

RESEARCH ARTICLE

A Combined Enrichment and Aptamer Pulldown Assay for *Francisella tularensis* Detection in Food and Environmental Matrices

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Abstract

Francisella tularensis, a Gram-negative bacterium and causative agent of tularemia, is categorized as a Class A select agent by the Centers for Disease Control and Prevention due to its ease of dissemination and ability to cause disease. Oropharyngeal and gastrointestinal tularemia may occur due to ingestion of contaminated food and water. Despite the concern to public health, little research is focused on *F. tularensis* detection in food and environmental matrices. Current diagnostics rely on host responses and amplification of *F. tularensis* genetic elements via Polymerase Chain Reaction; however, both tools are limited by development of an antibody response and limit of detection, respectively. During our investigation to develop an improved culture medium to aid *F. tularensis* diagnostics, we found enhanced *F. tularensis* growth using the spent culture filtrate. Addition of the spent culture filtrate allowed for increased detection of *F. tularensis* in mixed cultures of food and environmental matrices. Ultrapformance liquid chromatography (UPLC)/MS analysis identified several unique chemicals within the spent culture supernatant of which carnosine had a matching *m/z* ratio. Addition of 0.625 mg/mL of carnosine to conventional *F. tularensis* medium increased the growth of *F. tularensis* at low inoculums. In order to further enrich *F. tularensis* cells, we developed a DNA aptamer cocktail to physically separate *F. tularensis* from other bacteria present in food and environmental matrices. The combined enrichment steps resulted in a detection range of 1–10⁶ CFU/mL (starting

inoculums) in both soil and lettuce backgrounds. We propose that the two-step enrichment process may be utilized for easy field diagnostics and subtyping of suspected *F. tularensis* contamination as well as a tool to aid in basic research of *F. tularensis* ecology.

Introduction

Increased global processing and distribution of food has raised awareness of food safety in regards to accidental or purposeful introduction of a biological contaminant into the food network [1, 2]. *Francisella tularensis* subsp. *tularensis*, a Gram-negative bacterium responsible for tularemia in a wide range of hosts, is categorized as a class A agent by the Centers for Disease Control and Prevention (CDC) due to its high infectivity, ease of dissemination and ability to cause disease [3, 4]. Current models of *F. tularensis* infectivity and dissemination concern aerosolization leading to pneumonic tularemia; however, tularemia may exist as oropharyngeal and gastrointestinal clinical forms due to oral exposure and/or ingestion of contaminated food or water [4–7]. Clinical presentation of oropharyngeal and gastrointestinal tularemia may include lesions in the oropharynx, draining lymph nodes, and gastrointestinal tract [5, 8]. Progression from oropharyngeal to pneumonic tularemia (aspiration) may occur due to bacteremic spread into the lungs [9, 10].

Traditional diagnostic tools for *F. tularensis* have been developed for patient samples and consequently rely on host responses, including serum antibodies [11–15]. Serodiagnostics for *F. tularensis* require antibody levels that are achieved after 10 or more days of disease and would provide minimal information about the source of infection and how to best manage a potential outbreak [5]. Availability of *Francisella tularensis* genomes and comparative analyses against other members of the *Francisella* genus have allowed researchers to use specific *F. tularensis* genes in diagnostic formats such as Polymerase Chain Reaction (PCR) and real-time PCR [16–22]. It is important to note that the gold-standard to validate *F. tularensis* detection using serology and various PCR platforms remains cultivation of the organism, which requires growth on cysteine or thioglycolate enriched medium and incubation times of 2–4 days at 37°C [5, 23]. Studies utilizing these tools have been widely applied to *F. tularensis* detection in patients and animal carcasses; however, few techniques have been reported for identification of *F. tularensis* in food and environmental matrices [24–27]. Inasmuch as the potential for biocontamination with *F. tularensis* and the presence of resident microbes, which may outcompete *F. tularensis* growth and act as PCR inhibitors, there remains a critical need for improved cultivation and unambiguous detection of *F. tularensis* in food and environmental matrices.

In this study, we report on the development of a two-step enrichment process for improved cultivation and detection of *F. tularensis* in lettuce and soil. This

process first utilizes logarithmic-phase *F. tularensis* spent culture filtrate to supplement standard culture medium to enhance *F. tularensis* growth in the presence of resident bacteria from food and environmental matrices. Next, *F. tularensis* is further concentrated by physical separation from resident bacteria using a DNA aptamer cocktail capture assay.

Initial characterization of unique chemical entities found within the spent culture filtrate was carried out using UPLC/MS analysis with automated and manual database searches. Manual database searches identified carnosine as a chemical target that had similar *m/z* and retention time characteristics to those found by UPLC/MS. Addition of 0.625 mg/mL to conventional growth medium resulted in increased growth of *F. tularensis*. We propose that the application of the two-step enrichment process may be utilized in field diagnostics and molecular subtyping as well as extended to basic research in understanding the ecology of tularemia.

Results

F. tularensis spent medium increases the growth and detection of *F. tularensis* in mixed cultures of bacteria from food matrices

Intracellular pathogens frequently grow slower, have a long lag time, or fail to grow in vitro compared to replication within the host cell [28–31]. This poses a significant challenge for disease and bioweapon diagnostics, which rely on specific detection ranges of pathogen cells, toxin and/or other protein concentrations. Detectable levels from low starting inoculums of *F. tularensis* are difficult to achieve in vitro. Various studies have shown that the spent culture medium of pathogenic bacteria, such as *Mycobacterium tuberculosis*, stimulated enhanced growth of dormant and small inoculum cultures [32–34]. More importantly, studies conducted by Halmann and colleagues have described the presence of a growth-initiation substance (GIS) in the culture filtrate using small inocula of virulent strains of *F. tularensis* [35, 36]. Therefore, we tested if the spent culture filtrate of *F. tularensis* vaccine strain would stimulate and enhance the growth of a small number of *F. tularensis* cell. Overnight cultures of *F. tularensis* were serially diluted and spotted on standard culture medium (TSA and 0.1% L-cysteine) supplemented with and without spent culture filtrate (Fig. 1A). *F. tularensis* cultured on spent culture filtrate resulted in robust growth at all dilutions (10^{-1} – 10^{-6}) in contrast to standard culture medium, which only supported the growth of 10^{-1} – 10^{-2} dilutions (Fig. 1A).

The main challenge in diagnostics and pre-analytical processing of *F. tularensis* in food and environmental matrices is the presence of other bacteria, which may interfere with *F. tularensis* growth [7]. Furthermore, the possibility remained that *F. tularensis* spent culture filtrate may stimulate inter-species growth of bacteria found in food and would consequently limit its' utility as a diagnostic aid. To determine if *F. tularensis* spent culture medium promoted growth of other bacterial species, we compared the CFU/mL of bacteria found within lettuce and

