Evidence of vector borne transmission of Salmonella enterica subsp. enterica ser. Gallinarum and fowl typhoid mediated by the poultry red mite, Dermanyssus gallinae (De Geer, 1778)

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Research

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Abstract

Background: The poultry red mite Dermanyssus gallinae (De Geer 1778) is a major ectoparasite of poultry. Infestations are found in most laying hen farms in Europe, and breeder flocks have also been reported to be affected. Mite infestation has detrimental effects on animal welfare, are a cause of significant economic losses, and D. gallinae is often considered as a pathogen vector. Despite suspicion of a close relationship between the poultry red mite and Salmonella enterica subsp. enterica ser. Gallinarum biovar Gallinarum (S. Gallinarum), the causative agent of fowl typhoid (FT), there has been no definitive proof of mite-mediated transmission. Therefore, an investigation was conducted to determine if D. gallinae-mediated transmission of S. Gallinarum could be demonstrated among four different hen groups.

Methods: Two groups of 8 hens (A and B) were experimentally infected with S. Gallinarum in two isolators. After seven days, when birds showed signs of FT, about 25,000 mites were introduced. After 3 days, mites were harvested and used to infest other two hen groups of 8 (C and D), in two separate isolators. Health status of hens was constantly monitored; detection and quantification of S. Gallinarum were performed by PCR and qPCR from mites and organs of dead hens. The maximum likelihood estimation of the infection rate and mite vectorial capacity were calculated.

Results: Clinical disease was observed in groups infected with S. Gallinarum (A and B) and in hens of groups C and D infested with mites harvested from the isolators containing groups A and B. In all 4 groups, S. Gallinarum was detected from liver, spleen, ovary and cecum of hens, thus confirming the diagnosis of FT. Mite analysis demonstrated the presence of the pathogen, with an estimated infection rate ranging between 13.72 and 55.21 infected per thousand mites. Vectorial capacity was estimated to be 73.79.

Conclusion: Mites harvested from birds infected with S. Gallinarum were shown to carry the mite, and then to transfer S. Gallinarum to isolated groups of pathogen-free birds that subsequently showed signs of FT. Mite vectorial capacity was high, demonstrating that D. gallinae should be considered an effective vector of FT.

Background

The poultry red mite (PRM) Dermanyssus gallinae (De Geer, 1778) is a hematophagous ectoparasite of poultry that has a heavy impact on poultry farms worldwide [1]. Its life cycle, which consists of one non-feeding stage (larva) and three blood-sucking stages (protonymph, deutonymph, and adult, both male and female), usually occurs in two weeks or even less [2]. The effects of infestation on chickens include severe stress, irritation, reduction in weight gain and even death in case of massive infestation [3]. Detrimental effects are also observed in the quantity [4] and quality [5] of egg production. Overall, the costs associated with treatment, preventive measures and lost working days have been estimated at about 231 M€ per year in the EU [6].
Beyond the direct effects, many studies have surmised the role of *D. gallinae* as a vector of pathogens [7]. Association with *D. gallinae* has been reported for several viral and bacterial species, such as avian influenza virus [8], *Erysipelothrix rhusiopathiae* [9], *Chlamydia psittaci* [10], *Coxiella burnetii* and *Borrelia burgdorferi* sensu lato [11]. However, very few publications report the actual demonstration of transmission of pathogens mediated by the poultry red mite. Studies by Valiente Moro et al. [12,13] showed that *D. gallinae* could be infected by *Salmonella enterica* subsp. *enterica* ser. Enteritidis (*S. Enteritidis*), and that chicks could be infected through contaminated mites. Other than *S. Enteritidis*, the non-motile *Salmonella enterica* subsp. *enterica* ser. Gallinarum biovar Gallinarum (*S. Gallinarum*), belonging to the serogroup D according to the Kauffman-White scheme [14], has a direct impact on the poultry system. In fact, while *S. Enteritidis* is a threat to public health, *S. Gallinarum* is the etiological agent of fowl typhoid (FT), a septicemic disease that mainly affects chickens and turkeys [15].

Clinical signs of FT include diarrhea, depression, anorexia, ruffled feathers, and pale combs. Death may occur in four days. Other than mortality, decreased feed consumption and reduction in egg production are usually observed. At necropsy, the liver appears enlarged, bronze greenish and friable, often with scattered necrotic foci, and the spleen is enlarged and friable, too. Catarrhal enteritis is observed in most cases, and it is also possible to find enlargement of the heart with small pinhead-sized white foci on the myocardium, hemorrhages in pericardial fat, endocardium, and proventriculus. Severe congestion of lungs, necrotic ovaritis, catarrhal peritonitis, and congestion of kidneys may be observed [15, 16].

