment reduced bacterial burden by approximately 50%, indicating that

the intracellular environment becomes substantially less permissive for *C. burnetii* growth following acetate exposure. Because the inhibition observed intracellularly was less pronounced than that observed in axenic culture, the intracellular phenotype is likely determined by a combination of direct bacterial inhibition and acetate-induced changes in host-cell physiology. This interpretation is consistent with the complex dependence of *C. burnetii* on host metabolic pathways, vesicular trafficking, and membrane remodeling processes for establishment and maintenance of its replicative niche [28, 29, 37, 38]. Further, our data revealed that acetate disrupts two major aspects of CCV biology – expansion and acidity. We observed a marked reduction in CCV expansion following acetate treatment, suggesting that it negatively influences vacuolar maturation. Although CCV size alone does not directly correlate with bacterial burden, proper vacuolar expansion is required to accommodate bacterial replication and sustain long-term infection [58, 59]. The inability of CCVs to undergo normal expansion in SCFA-treated cells therefore suggests impairment of processes such as membrane acquisition and endosomal trafficking. Previous studies have demonstrated that *C. burnetii* actively manipulates host endocytic and recycling pathways to establish and expand the CCV [28, 29]. Thus, perturbation of these pathways by SCFAs may contribute to the reduced bacterial replication observed here.

Furthermore, acetate treatment decreased the CCV luminal acidity. Acetate is a weak organic acid and dissociates into Na^+ and CH_3COO^- ions (acetate ion) in aqueous medium, where the acetate ions act as conjugate base of a weak acid and therefore potentially elevates the pH of the solution. Because, acetate readily transports across biological membranes [60], we hypothesize that acetate transports into the CCV and increases the pH directly. Acetate treatment elevated the CCV pH from 5.2 to approximately 5.5–5.7. Although seemingly modest, this shift is biologically meaningful because *C. burnetii* relies on a narrow acidic window for optimal metabolic activation, activation of the Dot/Icm type IVB secretion system (T4BSS), and intracellular replication [24, 29]. Previous studies have shown that even relatively small increases in CCV pH negatively affect bacterial growth and survival [24]. Our findings therefore suggest that acetate-induced de-acidification of the CCV contributes directly to the attenuation of intracellular replication.

504 Transcriptomic analyses further support the idea that acetate induces a host-cell state that is unfavorable
505 for *C. burnetii* replication. Global gene expression patterns clustered according to treatment rather than
506 infection status, indicating that acetate was the dominant driver of transcriptional variation under these
507 conditions. Acetate exposure triggered widespread transcriptional remodeling characterized by activation
508 of inflammatory, stress-response, apoptotic, complement, coagulation, and IL-2/STAT5-associated
509 pathways. These responses were observed in both infected and uninfected cells, suggesting that acetate
510 broadly reprograms cellular physiology independent of pathogen presence. Such immunomodulatory
511 effects are consistent with the established ability of SCFAs to regulate host immune signaling,
512 inflammatory responses, and cellular metabolism [18, 60-62].

513 Among the most highly altered transcripts were genes involved in lipid metabolism, membrane
514 homeostasis, and vesicular trafficking. These pathways are particularly relevant because *C. burnetii*
515 extensively manipulates host membrane trafficking and lipid composition to establish and maintain the
516 CCV. Alterations in genes associated with endosomal transport and Rab-regulated trafficking may
517 therefore contribute to the reduced vacuolar expansion and impaired bacterial growth observed in
518 acetate-treated cells. Furthermore, induction of stress-associated regulators such as DACT3 and MT3
519 suggests activation of cellular programs that may oppose intracellular bacterial survival. Although the
520 current study was not designed to establish causality for individual pathways, these transcriptomic data
521 provide a valuable framework for future mechanistic investigations.

522 Several limitations should be considered when interpreting our findings. First, experiments were
523 performed in HeLa cells, which do not fully recapitulate the biology of primary alveolar macrophages, the
524 principal host cell targeted during natural infection. [11,12] Second, the acetate concentrations used in
525 this study exceed typical circulating systemic concentrations and were selected primarily to mimic an
526 average concentration found in human body. Third, prolonged treatment resulted in reduced host-cell
527 viability, restricting our analyses to relatively short exposure periods. Future studies utilizing primary
528 macrophages, physiologically relevant SCFA concentrations, and animal models will be necessary to
529 determine the relevance of these mechanisms during infection in vivo. Despite these limitations, our

findings establish that acetate restricts *C. burnetii* through a multifaceted mechanism involving direct antibacterial activity, disruption of CCV physiology, and extensive host-cell reprogramming. Rather than identifying acetate itself as an immediate therapeutic candidate, this study highlights metabolic sensitivity as an underappreciated vulnerability of *C. burnetii*. More broadly, these results emphasize the potential for host-derived metabolites to influence intracellular pathogen survival and complement the growing evidence that SCFAs can modulate infection outcomes through both direct antimicrobial and host-directed mechanisms.

Collectively, our study identifies acetate as a previously unrecognized inhibitor of *C. burnetii* and demonstrates that host-derived metabolites can profoundly influence both pathogen physiology and the intracellular environment required for bacterial replication. These findings expand our understanding of the metabolic factors governing *C. burnetii* pathogenesis and provide a foundation for future studies examining how microbially derived metabolites shape the outcome of intracellular bacterial infections.

Author contributions

YG and DS conceptualized the study. YG, AD, BAH, VDK, and DS performed the experiments and acquired data. YG, AD, BAH, VDK, and DS prepared the first draft of the manuscript. AQS assisted with writing the final draft of the manuscript. DS and AQS edited and finalized the manuscript. DS, BAH, and AQS organized and managed the data. DS acquired funding for the study.

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Data availability

556 The authors declare that the data are available on reasonable request.

557

558 **Clinical trial number:** Not applicable

559

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