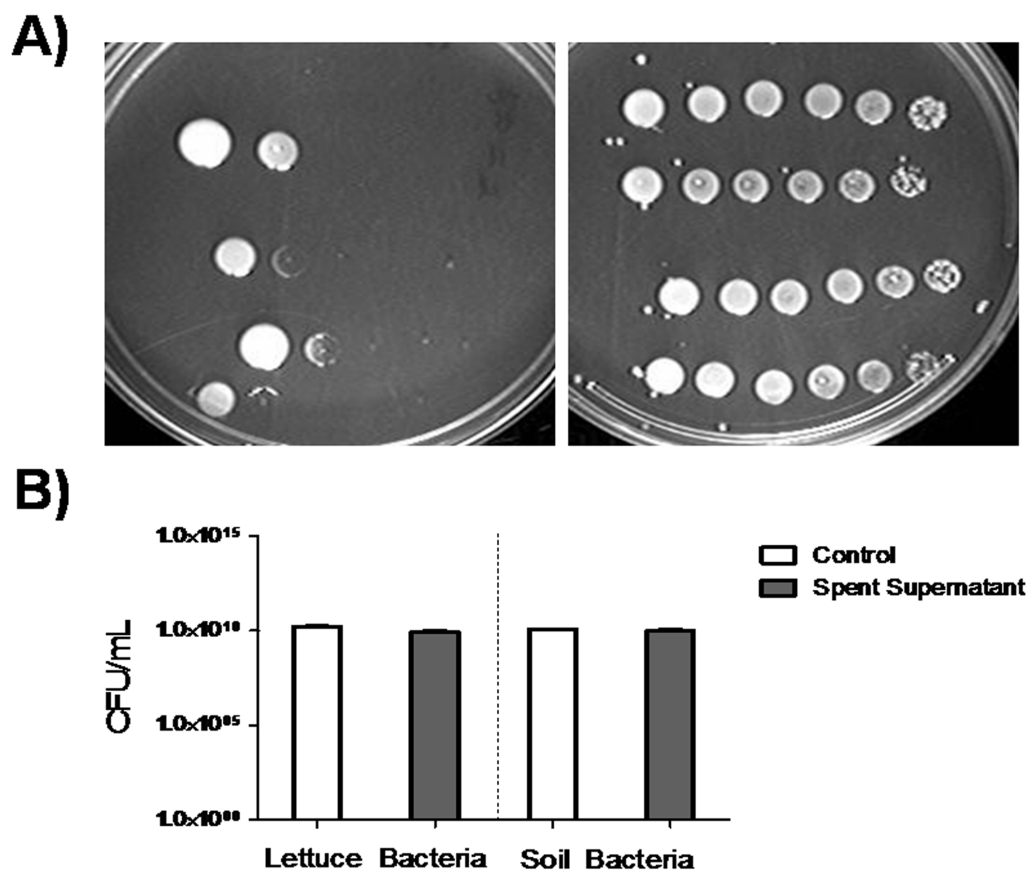


Fig. 1. *F. tularensis* spent medium enhances growth and is specific for *F. tularensis*. **A)** *F. tularensis* was cultured for 24 h and diluted on TSA containing 0.1% L-cysteine (left) and TSA containing 0.1% L-cysteine and 10% spent medium (right). A ten-fold dilution series of *F. tularensis* was created (10^{-1} – 10^{-6} , left to right). **B)** Lettuce bacteria were obtained from stomacher processing of 10 g of lettuce in 50 mL of TSB containing 0.1% L-cysteine. Soil bacteria were obtained from culturing of 1 g of soil, hay and dust in TSB containing 0.1% L-cysteine overnight at 37°C with shaking at 120 rpm. Bacteria from lettuce and soil sources were diluted to 10^{-6} and plated on TSA supplemented with 0.1% L-cysteine (control) and control agar supplemented with 10% *F. tularensis* spent filtrate. Samples were incubated overnight at 37°C and the CFU/mL was calculated. Legend: Control=white bar, Spent filtrate=medium grey bar.

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soil on spent filtrate supplemented medium and standard medium alone (Fig. 1B). We observed no difference in CFU/mL between standard medium supplemented with or without spent filtrate (Fig. 1B). However, the possibility remains that the spent culture filtrate may inhibit the growth of certain bacterial species, while other bacterial communities may continue to exist and fill the vacant niche. In fact, we did observe changes to colony phenotype when the spent culture filtrate was added to lettuce homogenates (data not shown). Next, we investigated our ability to detect *F. tularensis* by real-time PCR. Specifically, does the addition of spent culture filtrate in relation to the limit of detection and does the inclusion of soil and lettuce bacteria interfere with (Fig. 2)? *F. tularensis* cultured with spent filtrate showed a 2.5–3.5 fold increase in cells per mL at a starting inoculum of 1 bacterium per mL compared to standard medium alone (Fig. 2). Addition of soil bacteria to *F. tularensis* cultured with spent filtrate reduced the overnight growth of *F. tularensis* an average of 1.5 fold; however, a 2

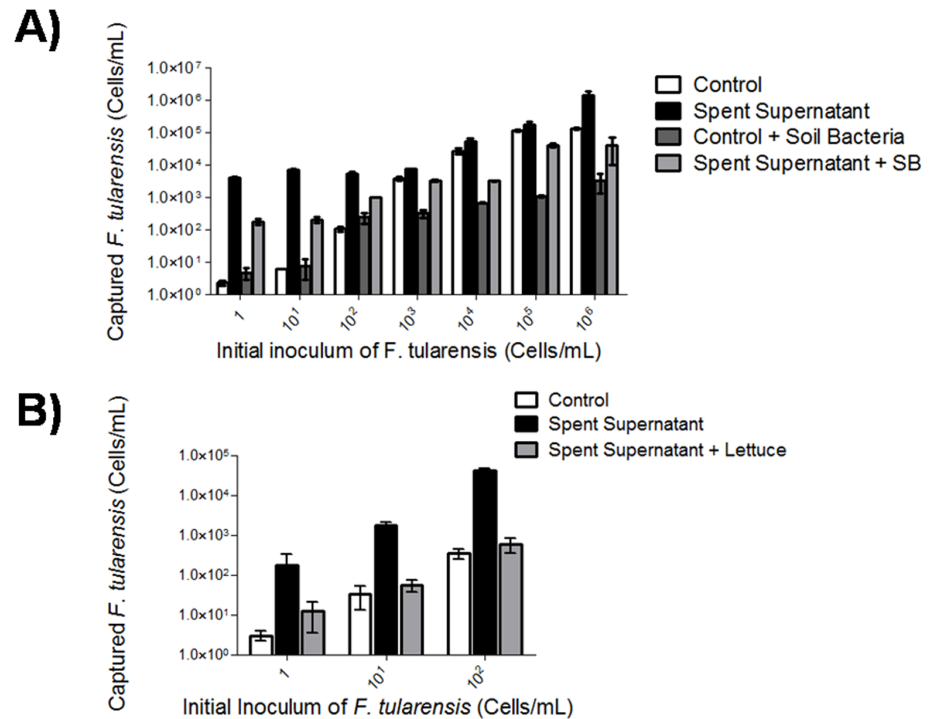


Fig. 2. Spent filtrate supplementation results in increased detection of *F. tularensis* in mixed cultures. **A)** *F. tularensis* was spiked in a ten-fold serial dilution in 10^9 CFU/mL of soil bacteria or inoculated as a pure culture and incubated in control broth (TSB with 0.1% L-cysteine) or spent filtrate (composition: TSB and 0.1% L-cysteine and 10% spent culture filtrate) overnight at 37°C with 120 rpm shaking speed. **B)** *F. tularensis* ($1-10^2$ cells/mL) was spiked in 10^9 CFU/mL of lettuce bacteria. Supplementation of TSB and 0.1% L-cysteine with spent culture filtrate resulted in *F. tularensis* detection at all inoculums ($1-10^2$ cells/mL). Control medium combined with lettuce showed Cp values greater than 34 and were considered negative for *F. tularensis*. All samples were conducted in triplicate with technical triplicates. Legend: Control=white bar, Spent filtrate=black bar, Control and soil bacteria=dark grey bar, and Spent filtrate and soil/lettuce bacteria=light grey bar.