Horizontal transmission of FT has been widely described [17, 18], and some authors have also hypothesized that a vector-mediated dissemination of the disease could be possible [19], but no evidence was further collected. Recently, a field investigation showed a close relationship between *D. gallinae* infestation and *S. Gallinarum* circulation in a hen flock [20]. That study also verified that mites remained infected by *S. Gallinarum* between two subsequent production cycles, even in the absence of hosts.

In the light of those considerations, this investigation was carried out to definitely demonstrate the vector-mediated transmission of FT by reproducing all steps of the process under conditions of secure hen isolation.

**Methods**

*Study design*

Thirty-two 10-month-old Hy-Lyne® Brown laying hens, *Salmonella* spp. free, were included in the study. Before the experimental phase, the animals were raised in a protected area at the facilities of the Department of Veterinary Medicine of the University of Bari. They were vaccinated against Marek's disease, Newcastle disease, and infectious bronchitis when one-day old, while no vaccines were administered for *S. Gallinarum* or other *S. enterica* serovars. Animals were not treated with acaricides or antibiotics.
Each hen, identified by a unique marked ring on the left leg, was randomly selected and assigned to one of four groups, each consisting of 8 birds. The first two groups, A and B, were housed in two isolators Bioflex® B40 Rigid Body Poultry Isolator (Bell Isolation Systems, Livingston, UK) and the other two, C and D, in two isolators HM 1500 (Montair Process Technology, Kronenberg, The Netherlands). Isolators were located in two different rooms under controlled environmental conditions (temperature 25 ± 3 °C, relative humidity 65-75%). Light cycle consisted of 12 hours of light per day, from 8:00 a.m. to 8:00 p.m. The birds were fed *ad libitum* with unmedicated commercial complete feed; drinking water was provided in commercial drinkers.

The *S. Gallinarum* strain used for the experimental infection was a field strain of *S. Gallinarum*, isolated in 2009 during an outbreak of FT in a poultry farm. It was stored in 15% glycerol tryptic soy broth at -80°C and, before use, it was revitalized by streaking a loopful on tryptic soy agar (TSA, Oxoid, Milan, Italy), and incubated at 42°C overnight. A single, well-isolated colony was selected and passaged on TSA. After overnight incubation at 42°C, a single, well-isolated colony was selected and inoculated in 25 mL tryptic soy broth. The suspension was washed three times in 0.9% NaCl solution, titrated by the plate count method, and administered after adjustment of the concentration to 6x10⁷ CFU/mL with 0.9% NaCl solution. One mL of suspension was administered by gavage to the hens of groups A and B (hereafter infected groups) on day D1, after they were acclimated for three days. Gavage was carried out using a soft plastic, sterile 1 mL Pasteur pipette for about two seconds. The study schedule is shown in Table S1.

After 7 days (D8), approximately 25,000 *D. gallinae* mites (mainly nymphs and adults) were introduced into each isolator, A and B, following a starvation period of three days to induce mites to more aggressively attack hosts to obtain a blood meal [21]. Contemporaneously, three mite traps were placed in each incubator to retrieve mites for transferring to isolators C and D, and to test for the presence of *S. Gallinarum*. Traps consisted of a pile of 5 wooden slats (200 mm long, 100 mm wide and 10 mm high) separated by 1 mm high wooden spacers and joint together by elastic bands. The mites came from a permanent colony reared at the facilities of MSD Animal Health MSD Innovation GmbH (Zur Propstei, Schwabenheim, Germany). The colony founders were collected from an industrial laying hen farm in Germany in 2001.

On day D10, all surviving hens of the infected group were humanely euthanized in accordance to the directive 2010/63/EU of the European Parliament and of the Council, and traps were removed from isolators A and B. Mites were recovered from the traps, starved for 24 h, and then, about 8,000 mites were introduced into each of isolators C and D, which held the other two groups of hens (hereafter, infested groups). The number of mites was estimated by weighing the mass of the mites, after establishing a standard by measuring a mass sample of five 5 aliquots consisting of 100 randomly selected mites.

Although a scheduled euthanasia program, the in-study deaths of 11 hens in the infested group meant that only five hens were euthanized at the end of the experimental program (the original schedule and its amendments are reported in Additional Table 1). Twenty-four days after the infestation of groups C and D, the experimental procedure was concluded by euthanizing the surviving hens. All remaining mites in all
four isolators were collected by removing traps and manually recovering any mites present on the walls and floors of the isolators.

*Health status of hens and clinical score*

The health status of the hens was monitored twice a day for the duration of the experimental procedure and a score was assigned according to the observed clinical signs, as detailed in Additional Table 2. The sum of the two daily observations was considered as the daily score. Hens with daily scores greater than 4 were considered sick.

*Post-mortem examination*

All hens that died (spontaneously or euthanized) were necropsied, and the gross lesions recorded. Liver, spleen, ovary, and cecum were excised from all birds by using sterile scissors and surgical blades.

*Mite samples*

On D10, when traps were collected from isolators A and B, two 100-mite aliquots were prepared for analysis, and the other mites were starved for the infestation of groups C and D, as above described.