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fold increase was observed when compared against *F. tularensis* mixed with soil bacteria in standard medium (Fig. 2A). Given that the spent culture filtrate resulted in enhanced detection at low inoculums ($1-10^2$ cells/mL), we focused on detection of *F. tularensis* at $1-10^2$ cells/mL spiked in lettuce bacteria (Fig. 2B). Supplementation of TSB and 0.1% L-cysteine with spent culture filtrate resulted in increased growth of *F. tularensis* mixed with lettuce bacteria at all inoculums ($1-10^2$ cells/mL) in comparison to standard medium (Fig. 2B). It is interesting to note that unlike soil bacteria, inclusion of lettuce bacteria inhibited *F. tularensis* in control samples (Fig. 2B). Together these data confirm the findings presented by Halmann et al. and suggest that the increased growth of *F. tularensis* due to the addition of spent culture filtrate is unhindered by the presence of other bacteria [35].

DNA aptamer cocktail captures *F. tularensis* in mixed cultures of bacteria

In order to further lower the range of detection, we generated DNA aptamers against *F. tularensis*, which may be applied to diagnostics in food matrices to achieve physical separation of *F. tularensis* from background bacteria. Aptamers, short RNA and single stranded DNA sequences, have high affinities to their selected receptors, show limited cross-reactivity to homologous targets, and may serve as pre-analytical tools and biosensors for food surveillance [37–41]. DNA aptamers against *F. tularensis* were enriched through 11 iterations of SELEX and 4 iterations of counter-SELEX. Confirmation of *F. tularensis* binding to the selected aptamer pool (from SELEX round 8) was determined by Southwestern blot analysis. The selected aptamer pool showed binding to 10^3 cells of *F. tularensis* as opposed to the unselected aptamer library which could not detect the presence of *F. tularensis* at this concentration (*data not shown*). Sequencing of 188 transformants containing aptamer inserts revealed 10 redundant DNA motif sequence groups (S1 Fig.). A single aptamer (those with the highest number of repeat clones) were selected from each motif group (Table 1) for further testing against *F. tularensis*.

Individual aptamers were tested for *F. tularensis* binding using *fopA* real-time PCR analysis. Cp values did not significantly differ from one another; therefore, the 10 aptamers were combined into a cocktail to increase binding efficiency (*data not shown*). The aptamer cocktail bound to M-280 Dynabeads was separately incubated with *F. tularensis* control and spiked lettuce or soil solutions. Using pure cultures of *F. tularensis* as well as mixed cultures (both soil and lettuce solutions), the range of *F. tularensis* cell capture was 10^2 – 10^6 cells/mL (Fig. 3A and B). Furthermore, *F. tularensis* spiked with lettuce bacteria was fully recovered at 10^2 – 10^6 cells/mL (Fig. 3B) while bead controls did not capture *F. tularensis* in pure or mixed cultures (Fig. 3). However, aptamers failed to capture *F. tularensis* at low inoculums (1–10 cells/mL) (Fig. 3). In summary, we have developed a DNA aptamer cocktail to enrich for *F. tularensis* from resident bacteria in food and environmental matrices at 10^2 – 10^6 cells/mL.

Two-step enrichment process provides optimal recovery of *F. tularensis* in mixed cultures

In order to achieve *F. tularensis* recovery from small inoculums and physical separation in the presence of complex food and environmental matrices, we combined growth enhancement and DNA aptamer capture. Resident bacteria from lettuce and soil were utilized in this study as these sources represent food and environments that rabbits, reservoirs for *F. tularensis*, would encounter [8]. First, *F. tularensis* growth was increased by the addition of spent culture filtrate to standard medium during overnight incubation. This was subsequently followed with *F. tularensis* capture and separation from resident bacteria using the DNA aptamer cocktail. This two-step approach resulted in a lower limit of detection from a starting inoculum of 1 cell/mL (Fig. 4). Data suggests that enrichment with

Table 1. Repeat DNA sequences and number of clones from sequenced aptamer pool^a.

DNA Aptamer	Sequence (5'---3')	No. of Repeats
FT17	CGCGTCAGAGGTGTGTCGGGGCTGTGTAGATCTACATGGG	17
FT12	CAGTCGCTTTCCGTTCTCCGGCAGGTTTCATTGTGGTTTCG	12
FT11	CATATCAGGTCGTCACCGTAACAGAGCTCTCGCAATCACG	11
FT9	CAAAGGCAGCAGTAGCATGGCGATTTACATCAATTATTGG	9
FT8	CAATATCAGAAGTAGCGCGAAGGACGACATGTCAGGAAGG	8
FT7	CACACATCCTCGCAGCCTCGTACCTGATTCCAGTCTATTG	7
FT6	CCAGGAAGGCGAGAGCCGAGAAGCGATCCTTGGGTATAGG	6
FT5	CGGCATCCGTTACAGTACCTGTCTAGTTATCACCGTTTG	5
FT5.1	CAAGCAGAGTTCCGAGACACAGTACCACACGCATATCCGG	5
FT4	CACACAGAGACGGTGAAGGCGCCGACCAGTTCCTAAAGAG	4

^aSequence aptamer pool belonged to the 11th round of SELEX.

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spent culture filtrate followed by physical separation and capture by the DNA aptamer cocktail can be utilized alongside current diagnostics as a pre-analytical processing tool.

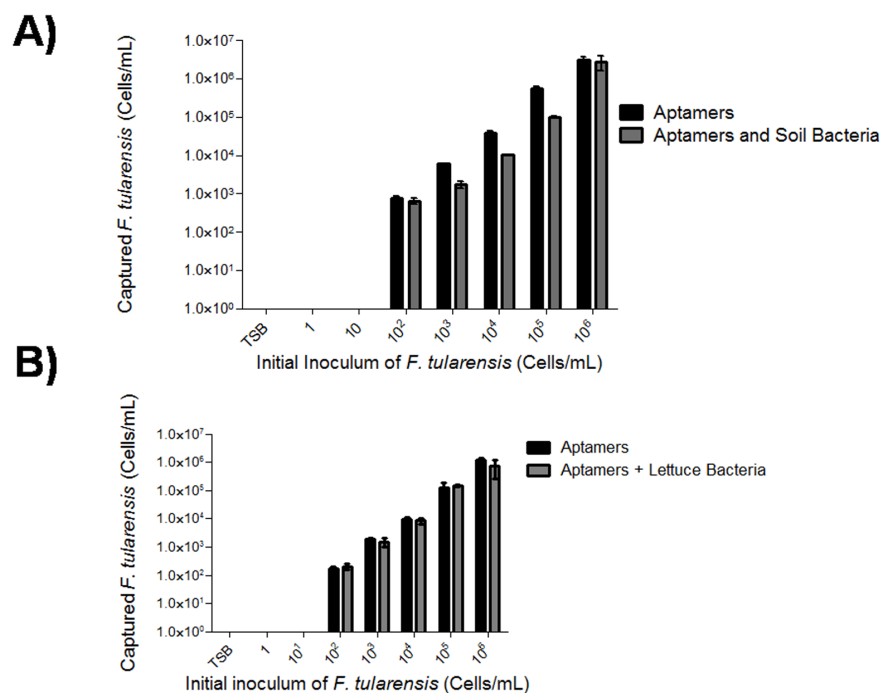


Fig. 3. DNA aptamers against *F. tularensis* specifically capture *F. tularensis* in mixed bacteria samples. A biotinylated aptamer cocktail composed of 10 aptamers (0.4 pmol each) was bound to M-280 streptavidin Dynabeads. *F. tularensis* (1–10⁶ cells/mL) was separately mixed with **A)** 10⁹ CFU/mL of soil bacteria or **B)** lettuce bacteria and aptamer cocktail beads or control beads (beads without aptamers) and incubated for 1 h at room temperature. Samples were denatured and the supernatant was subjected to real-time PCR analysis. Bead controls showed Cp values greater than 34 and were considered negative for *F. tularensis*. Legend: Aptamers=black bar, Aptamers and Soil Bacteria=dark grey bar.

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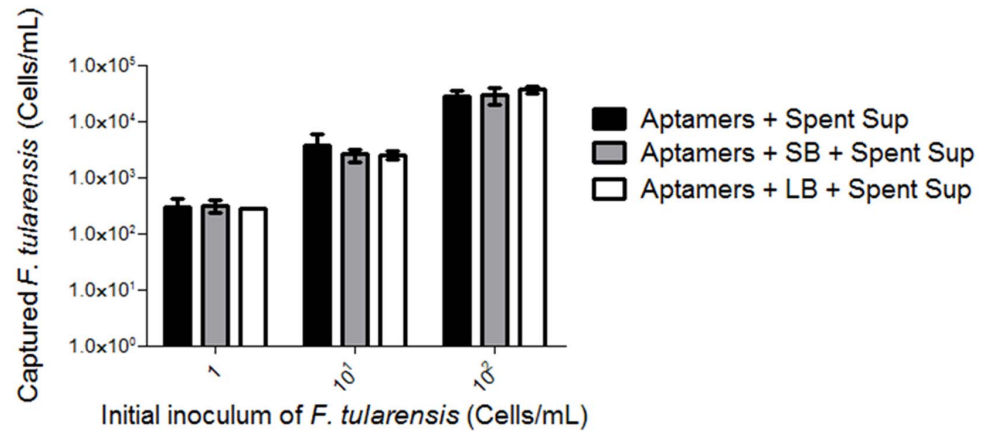


Fig. 4. Combined enrichment with spent culture supernatant and DNA aptamer capture leads to complete isolation of *F. tularensis*. *F. tularensis* ($1-10^2$ cells/mL) mixed samples in soil and/or lettuce homogenates were enriched for by overnight incubation with 10% spent culture supernatant and *F. tularensis* cells were subsequently captured by the DNA aptamer pool. Samples were denatured and the supernatant was subjected to real-time PCR analysis. Aptamers combined with either soil or lettuce bacteria alone (controls) showed Cp values greater than 34 and were considered negative for *F. tularensis*. Legend: Aptamers and Spent Sup (Spent supernatant)=black bar, Aptamers and SB and Spent Sup=grey bar, and Aptamers and LB and Spent Sup=white bar.

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Carnosine yields enhanced growth for *F. tularensis* growth

Although the two-step enrichment process is an easy method that does not require specialized equipment for *F. tularensis* detection, it is not advisable to collect liter amounts of *F. tularensis* spent culture filtrate for multiple sample testing. Therefore, we sought to identify the GIS component within the *F. tularensis* spent supernatant to enable chemical synthesis and subsequent supplementation to control medium. We hypothesized that the GIS accumulates overtime; therefore, we selected spent culture filtrates at 0.5, 2, 8 and 24 h for UPLC/MS analyses. The analytical workflow to identify chemical targets specific growth enhancement entailed untargeted UPLC/MS profiling of spent culture filtrates; PCA analyses, chemometrics OPLS-DA; evaluation of extracted ion chromatograms (XICs) for each GIS specific ion; database searches to provide chemical identities of GIS features; mass accuracy confirmation and evaluation of fragmentation patterns for each target of MS simultaneously acquired high-collision energy mass spectra. PCA showed that the greatest separation between time points for both positive and negative ionization was achieved when comparing 0.5 and 2 h separately to 8 and 24 h post incubation (S2 Fig.). Therefore, the following comparisons were evaluated by OPLS-DA: 0.5 h v 8 or 24 h and 2 h v 8 or 24 h. OPLS-DA data were visualized by S-plots and those features unique to early (0.5 and 2 h) or late (8 or 24 h) were selected for further investigation. S-plot examples for positive and negative ionization and target selection for 2 v. 24 h are shown in S3 Fig. Similar S-plots were generated for 0.5 v 24 h and early time points v. 8 h (*data not shown*). Chemical entities with high confidence (high correlation) were defined as being unique to either early (-1.0) or late (1.0) time points and selected in lower

left and upper right quadrants, respectively (S3 Fig.; selection represented by fuchsia boxes). Trend plots for each selected feature were created to determine abundance at comparison time points (S4A Fig.) with manual evaluation of XICs (S4B Fig.). Manual validation of tentative chemical entities (mined using Yeast, ChEBI, ChemSpider, KEGG, and LIPID MAPS databases) using UPLC/MS raw data and chemical standards failed. However, manual database searches (limited to parent ions and mass fractionation patterns of m/z of 397 and 297) as well as extensive literature based searches uncovered two potential pathways for enhanced growth 1) an autoinducer (such as N-acetyl-homoserine lactones (AHLs) or 2) carnosine, a chemical previously reported to cause enhanced growth and biofilm formation in *E. coli* [42]. Addition of commercially available AHLs to control broth did not yield enhanced growth of overnight cultures of *F. tularensis* (data not shown). Carnosine supplementation at concentrations of 0.625–2.5 mg/mL to control broth resulted in increased growth of *F. tularensis* albeit to a lesser extent (10 fold) than the spent culture filtrate (Fig. 5). Higher concentrations of carnosine, particularly at 10 mg/mL, had a bacteriocidal effect on *F. tularensis* (Fig. 5).

Discussion

Reliable and time-sensitive detection of a biological contaminant within the food network is essential to advise management and treatment procedures during a potential outbreak. The gold standard for microbe detection is successful cultivation of the agent in vitro; however, this remains a challenge for intracellular pathogens as many are either slow-growers or dormant in vitro. *F. tularensis*, a possible aerosol and biocontaminant in food, requires incubation for 2–4 days at 37°C when supplemented with cysteine, thioglycolate and/or blood. Furthermore, culture of *F. tularensis* in biological samples is also difficult to achieve in broth culture, unless a high starting inoculum is used.

Few studies have focused on the development of tools capable of detecting *F. tularensis* in food and the environment [24–27]. This is a significant concern given the potential of *F. tularensis* to contaminate food (accidentally or deliberately). A 2009 study by Day and Whiting utilized a macrophage cell culture model (RAW 264.7) to isolate and enrich for *F. tularensis* in infant formula, liquid egg whites, and lettuce. Day's and Whiting's study was unique in the fact that they took advantage of *F. tularensis*' niche cell, where the pathogen would efficiently replicate inside [24]. Once noninvasive resident bacteria were discarded from the cell culture medium and sufficient time was allowed for *F. tularensis* growth, real-time PCR analysis could identify as little as 10 CFU/mL of *F. tularensis* in infant formula and lettuce (5 h post-infection) and liquid egg whites (18 h post-infection) [24]. Although this method successfully enhanced the growth of and separated *F. tularensis* from food bacteria, it required specialized training in cell culture maintenance and access to equipment. This may not be feasible for field testing.