In addition, on D9 and D11, mites within the isolators A and B (infected groups) but outside the traps were collected, obtaining another 100-mite aliquot form each of the two isolators. No more aliquots were obtained from isolators A and B in order to avoid loss of mites for the infestations of isolators C and D.

Further mite samples were collected on D36, at the end of the procedures. Specifically, two 100-mite aliquots were retrieved from residual mites in isolators A and B, while ten 100-mite aliquots were prepared from isolator C, and seven 100-mite aliquots from isolator D. Six out of the aliquots from isolator C and four from isolator D were washed by formaldehyde 4% and rinsed with sterile distilled water three times. All aliquots underwent molecular detection and quantification of *S. Gallinarum* immediately after preparation.

*Molecular detection and quantification of Salmonella entericasubsp.entericaser. Gallinarum*

In order to carry out the total genomic DNA extraction, 30 mg of each collected tissue and the 100-mite pools were carefully ground with sterile mortar and pestle, and then processed by the means of the PureLink Genomic DNA kit (Thermo Scientific, Milan, Italy), according to the manufacturer's instructions. The quantification of DNA solutions was achieved by measuring optical density at 260 nm with NanoDrop 1000 spectrophotometer (Thermo Scientific).

The detection of *S. Gallinarum* was performed by seminested PCR (snPCR) as previously described [22]. The quantification of *S. Gallinarum* was carried out by real-time PCR (qPCR) from the snPCR-positive mite samples, according to the previously described protocol [20]. Briefly, amplification was carried out in 20 µL of mixture containing 1X SsoFast™ Probes Supermix with ROX (Bio-Rad, Milan Italy), 100 nm of each primer, 400 nm of FAM-labelled probe and 1 µL of template. Non-template controls were included in each
run by adding sterile distilled water instead of DNA. The conditions were: one cycle at 95 °C for 5 min for Taq polymerase activation, followed by 45 cycles of 95 °C for 30 s, 55 °C for 30 s and 65 °C for 30 s. Fluorescence was acquired during each extension step. Data were acquired and treated by the mean of the Sequence Detection Software Version 1.2.3 (Applied Biosystems, Milan, Italy). Cycle threshold and baseline were calculated automatically by the software and checked by the operators to ascertain possible inconsistencies. For each plate, the standard curve was set up by including 7 serial dilutions (specifically, 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10 000, 1:50 000) of purified DNA from a pure culture of S. Gallinarum. The initial concentration of the DNA solution was determined using the NanoDrop 1000 (Thermo Scientific). All unknown samples, non-template controls, and standards were analyzed in triplicate. The R^2 value of the standard curve in all experiments was higher than 0.98.

Considering that 1 µL of DNA solution was included in each reaction, that DNA was extracted from 100 mites, and that it was eluted in a final volume of 200 µL, the S. Gallinarum genome size and the unicity of the target locus in the pathogen genome, the results have been normalized and expressed as S. Gallinarum cells/mite [20]. Considering the sample size, values below 0.01 S. Gallinarum cells/mite (corresponding to one cell per 100 mites) were treated as 0.

**Serological investigation**

One mL of venous blood was collected from hens on D-1 for confirming that there had been no prior contact with S. Gallinarum, by the mean of an indirect enzyme-linked immunosorbent assay (ELISA). Serum was separated soon after collection and anti-group-D *Salmonella* antibodies were measured using the Chicken Salmonella Antibody Test Kit (BioChek, Reeuwijk, Netherlands), according to the manufacturer’s instructions. Data were analyzed by BioChek II Version 2013.0.07. A sample to positive (S/P) ratio higher than 0.5 was considered positive.

**Statistical analysis**

The normal distribution of quantitative datasets was verified by the Shapiro–Wilk test, with a threshold of \( P = 0.05 \). Considering that no dataset contained normally distributed data, non-parametric analysis was performed. Therefore, the Hodges-Lehmann location estimator [23, 24] has been used to calculate the central values and the 95% confidence interval (CI). Datasets were compared by the Mann-Whitney U test. Mortality and morbidity of infested and infected groups, as well as the number of positive organs collected from the two groups, were compared by the Fisher’s exact test. In both cases, \( P = 0.05 \) was assumed as the significance threshold.

All statistical analyses were performed by the mean of R software v. 3.6.1 [25] and DescTools package v. 0.99.28.

**Determination of the infection rate, of the entomological infection rate and of the vectorial capacity**
The maximum likelihood estimation (MLE) of the infection rate (IR) [26] of *D. gallinae* was calculated by the PooledInfRate v. 4.0, an algorithm that takes into account the number of *S. Gallinarum* positive mite aliquots and the size of aliquots [27].

The IR was calculated using a combination of pathogen location within and on mites. To avoid potential bias by inclusion of washed samples (eliminating pathogens on the mite surface), only unwashed aliquots were considered in the IR calculation.

The entomological inoculum rate (EIR) was obtained by the equation:

\[ EIR = CpH \cdot IR \]

where *CpH* is the number of mites that came in contact with each host and *IR* the infection rate (modified from [28]). The number of contacts was calculated by considering that, from previous observations, about 90% of starved mites were found to feed when they can, and that about 1,000 mites per hens were introduced in isolators C and D.

The vectorial capacity (VC) was calculated as previously described [29], with the equation:

\[ VC = ma^2bp^n/ - \log_p \]

where *m* is the number of total mites per host, *a* is the daily blood feeding rate, *b* is the transmission rate among exposed mites, *p* is the probability of daily survival and *n* the extrinsic incubation period in days. For this study, it was assumed that *m* = 1,000 (the number of mites per hen in incubators C and D); *a* = 0.9 (as above described); *b* = IR; *n* = 3 (as mites were introduced into the isolators C and D 3 days after their first contact with infected hens in isolators A and B). It should be underlined that *m* is a value that describes the mite population as a mix of stages and sexes, all but larvae capable of feeding on hosts. The assumed survival rate of mites following a blood meal was 0.836, derived from the mean of three earlier values: 89.1% of protonymphs, and 66% of deutonymphs [30] and our own unpublished observations of adult mite survival rate of 95.83%.

Since mites were found to be positive as soon as one day after exposure to infected hens, *n* could also be assumed equal to 1, but the most stringent parameters were used. Vectorial capacity quantifies the probability that an arthropod may transmit a pathogen following exposure to an infected host [31, 32].

**Results**

*Occurrence of fowl typhoid*

Before experimental infection, all hens were seronegative for anti-group-D-*Salmonella* antibodies, as the S/P ratio was below 0.5 in all animals (Additional Table 3). Additionally, no detection of *S. enterica* was obtained from fecal samples collected on D-1.
At 6 days post infection, all hens of the infected groups (A and B) exhibited signs of FT, including watery diarrhea that became catarrhal after a few days, depression, lethargy, and a reduction in food intake and oviposition. Similarly, in the infested groups (C and D), clinical signs were observed in 7 of the 16 animals 8 days after the exposure to the starved PRMs collected from isolators A and B. Three other hens became sick the next day (9 days post infestation). Overall, 15 and 13 hens out of 16 developed signs of FT in infected (A-B) and infested (C-D) groups, respectively (Table 1).

No significant difference in morbidity was observed between infected and infested groups ($P = 0.600$). Mortality was numerically higher in the infested groups (13/18) than in infected (6/12), but the difference was not found to be statistically significant ($P = 0.181$).

At necropsy, the hens of both infected and infested groups presented similar anatomopathological patterns (Fig. 1). Discoloration of comb and wattle associated with petechial hemorrhages was evident. Greenish fecal residuals soiled the pericloacal region of most hens. The livers appeared enlarged and congested, with hemorrhagic or necrotic foci. Splenomegaly was evident in all hens, often associated with ovaritis and congestion of the kidneys. The diagnosis of FT was confirmed by the detection of $S. Gallinarum$ from target organs. In all four groups, at least one organ from each hen was positive for $S. Gallinarum$, which was also detected from almost all livers and spleens, 8 ovaries and 3 ceca of the infected groups, and 13 ovaries and 11 ceca from the infested groups (Table 1; Additional Table 6).

While no difference between infected and infested groups was observed in the proportion of positive livers and spleens ($P = 1.000$ in both cases), the number of positive ovaries was numerically but not significantly higher in the infested groups ($P = 0.135$). Conversely, the number of positive ceca from C-D groups was significantly higher than from A-B groups ($P = 0.017$).

The mite samples tested by snPCR at the beginning of the experiment were negative to $S. Gallinarum$. Following exposure to infected hens, all but one of the tested mite aliquots collected on D9, D10 and D11 (24, 48 and 72 hours after exposure to the infected groups) were positive for $S. Gallinarum$ (Table 2). The negative aliquot was collected on D11 from isolator B.

The estimated pathogen load in mites collected from isolators A and B from D9 to D11 was 12.97 cells per mite (95% CI: 0.88 – 64.50). The estimated $S. Gallinarum$ load of mites collected from those isolators on D36 was 21.50 cells per mite, but the limited size of the latter dataset ($n = 2$) did not allow further analysis.

The pathogen was also detected in all pools of $D. gallinae$ collected from isolators C and D at the end of investigation (D36), and wide fluctuations were observed in the contamination level (Table 2), which ranged between 5.25 and 629.05 cells/mite, with an estimated value of 68.26 cells/mite (95% CI: 5.96-389.27). The increase in pathogen load was found to be at the significance limit ($P = 0.073$).

Positivity to snPCR was returned by all formaldehyde-washed samples. Wide fluctuations were observed in the amount of $S. Gallinarum$ cells that were harbored. The estimated value was 16.99 cells/mite (95%
The difference in the pathogen load between formaldehyde-washed and unwashed pools of mites was not statistically significant ($P = 0.222$).