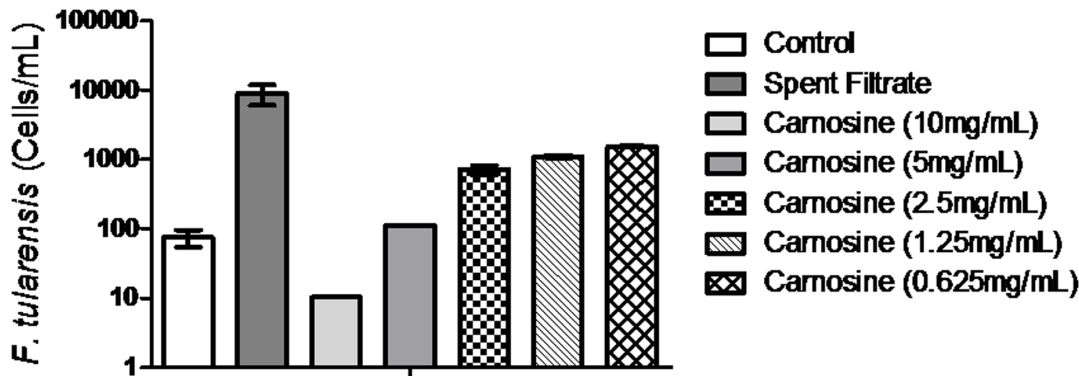


Fig. 5. Carnosine increases *F. tularensis* growth. *F. tularensis* (10^2 cells/mL) were incubated overnight at 37°C with agitation in control broth alone or supplemented with 10% spent culture filtrate or various concentrations of carnosine (0.625 mg/mL–10 mg/mL). DNA was extracted from all samples and analyzed by *fopA* real-time PCR. All samples were conducted in triplicate with technical triplicates. Legend: Control=white bar, Spent filtrate=dark grey bar, [carnosine=10 mg/mL (light grey bar), 5.0 mg/mL (medium grey bar), 2.5 mg/mL (checkered pattern bar), 1.25 mg/mL (diagonal pattern bar) and 0.625 mg/mL (diamond pattern bar)].

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During the course of our investigation to create an improved cultivation medium for *F. tularensis*, we rediscovered initial experiments and subsequent observations reported by Halmann et al. [35,36]. Halmann et al. report the presence of a Growth Initiating Substance (GIS) from low inocula of *F. tularensis* (strains Schu, Schu-M16, Vavenly, 425-F4G, 301I0, and LVS) that enhanced the growth of dormant *F. tularensis* cells when supplemented in traditional culture medium [36]. Similar to the Halmann studies, we have also shown that supplementation of standard medium with 10% spent culture filtrate results in *F. tularensis* enhanced growth in pure and mixed (lettuce and soil homogenates) cultures (Fig. 1A and Fig. 2). This data suggests that the majority of *F. tularensis* cells are composed of dormant cells capable of resuscitation with the addition of spent culture filtrate. Through a series of Sephadex gel filtration and ion-exchange chromatography, Halmann et al. characterized the GIS as having a low-molecular weight and heat stable at neutral pH but destroyable if heated in an acidic solution [36]. Furthermore, GIS was shown to form metal complexes (iron and copper complexes) and have enhanced activity when supplemented with ornithine [36]. Halmann and colleagues conjectured that ornithine may be a precursor to the GIS and that the GIS may be a sideramine or related to sideramine production. However, our analysis of the spent culture filtrate using UPLC/MS analysis and subsequent extensive database searches did not find any chemical entities related to sideramine function. UPLC/MS trend plots uncovered unique chemical signatures that had *m/z* ratios of 297 and 397; however, database searches were unable to provide a definitive identification. Manual database and literature searches related to bacterial growth and chemical entities with a *m/z* of 297–397 revealed AHLs or carnosine as suitable candidates. While available AHLs did not result in enhanced *F. tularensis* growth, carnosine (0.625 mg–2.5 mg/mL) promoted a 10-fold increase in *F. tularensis* cells/mL (Fig. 5). However, increased growth by carnosine did not fully recapitulate growth seen by the spent culture

filtrate (Fig. 5). It is possible that other chemicals found within the spent filtrate act in concert with carnosine to increase *F. tularensis* growth or that the GIS component is a chemical related to carnosine and that additional side-chain components are responsible for the 10-fold difference. Higher concentrations of carnosine, specifically at 10 mg/mL, had a bacteriocidal effect (Fig. 5). This may be due to its ability to bind zinc and ferrous ions, which high carnosine concentrations may lead to iron toxicity or depletion of iron from the media [43]. Therefore, it is likely that *F. tularensis* may have a “Goldilocks” mechanism to regulate an appropriate amount of carnosine. These data suggest that carnosine functions both as a growth promoter and bactericide and that careful regulation by the bacterium must be conducted to ensure persistence. Further studies will need to revisit chemical entities identified by UPLC/MS to determine the exact GIS compound found in the spent supernatant and *F. tularensis* gene regulation in response to carnosine.

The second part of our *F. tularensis* enrichment process involves physical separation of *F. tularensis* from resident bacteria using DNA aptamer capture. Aptamers, short RNA or single-stranded DNA sequences, have high affinities to their target molecules and are sought after as an alternative to antibodies due to their ease of generation, amenability to work with various platforms and cost-effectiveness [38, 40, 44, 45]. We and others have shown that aptamers are effective tools to achieve separation of target bacteria from closely related microbes [37, 41, 46, 47]. We generated 10 redundant DNA aptamers, which were utilized in a cocktail to capture *F. tularensis* after growth enhancement. Physical separation of *F. tularensis* from resident bacteria by DNA aptamer capture increased the assay limit of detection and the recovered cell population was similar in number to that of pure cultures supplemented with spent culture filtrate (Figs. 3 and 4). Previous to our report, a study by Vivekananda and Kiel reported the generation of 25 unique DNA aptamer sequences specific for *F. tularensis* [48]. It is important to note that there are several key differences between the two study designs as well as data interpretation. In our study, we sequenced 188 transformants to reliably identify redundant motif sequences. Vivekananda and Kiel sequenced a total of thirty-five aptamer transformants and observed 10 duplications [48]. While we utilized the intact *F. tularensis* cell in rounds of SELEX and soil bacteria for counter-SELEX, Vivekananda and Kiel generated aptamers against a commercially available *F. tularensis* antigen and performed counter-SELEX against nitrocellulose membranes [48]. Although counter-SELEX would discard aptamers that bound to the nitrocellulose membrane, it is unclear if specificity for *F. tularensis* is greatly increased. However, the 2006 study does examine cross-reactivity of the 25 aptamers (combined in a cocktail) to lysozyme, albumin and *B. henselae* of which there is some reactivity to *B. henselae* [48]. Given that the optical density (405 nm) of the aptamers against the *F. tularensis* antigen in an Aptamer-Linked Immobilized Sorbent Assay (ALISA) was reported to be ~ 0.1 , it would be impossible to distinguish from *B. henselae* (~ 0.3). DNA aptamers generated for this study failed to capture soil and lettuce bacteria and when combined with

spent culture filtrate and real-time PCR had an increased detection range for *F. tularensis* (Fig. 4).

The combination of spent culture filtrate or carnosine and DNA aptamer capture as a two-part enrichment method for *F. tularensis* may be amendable for field diagnostics. For example, this method may be used in tandem with a portable real-time PCR device for rapid detection of *F. tularensis*. Furthermore, this method is advantageous to concentrate *F. tularensis* cells for subtyping, which may impact decision making in a potential outbreak. Benefits of such a diagnostic tool may be further extended in bench research investigating the ecology of *F. tularensis* by screening of animal carcass samples collected from multiple sites and varying years. Current investigations in our laboratory seek to determine if aptamer captured *F. tularensis* can be re-cultured. Pure cultures of *F. tularensis* isolates from the environment would allow investigators to pursue downstream studies that go beyond diagnostics.