**Vectorial parameters**

The MLE of the IR was 27.93 infected mites per thousand. Consequently, the estimated EIR was 25.11 infectious contacts per thousand and the estimated VC was 73.79 (Table 3).

**Discussion**

There are two broadly accepted definitions of vectors. The first considers a vector an arthropod that is responsible for the transmission of a pathogen among vertebrate hosts [33], while the World Organisation for Animal Health (OIE) gives a more stringent definition, that is a living organism capable not only to transmit the pathogen but also to disseminate the related disease [34].

On those bases, the gathered results provide robust evidence of transmission of *S. Gallinarum* mediated by *D. gallinae*, thus supporting the vectorial competence of the mite. The investigation also proved that mites were able to transmit not only the pathogen but also the disease, as no differences were found in terms of incidence and severity of FT between infected and infested groups. Since all experimental procedures were carried out in isolators, *D. gallinae* was the only contact point between infected (A and B) and infested (C and D) groups, the poultry red mite was the only possible route for the introduction of FT. As most of the diseased hens in the infested group developed signs of FT almost simultaneously, 8 and 9 days after the exposure to mites, it is possible to assume that the contribution of the horizontal transmission in spreading FT within the infested groups was minimal. It is not possible to exclude that horizontal transmission occurred, but this could be a secondary event, which took place after the initial transmission from mites.

The capability of *D. gallinae* to transmit the disease among hens implies that it may inoculate a high enough dose of *S. Gallinarum*. Several studies were carried out to establish the infectious dose, and they reported that no less than $10^4$ CFU of *S. Gallinarum* must be administered orally to trigger FT [35, 36]. Such an infectious dose dramatically drops when the entry point of the pathogen is not linked to the oral route. Previous studies reported that the intramuscular LD50 of *S. Gallinarum* was less than 10 cells (namely, $0.6 \log_{10}$), therefore sensibly lower [37].

Therefore, the number of pathogen cells that *D. gallinae* may harbor and inoculate becomes pivotal. In the current trial, as well as in previous studies [20], mites were found to harbor a highly variable amount of *S. Gallinarum*. This may be explained by considering that mites ingest about 0.3 mg of blood [38] and that the number of bacterial cells in such a small quantity may be far from uniform. However, the estimated pathogen load was quite low after the first infestation, insomuch that, by combining the pathogen load and the EIR, it is reasonable to assume that hens of groups C and D came in contact with a small amount
of *S. Gallinarum*. Nonetheless, such a low quantity was likewise capable to cause disease in most of animals.

Several factors may account for such an apparent inconsistency. First, there is the possibility that the algorithm used to calculate the IR might underrate that parameter when aliquot size is too large, having been devised for mosquitoes, which are usually investigated in smaller pools, or even individually [39]. Then, the amplification factor should also be considered, since the present study, as well as previous investigations [20], revealed that the concurrent presence of *D. gallinae*, *S. Gallinarum*, and chickens may increase the circulation of *S. Gallinarum* among hens and the consequent increment in the pathogen load of mites.

Finally, the transmission route from mites to hosts should be analyzed. As detailed above, if the pathogen penetration occurs other than orally (intramuscularly, e.g.), a lower dose is required to evoke FT, probably because the bacterial cells do not have to transit through the gastric barrier to reach the target organs. The herein presented results do not establish whether *D. gallinae* transmits *S. Gallinarum* while biting or by being ingested with hens’ picking. However, the capability to transmit FT despite the low EIR indirectly suggests that *S. Gallinarum* inoculum of chickens may result from the mite’s feeding behavior, which could lead to the introduction of the pathogen directly into the bloodstream. However, specific investigations should be carried out to confirm this point.

Whatever the route, transmission of FT mediated by *D. gallinae* may have a deep impact on poultry systems. It is known that, in case of severe infestation, up to 50,000 mites can attack a host each night [40], therefore the EIR can reach very high values. Additionally, weighing further biological features of *D. gallinae*, the VC reached the remarkable value of 73.79, with a wide CI. That is due to the high number of poultry red mites that may attack each host, the high mite survival potential and high feeding rate. To our knowledge, no data are available about the VC of *D. gallinae* or other mite species, therefore a direct comparison seems not possible. However, the datum becomes remarkable when compared to the VC of mosquitoes in transmitting vector-borne pathogens such as Zika virus, dengue virus and chikungunya virus. In those cases, VC was 0.35 [41], 18.60 and 13.99 [both recalculated from 32], respectively, more than 30 times lower than the value calculated for *D. gallinae*, even considering the wide CI. About this last point, it should be underlined that the large majority of those models have been developed for insects or ticks, which have population dynamics and biology that can differ substantially from mites. Additionally, despite carried out in strictly controlled conditions, the present study is based on the analysis of two groups, and further studies might be aimed to provide more accurate esteems of vectorial parametes. In the light of those consideration, the development and implementation of statistical and mathematical tools for the assessment and quantification of vectorial properties of mites are imperative in building understanding of the great volume of data that has been produced about mites.