Materials and Methods

Bacterial Culture

F. tularensis subsp. *holarctica* Live Vaccine Strain (LVS) NR-14 (Biodefense and Emerging Infections Research Resources Repositories, NAID, NIH; herein referred to as *F. tularensis*) was maintained in tryptic soy agar (TSA) supplemented with 0.1% L-cysteine at 37°C. *F. tularensis* was subcultured in tryptic soy broth (TSB) supplemented with 0.1% L-cysteine (Sigma-Aldrich, St. Louis, MO) at 37°C with 120 rpm shaking. Environmental bacteria (herein referred to as soil bacteria) were obtained from one gram (g) of soil, hay and dust and cultured in TSB and diluted to 10^{-1} in TSB containing 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to represent low and high nutrition conditions at room temperature and 37°C under constant shaking at 120 rpm. Lettuce bacteria were cultured from bagged lettuce purchased from a local grocery store. Approximately 10 g of lettuce were mixed with 50 mL of TSB supplemented with 0.1% L-cysteine inside a sterile Stomacher 400 classic bag (Seward Laboratory Systems Inc., Port Saint Lucie, FL) and homogenized for 2 min at high speed in a Stomacher. Homogenate was subcultured at 10^{-1} in TSB supplemented with 0.1% L-cysteine at 37°C with 120 rpm shaking.

F. tularensis Growth Enrichment

F. tularensis was grown as described overnight at 37°C with shaking at 120 rpm. Upon completion of incubation, *F. tularensis* was pelleted at $7000 \times g$ for 10 min and the supernatant was filtered through a 0.22 μm syringe-driven filter (EMD Millipore, Billerica, MA) to remove any residual bacteria. An overnight culture of *F. tularensis* was serially diluted ten-fold and spotted on TSA supplemented with 0.1% L-cysteine (control) or control medium with 10% (v/v) spent filtrate, carnosine (0.625 mg/mL–10 mg/mL) (Sigma-Aldrich, St. Louis, MO), and N-Acetyl-homoserine lactones (AHLs) (Sigma-Aldrich, St. Louis, MO). All samples

were incubated overnight at 37°C and differences in growth were recorded the following day. The number of *F. tularensis* cells per mL were determined by *fopA* real-time PCR analysis described below.

Selection of DNA aptamers against *F. tularensis* using Systemic Evolution of Ligands by Exponential Enrichment (SELEX)

Aptamer candidates against *F. tularensis* were selected using a SELEX protocol. Briefly, a modified single-stranded aptamer library (WAP40m) consisting of a 40-mer randomized regions flanked by constant-primer binding regions (Integrated DNA Technologies, Inc., Coralville, IA) underwent four rounds of counter-SELEX iterations. The aptamer library was incubated for 30 min with 10^8 CFU/mL of soil bacteria (previously cultured overnight at both 25°C or 37°C) in TSB containing 0.1% L-cysteine at room temperature with gentle shaking (Labquake tube shaker) [45, 49]. Aptamers bound to soil bacteria were discarded and the filtrate was collected, which was used for positive rounds of SELEX against *F. tularensis*. Positive SELEX iterations involved exposure of the remaining aptamer library to 10^8 cells/mL of *F. tularensis* at room temperature with gentle shaking for 30 min. Unbound or weak-binding aptamers were removed from further rounds of SELEX by washing cells 6 times in 1X PBS containing 0.025% Tween-80 and twice with nuclease-free water. Aptamers bound to *F. tularensis* cells were resuspended in 100 μ L of nuclease-free water and subjected to Polymerase Chain Reaction (PCR) after every round of SELEX to amplify aptamer candidates. PCR was performed using a biotin-labeled on the primer's anti-sense strand (WP20R1; 5 pmol), an unlabeled primer (WP20F1; 5 pmol), 2X Hotstar taq polymerase (Qiagen, Valencia, CA) and the following program: 95°C for 15 min, followed by 15 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 7 min (Table 1). In order to enrich for aptamer sense strands needed for further rounds of SELEX, amplicons were applied as templates at 1:10 and 1:25 dilutions for asymmetric-touchdown PCR using 30 pmol of WP20F1 and 1.2 pmol of biotin labeled WP20R1. The asymmetric-touchdown program was 95°C for 15 min, followed by 9 cycles of 95°C for 15 s, 72°C for 15 s (gradually decreasing by 1°C each cycle), and 72°C for 15 s, followed by 11 cycles of 95°C for 15 s, 63°C for 15 s, and 72°C for 15 s, and a final extension at 72°C for 3 min. PCR amplicons were pooled and anti-sense strands were removed using a snap cool method with 50 μ L of Dynabeads M-280 streptavidin coated beads (Invitrogen, Carlsbad, CA). The amplicon sense strand was applied to the remaining rounds of SELEX. A total of 11 SELEX iterations were performed.

Cloning, sequencing and characterization of DNA aptamers against *F. tularensis*

Four hundred μ L of PCR amplicons from the 11th round of SELEX were purified using the MiniElute PCR purification kit (Qiagen, Valencia, CA). The purified PCR product was cloned into the TA-TOPO pCR 2.1 vector (Invitrogen,

Carlsbad, CA) and later transformed into TOP10 chemically competent *E. coli* cells per manufacturer's instructions. Transformed TOP10 *E. coli* cells (100 μ L) were plated on Luria-Bertani agar containing ampicillin (50 μ g/mL) and X-gal. Successful transformants were selected based on blue/white screening for disruption of *lacZ*. Colony PCR was performed on selected transformants using M13 primers and the following PCR conditions: 95°C for 15 min and 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min, and final extension for 72°C for 5 min (Table 2). One hundred and eighty-eight transformants with the correct amplicon size were sequenced and analyzed for redundancy using Sequencher (GeneCodes, Ann Arbor, MI) and CLUSTAL W in MEGA 4.0 (<http://www.megasoftware.net/mega4/mega.html>) (Table 2 and S1 Fig.). Redundant aptamers were further characterized using the m-fold program (<http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form>) available from the RNA Institute.

Spike and recovery of *F. tularensis*

An overnight broth culture of *F. tularensis* was washed three times in PBS, serially diluted ten-fold ($1-10^6$ cells/mL) in TSB supplemented with 0.1% L-cysteine and spiked in 10^9 CFU/mL of soil or lettuce bacteria (homogenized solutions) or medium alone. Dilutions in medium alone and those mixed with soil or lettuce bacteria were supplemented with 10% filtered spent medium. Control samples did not include supplementation with spent filtrate. All samples were incubated overnight at 37°C with shaking at 120 rpm. After incubation, samples were pelleted at 8,000 rpm and filtrates were discarded. Genomic DNA was extracted from samples using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) per manufacturer's instructions. DNA was treated with 4.0 μ L of RNase A (Qiagen, Valencia, CA) for 2 min at room temperature. Genomic DNA was suspended in 50 μ L of nuclease-free water. All treatments were conducted in triplicate. All experiments were conducted in triplicate. Genomic DNA was stored at -20°C until real-time PCR analysis.

Spike and aptamer cocktail recovery of *F. tularensis*

An overnight broth culture of *F. tularensis* was prepared and spiked in soil or lettuce bacteria (homogenized solutions) or medium alone as described above. Approximately 0.4 pmol of each selected biotin tagged aptamer (Table 1) was mixed together as a cocktail and bound to Dynabeads M-280 streptavidin coated beads (Invitrogen, Carlsbad, CA) using a modified protocol [38]. Briefly, 25 μ L of M-280 streptavidin beads were loaded into individual wells in a 96 well PCR plate, washed 3 times in PBS and incubated with the biotin labeled aptamer cocktail overnight at 37°C with shaking at 200 rpm. After incubation, aptamers bound to M-280 streptavidin beads were washed thrice in 200 μ L of PBS and blocked for 2 h at room temperature with shaking at 200 rpm using 100 μ L of PBS containing 0.05% Tween-80 and 0.05% bovine serum albumin (BSA). Aptamer M-280 streptavidin beads were later washed twice in 200 μ L of PBS and once with 200 μ L

Table 2. Primers used in this study.

Primer	Sequence (5'---3')
WP20F1	AGTGCAAGCAGTATTCCGGTC
WP20R1	TAAAGCTGATGCGTGATGCC
M13F	GTTTTCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC
<i>fopA</i> _F	GTTCAAGGTGCTTGGATG
<i>fopA</i> _R	TGCAACAGCGCTAAGAGTTTT

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of nuclease-free water. One hundred μL of *F. tularensis* control and spiked samples (soil or lettuce bacteria) were mixed separately in individual wells containing aptamer M-280 beads and incubated for 1 h at room temperature with 200 rpm shaking. Aptamer M-280 beads were washed 5 times in 200 μL of PBS containing 0.05% Tween-80 and thrice in 200 μL of nuclease-free water and resuspended in 100 μL of nuclease-free water. In order to release the genomic DNA from captured bacterial cells, they were heated to 95°C for 10 min. All treatments were conducted in triplicate and each experiment was performed thrice.

Two-step enrichment of *F. tularensis* in Mixed Bacterial Cultures

An overnight culture of *F. tularensis* was washed thrice in PBS and diluted to $1-10^2$ cells/mL in TSB supplemented with 0.1% L-cysteine. *F. tularensis* dilutions were either mixed in medium with or without 10% spent culture filtrate alone or with 10^9 CFU/mL of lettuce or soil bacteria. All samples were incubated overnight at 37°C with shaking at 120 rpm and later mixed separately with aptamer M-280 streptavidin beads. All treatments were conducted in triplicate and each experiment was performed thrice.

Real-time PCR Analysis of *F. tularensis* in Pure and Mixed Bacterial Cultures

Real-time PCR of the *fopA* gene was performed using *F. tularensis* spiked with soil or lettuce bacteria and control samples using a Lightcycler 480 Probes Master Mix and Universal ProbeLibrary Probe #10 (Roche, Indianapolis, IN) per manufacturer's recommendations (Table 2). All samples were analyzed on the Roche Lightcycler 480II and corresponding software (Roche, Indianapolis, IN). The following real-time PCR conditions were applied: 95°C for 5 min and 45 cycles of 95°C for 10 s, 50°C for 30 s and 72°C for 1 s. Primers were designed using the Universal ProbeLibrary Assay Design Center (<https://www.roche-applied-science.com/>). The number of cells/mL was calculated for each sample based on a *F. tularensis* cells/mL standard curve. The standard curve was generated by a ten-fold serial dilution of *F. tularensis* genomic DNA with a known cells/mL ($10-10^7$ cells/mL). Cross-point (Cp) values were calculated using the second derivative

maximum method available in the 480 II lightcycler software (Roche, Indianapolis, IN) for each dilution in the standard curve and for all test samples [50]. C_p values from the standard curve ranged 13–34 cycles. Sample C_p values outside of the standard curve range were designated as negative for *F. tularensis*. T_m Calling analysis was used to verify negative samples [50]. In the case of a negative sample, T_m Calling results showed two-shouldered or no melting peaks. The former indicated the production of primer dimers. All samples were conducted in triplicate.

Supporting Information

S1 Fig. DNA Aptamer Sequence Motifs. The DNA aptamer pool was sequenced after the 11th round of SELEX and sequences were aligned using CLUSTAL W in MEGA 4.0. Ten repeated motifs were identified and 1 DNA aptamer was selected from each motif group for further characterization.

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S2 Fig. Principal Component Analyses (PCA) of *F. tularensis* spent culture filtrate. MarkerLynx software was used to generate PCA plots of UPLC/MS data. PCA plots include positive (*right*) and (*negative*) ionization at 0.5 h (blue circles) v 24 h (seafoam green) and 2 h (blue circles) v 24 h.

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S3 Fig. OPLS-DA Generated S-Plots. Chemometric S plot resulting from OPLS-DA of UPLC/MS analysis of *F. tularensis* spent culture filtrates at 2 h versus 24 h with positive and negative ionization. Similar S-plots were generated for 0.5 h versus 24 h. The y-axis represents correlation (confidence; time point specific), while the x-axis represents the coefficient (specificity). The selection boxes represent features that are highly specific to each time point. Selection boxes are noted in fuchsia.

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S4 Fig. UPLC/MS Characterization. A) A chemometric trend plot depicting the relative abundance of the chemical entity found at 0.5 h and 24 h. B) Extracted ion chromatogram (XIC) for m/z 397.2205.

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Author Contributions

Conceived and designed the experiments: EAL PW SE REI SS. Performed the experiments: EAL PW SE AA KB. Analyzed the data: EAL SS PW SE REI.

Contributed to the writing of the manuscript: SS EAL PW SE KB AA REI. Voted on the manuscript: EAL SS.

References

1. **Hartnett E, Paoli GM, Schaffner DW** (2009) Modeling the public health system response to a terrorist event in the food supply. *Risk Anal* 29: 1506–1520.
2. **Hennessy DA** (2008) Economic aspects of agricultural and food biosecurity. *Biosecur Bioterror* 6: 66–77.
3. **Day JB, Nguyen H, Sharma SK, Al-Khalidi SF, Hao YY** (2009) Effect of dehydrated storage on the survival of *Francisella tularensis* in infant formula. *Food Microbiol* 26: 932–935.
4. **Willke A, Meric M, Grunow R, Sayan M, Finke EJ, et al.** (2009) An outbreak of oropharyngeal tularemia linked to natural spring water. *J Med Microbiol* 58: 112–116.
5. **Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, et al.** (2001) Tularemia as a biological weapon: medical and public health management. *JAMA* 285: 2763–2773.
6. **Egan JR, Hall IM, Leach S** (2011) Modeling inhalational tularemia: deliberate release and public health response. *Biosecur Bioterror* 9: 331–343.
7. **Oyston PC, Sjostedt A, Titball RW** (2004) Tularemia: bioterrorism defence renews interest in *Francisella tularensis*. *Nat Rev Microbiol* 2: 967–978.
8. **Foley JE, Nieto NC** (2010) Tularemia. *Vet Microbiol* 140: 332–338.
9. **Gill V, Cunha BA** (1997) Tularemia pneumonia. *Semin Respir Infect* 12: 61–67.
10. **Ojeda SS, Wang ZJ, Mares CA, Chang TA, Li Q, et al.** (2008) Rapid dissemination of *Francisella tularensis* and the effect of route of infection. *BMC Microbiol* 8: 215.
11. **Bevanger L, Maeland JA, Naess AI** (1988) Agglutinins and antibodies to *Francisella tularensis* outer membrane antigens in the early diagnosis of disease during an outbreak of tularemia. *J Clin Microbiol* 26: 433–437.
12. **Sharma N, Hotta A, Yamamoto Y, Fujita O, Uda A, et al.** (2013) Detection of *Francisella tularensis*-specific antibodies in patients with tularemia by a novel competitive enzyme-linked immunosorbent assay. *Clin Vaccine Immunol* 20: 9–16.
13. **Massey ED, Mangiafico JA** (1974) Microagglutination test for detecting and measuring serum agglutinins of *Francisella tularensis*. *Appl Microbiol* 27: 25–27.
14. **Bevanger L, Maeland JA, Kvan AI** (1994) Comparative analysis of antibodies to *Francisella tularensis* antigens during the acute phase of tularemia and eight years later. *Clin Diagn Lab Immunol* 1: 238–240.
15. **Martin-Serradilla JI, Sanchez Navarro JJ, Arias Giralda M, San Jose Alonso J** (2004) [Evolution of serological characteristics in 26 patients with tularemia three years after the outbreak]. *Rev Clin Esp* 204: 351–354.
16. **Antwerpen MH, Schacht E, Kaysser P, Splettsjoesser WD** (2013) Complete Genome Sequence of a *Francisella tularensis* subsp. *holarctica* Strain from Germany Causing Lethal Infection in Common Marmosets. *Genome Announc* 1.
17. **Chaudhuri RR, Ren CP, Desmond L, Vincent GA, Silman NJ, et al.** (2007) Genome sequencing shows that European isolates of *Francisella tularensis* subspecies *tularensis* are almost identical to US laboratory strain Schu S4. *PLoS One* 2: e352.
18. **Broekhuijsen M, Larsson P, Johansson A, Bystrom M, Eriksson U, et al.** (2003) Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. *J Clin Microbiol* 41: 2924–2931.
19. **Petrosino JF, Xiang Q, Karpathy SE, Jiang H, Yerrapragada S, et al.** (2006) Chromosome rearrangement and diversification of *Francisella tularensis* revealed by the type B (OSU18) genome sequence. *J Bacteriol* 188: 6977–6985.