On the other side, more concrete data are available to classify *D. gallinae* as a biological or a mechanical vector. The difference resides in the possibility for the pathogen to replicate or not during the vector infection, respectively [42], despite authoritative Authors consider this definition as inadequate [43].
Actually, *S. Gallinarum* was early found to persist in association with *D. gallinae* for a long time, even in absence of hosts [19]. The present study, consistently with the recent one [20], confirmed such a subsistence but without detecting an increase in the pathogen load in the absence of a host. Those observations strongly suggest that *D. gallinae* could influence the life cycle of *S. Gallinarum* that may at least survive when associated with the poultry red mite, and this would include the arthropod among the biological vectors.

The survival potential of *S. Gallinarum* might be enhanced by the fact it can survive within *D. gallinae*, as demonstrated by the positivity of the formaldehyde-washed aliquots. This fact could contribute to protecting the pathogen from adverse environmental conditions such as dehydration, excessive level of ammonium compounds, lack of nutrients, and even the contact with antimicrobial substances.

Those considerations have important outcomes when transposed into the field. It has been already postulated that the infection of *D. gallinae* could contribute to the reduction in the efficacy of antibiotic therapies during foci of FT [20], since the mites may offer a drug-free environment where *S. Gallinarum* can survive. The capability of mites to re-transmit the infection to chickens, along with the ubiquitous diffusion of *D. gallinae*, may provide an additional explanation for the limited efficacy of antibiotic treatments of FT, which has often been described [44].

Altogether, those data reinforce the need for a comprehensive strategy for the control of both infestations and infections. The importance of an integrated pest management approach for controlling *D. gallinae* infestations has been widely proposed. The approach consists of a coordinated application of good hygiene practices, effective protection against external contaminations, optimization of the chickens’ population density, proper farm design, and the proper administration of the most effective authorized and available acaricide drugs, including a recently introduced drinking water application of fluralaner that offers the potential for improved control of the poultry red mite [45, 46]. A similar approach should be advisable to control and potentially, eradicate FT in affected poultry farms. The presence of *D. gallinae* may not only provide shelter for *S. Gallinarum* to avoid the contact with antibiotics, but it also reduces or eliminates the effectiveness of sanitary breaks, considering the high persistence of the pathogen in association with the mite. Therefore, considering current knowledge, the treatment of FT cannot obviate the need for simultaneous actions to remove *D. gallinae* infestations.

**Conclusion**

The investigation demonstrated that the poultry red mite *D. gallinae* acts as a vector of *S. Gallinarum* and FT. That has important consequence in the management of FT outbreaks since the strategies devised for the eradication of the disease have to take into account the control of *D. gallinae* infestation, as a necessary measure to make antibiotic treatments more effective, and to prevent the onset of FT in consecutive production cycles, even after the sanitary break.

**Additional Files**
Additional file 1: Table S1. Schedule of the study.

Additional file 2: Table S2. Clinical score assigned to hens on the bases of the observed clinical signs.

Additional file 3: Table S3. Level of anti-Group-D-Salmonella antibodies detected in chickens on D-1, before the beginning of the experimental procedures. Values are expressed as sample to positive (S/P) ratio.

Additional file 4: Table S4. Daily clinical scores of the hens belonging to groups A and B.

Additional file 5: Table S5. Daily clinical scores of the hens belonging to groups C and D.

Additional file 6: Table S6. Detection of Salmonella enterica subsp. enterica ser. Gallinarum from the target organs of hens included in the experimental trial. +: Positive organ; -: negative organ.

Abbreviations

CFU: Colony forming units

CI: Confidence interval

EIR: Entomological infection rate

ELISA: Enzyme-linked immunosorbent assay

IR: infection rate

FT: Fowl typhoid

IPM: Integrated pest management

LD50: median lethal dose

MLE: Maximum likelihood estimation

OIE: World Organization for Animal Health

PRM: Poultry red mite

qPCR: real-time polymerase chain reaction

snPCR: Seminested polymerase chain reaction

VC: vectorial capacity

Declarations
Acknowledgments

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Ethical approval and consent to participate

The trial has been approved by the Italian Ministry of Health with the authorization number 718/2017-PR, released on 2-10-2017. All operations complied with the Directive 2010/63/EU.

Consent for publication

Not applicable.

Availability of data and materials

Most of the data used and/or analyzed during the current study are included in this published article and its supplementary information files. The raw data are properties of MSD Animal Health and restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of MSD Animal Health.

Competing interests

The authors declare they have no competing interests.