20. **Sridhar S, Sharma A, Kongshaug H, Nilsen F, Jonassen I** (2012) Whole genome sequencing of the fish pathogen *Francisella noatunensis* subsp. *orientalis* Toba04 gives novel insights into *Francisella* evolution and pathogenicity. *BMC Genomics* 13: 598.
21. **Fulop M, Leslie D, Titball R** (1996) A rapid, highly sensitive method for the detection of *Francisella tularensis* in clinical samples using the polymerase chain reaction. *Am J Trop Med Hyg* 54: 364–366.
22. **Versage JL, Severin DD, Chu MC, Petersen JM** (2003) Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. *J Clin Microbiol* 41: 5492–5499.
23. **Ellis J, Oyston PC, Green M, Titball RW** (2002) Tularemia. *Clin Microbiol Rev* 15: 631–646.
24. **Day JB, Whiting RC** (2009) Development of a macrophage cell culture method to isolate and enrich *Francisella tularensis* from food matrices for subsequent detection by real-time PCR. *J Food Prot* 72: 1156–1164.
25. **Whitehouse CA, Kesterson KE, Duncan DD, Eshoo MW, Wolcott M** (2012) Identification and characterization of *Francisella* species from natural warm springs in Utah, USA. *Lett Appl Microbiol* 54: 313–324.
26. **Humrighouse BW, Adcock NJ, Rice EW** (2011) Use of acid treatment and a selective medium to enhance the recovery of *Francisella tularensis* from water. *Appl Environ Microbiol* 77: 6729–6732.
27. **Petersen JM, Carlson J, Yockey B, Pillai S, Kuske C, et al.** (2009) Direct isolation of *Francisella* spp. from environmental samples. *Lett Appl Microbiol* 48: 663–667.
28. **Storrs EE, Walsh GP, Burchfield HP, Binford CH** (1974) Leprosy in the armadillo: new model for biomedical research. *Science* 183: 851–852.
29. **Merriott J, Shoemaker A, Downs CM** (1961) Growth of *Pasteurella tularensis* in cultured cells. *J Infect Dis* 108: 136–150.
30. **Abd H, Johansson T, Golovliov I, Sandstrom G, Forsman M** (2003) Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. *Appl Environ Microbiol* 69: 600–606.
31. **Sansonetti PJ, Ryter A, Clerc P, Maurelli AT, Mounier J** (1986) Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infect Immun* 51: 461–469.
32. **Sun Z, Zhang Y** (1999) Spent culture supernatant of *Mycobacterium tuberculosis* H37Ra improves viability of aged cultures of this strain and allows small inocula to initiate growth. *J Bacteriol* 181: 7626–7628.
33. **Shleeva MO, Bagramyan K, Telkov MV, Mukamolova GV, Young M, et al.** (2002) Formation and resuscitation of “non-culturable” cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase. *Microbiology* 148: 1581–1591.
34. **Mukamolova GV, Kormer SS, Kell DB, Kaprelyants AS** (1999) Stimulation of the multiplication of *Micrococcus luteus* by an autocrine growth factor. *Arch Microbiol* 172: 9–14.
35. **Halmann M, Benedict M, Mager J** (1967) Nutritional requirements of *Pasteurella tularensis* for growth from small inocula. *Journal of General Microbiology* 49: 451–460.
36. **Halmann M, Mager J** (1967) An endogenously produced substance essential for growth initiation of *Pasteurella tularensis*. *Journal of General Microbiology* 49: 461–468.
37. **Joshi R, Janagama H, Dwivedi HP, Senthil Kumar TM, Jaykus LA, et al.** (2009) Selection, characterization, and application of DNA aptamers for the capture and detection of *Salmonella enterica* serovars. *Mol Cell Probes* 23: 20–28.
38. **Lamont EA, He L, Warriner K, Labuza TP, Sreevatsan S** (2011) A single DNA aptamer functions as a biosensor for ricin. *Analyst* 136: 3884–3895.
39. **Ellington AD, Szostak JW** (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature* 346: 818–822.
40. **Gold L** (1995) The SELEX process: a surprising source of therapeutic and diagnostic compounds. *Harvey Lect* 91: 47–57.
41. **Chang YC, Yang CY, Sun RL, Cheng YF, Kao WC, et al.** (2013) Rapid single cell detection of *Staphylococcus aureus* by aptamer-conjugated gold nanoparticles. *Sci Rep* 3: 1863.

42. **Brombacher E, Dorel C, Zehnder AJ, Landini P** (2003) The curli biosynthesis regulator CsgD coordinates the expression of both positive and negative determinants for biofilm formation in *Escherichia coli*. *Microbiology* 149: 2847–2857.
43. **Guiotto A, Calderan A, Ruzza P, Borin G** (2005) Carnosine and carnosine-related antioxidants: a review. *Current medicinal chemistry* 12: 2293–2315.
44. **Tian Y, Adya N, Wagner S, Giam CZ, Green MR, et al.** (1995) Dissecting protein:protein interactions between transcription factors with an RNA aptamer. *RNA* 1: 317–326.
45. **Wongphatcharachai M, Wang P, Enomoto S, Webby RJ, Gramer MR, et al.** (2013) Neutralizing DNA aptamers against swine influenza H3N2 viruses. *J Clin Microbiol* 51: 46–54.
46. **Zelada-Guillen GA, Riu J, Duzgun A, Rius FX** (2009) Immediate detection of living bacteria at ultralow concentrations using a carbon nanotube based potentiometric aptasensor. *Angew Chem Int Ed Engl* 48: 7334–7337.
47. **Duan N, Ding X, Wu S, Xia Y, Ma X, et al.** (2013) In vitro selection of a DNA aptamer targeted against *Shigella dysenteriae*. *J Microbiol Methods*.
48. **Vivekananda J, Kiel JL** (2006) Anti-*Francisella tularensis* DNA aptamers detect tularemia antigen from different subspecies by Aptamer-Linked Immobilized Sorbent Assay. *Lab Invest* 86: 610–618.
49. **Wang P, Hatcher KL, Bartz JC, Chen SG, Skinner P, et al.** (2011) Selection and characterization of DNA aptamers against PrP(Sc). *Exp Biol Med (Maywood)* 236: 466–476.
50. **Rasmussen R** (2001) Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K, editors. *Rapid Cycle Real-Time PCR: Methods and Applications*. Berlin: Springer. 21–34.