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Authors’ contribution

N.P., E. Circella, E.T. and A.C designed research; G.C., E. Catelli, E. Circella, C. Lupini, and G. Mescolini carried out the experimental procedures; H.Z. organized the shipment of mites; M.B.-S. carried out raw data check; N.P. performed formal analysis of data; A.C. and E.T. provided funding acquisition; A.C. and E. Catelli provided project administration, G.C. and N.P. wrote the paper, A.C. supervised all phases of the project.

References

1. Hinkle NC, Corrigan RM. External parasites and poultry pests. In: Swine DE, editor. Diseases of Poultry, thirteenth edition. Ames: John Wiley & Sons; 2013. p. 1099-1116.
2. George DR, Finn RD, Graham KM, Mul MF, Maurer V, Moro CV, Sparagano OA. Should the poultry red mite *Dermanyssus gallinae* be of wider concern for veterinary and medical science? Parasit Vectors.
3. Kilpinen O, Roepstorff A, Permin A, Nørgaard-Nielsen G, Lawson LG, Simonsen HB. Influence of *Dermanyssus gallinae* and *Ascaridia galli* infections on behavior and health of laying hens (*Gallus gallus domesticus*). Br Poult Sci. 2005;46:26-34.

4. Cosoroabă I. Observation d’invasion massives par *Dermanyssus gallinae* (De Geer 1778), chez les poules élevées en batterie en Roumanie. Rev Med Vet. 2001;1:89-96.

5. Chauve C. The poultry red mite *Dermanyssus gallinae* (De Geer, 1778): current situation and future prospects for control. Vet Parasitol. 1998;79:239-245.

6. Sigougnault-Flochlay A, Thomas E, Sparagano O. Poultry red mite (*Dermanyssus gallinae*) infestation: a broad impact parasitological disease that still remains a significant challenge for the egg-laying industry in Europe. Parasit Vectors. 2017;10:357.

7. Valiente Moro C, De Luna CJ, Tod A, Guy JH, Sparagano OAE, Zenner L. The poultry red mite (*Dermanyssus gallinae*): a potential vector of pathogenic agents. Exp Appl Acarol. 2009;48:93-104.

8. Sommer D, Heffels-Redmann U, Köhler K, Lierz M. Kaleta EF. Rolle der Roten Vogelmilbe (*Dermanyssus gallinae*) bei der Übertragung von aviärem Influenza-A-Virus. Tierarztl Prax Ausg G Grosstiere Nutztiere. 2016;44:26-33.

9. Chirico J, Eriksson H, Fossum O, Jansson D. The poultry red mite, *Dermanyssus gallinae*, a potential vector of *Erysipelothrix rhusiopathiae* causing erysipelas in hens. Med Vet Entomol. 2003;17:232-234.

10. Circella E, Pugliese N, Todisco G, Cafiero MA, Sparagano OAE, Camarda A. *Chlamydia psittaci* infection in canaries heavily infested by *Dermanyssus gallinae*. Exp Appl Acarol. 2011;55:329-338.

11. Raele DA, Galante D, Pugliese N, La Salandra G, Lomuto M, Cafiero MA. First report of *Coxiella burnetii* and *Borrelia burgdorferi* sensu lato in poultry red mites, *Dermanyssus gallinae* (*Mesostigmata, Acari*), related to urban outbreaks of dermatitis in Italy. New Microbes New Infect. 2018;23:103-109.

12. Valiente Moro C, Chauve C, Zenner L. Experimental infection of *Salmonella* Enteritidis by the poultry red mite, *Dermanyssus gallinae*. Vet Parasitol. 2007;146:329-336.

13. Valiente Moro C, Fravalo P, Amelot M, Chauve C, Zenner L, Salvat G. Colonization and organ invasion in chick experimentally infected with *Dermanyssus gallinae* contaminated by *Salmonella* Enteritidis. Avian Pathol. 2007;36:307-311.

14. Popoff MY, Le Minor LE. Genus XXXIII. *Salmonella* Ligneres 1900, 389AL. In: Garrity GM, Brenner DJ, Krieg NR, Staley JT, editors. Bergey's manual of systematic bacteriology, second edition, volume two The *Proteobacteria*, part B The *Gammaproteobacteria*. New York: Springer; 2005. p. 764-799.

15. Shivaprasad HL, Barrow PA. Pullorum disease and fowl typhoid. In: Swine DE, editor. Diseases of Poultry, thirteenth edition. Ames: John Wiley & Sons; 2013. p. 678-693.

16. OIE (World Organisation for Animal Health). Fowl typhoid and pullorum disease. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.11_FOWL_TYPHOID.pdf
(2018) Accessed 16 Apr 2019.

17. Shivaprasad HL. Fowl typhoid and pullorum disease. Rev Sci Tech. 2000;19:405-424.

18. Berchieri Jr A, Murphy CK, Marston K, Barrow PA. Observations on the persistence and vertical transmission of *Salmonella enterica* serovars Pullorum and Gallinarum in chickens: effects of bacterial and host genetic background. Avian Pathol. 2001;30:221-231.

19. Zeman P, Štika V, Skalka B, Bártik, Dusbábek F, Lávičková M. Potential role of *Dermanyssus gallinae* De Geer, 1778 in the circulation of the agent of pullurosis-typhus in hens. Folia Parasitol (Praha). 1982;29:371-374.

20. Pugliese N, Circella E, Marino M, De Virgilio C, Coccoilo G, Lozito P, et al. Circulation dynamics of *Salmonella enterica* subsp. *enterica* ser. Gallinarum biovar Gallinarum in a poultry farm infested by *Dermanyssus gallinae*. Med Vet Entomol. 2019;33:162-170.

21. Kilpinen O, Mullens BA. Effect of food deprivation on response of the mite, *Dermanyssus gallinae*, to heat. Med Vet Entomol 2004;18:368-371.

22. Pugliese N, Circella E, Pazzani C, Pupillo A, Camarda A. Validation of a seminested PCR approach for rapid detection of *Salmonella enterica* subsp. *enterica* ser. Gallinarum. J Microbiol Methods 2011;85:22-27.

23. Serfling R. Asymptotic relative efficiency in estimation. In: Lovric M, Editor. International Encyclopedia of Statistical Science. Berlin: Springer; 2011. P. 68-72.

24. Hodges JL, Lehmann EL. Estimates of location based on rank test. Ann Math Statist 1963;34:598-611.

25. R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical computing, Vienna, Austria. 2015. http://www.r-project.org

26. Gu W, Lampman R, Novak RJ. Problems in estimating mosquito infection rates using minimum infection rate. J med Entomol. 2003;40:595-596.

27. Biggerstaff BJ. 2009. PooledInfRate, version 4.0: a Microsoft® Office Excel® add-in to compute prevalence estimates from pooled samples. Centers for Disease Control and Prevention. Fort Collins, USA. https://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html Accessed 12 Jul 2019.

28. Nepomichene TNJJ, Tata E, Boyer S. Malaria case in Madagascar, probable implication of a new vector, *Anopheles coustani*. Malar J. 2015;14:475.

29. Kramer LD, Ciota AT. Dissecting vectorial capacity for mosquito-borne viruses. Curr Opin Virol 2015;15:112-118.

30. Tucci EC, Prado AP, Araújo RP. Development of *Dermanyssus gallinae* (Acari: Dermanyssidae) at different temperatures. Vet Parasitol 2008;155:127-132.

31. Macdonald G. Epidemiologic models in studies of vector-borne diseases. Public Health Rep 1961;76:753-764.

32. Ciota AT, Chin PA, Ehrbar DJ, Micieli MV, Fonseca DM, Kramer LD. Differential effects of temperature and mosquito genetics determine transmissibility of Arboviruses by *Aedes aegypti* in Argentina. Am
J Trop Med Hyg 2018;99:417-424.

33. Reisen WK. Epidemiology of vector-borne diseases. In: Mullen GR, Durden LA, editors. Medical and Veterinary Entomology. Cambridge: Academic Press; 2002. p. 15-27.

34. Verwoerd DW. Definition of a vector and a vector-borne disease. Rev Sci Tech Off Int Epiz 2015;34:29-31.

35. Berchieri Jr A, Murphy, CK, Marston K, Barrow, PA. Observations of the persistence and vertical transmission of *Salmonella enterica* serovars Pullorum and Gallinarum in chickens: effect of bacterial and host genetic background. Avian Pathol 2011;30:221-231.

36. de Oliveira GH, Berchieri Junior A, Fernandes AC. Experimental infection of laying hens with *Salmonella enterica* serovar Gallinarum. Braz J Microbiol 2005;36:51-56.

37. Barrow PA, Simpson JM, Lovell MA, Binns MM. Contribution of *Salmonella gallinarum* large plasmid toward virulence in fowl typhoid. Infect Immun 1987;55:388-392.

38. Sikes RK, Chamberlain RW. Laboratory observations on three species of bird mites. J Parasitol 1954;40:533-535.

39. Condotta SA, Hunter FF, Bidochka MJ. West Nile virus infection rates in pooled and individual mosquito samples. Vector Borne Zoonotic Dis 2004;4:198-203.

40. Sparagano OAE, George DR, Harrington DWJ, Giangaspero A. Significance and control of the poultry red mite, *Dermanyssus gallinae*. Annu Rev Entomol 2014;59:447-466.

41. Lozano-Fuentes S, Kenney JL, Varnado W, Byrd BD, Burkhalter KL, Savage HM. Susceptibility and vectorial capacity of American *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae) to American Zika virus strains. J Med Entomol 2019;56:233-240.

42. Carn VM. The role of dipterous insects in the mechanical transmission of animal viruses. Br Vet J 1996;152:377-393.

43. Gray SM, Benerjee N. Mechanisms of arthropod transmission of plant and animal viruses. Microbiol Mol Biol Rev 1999;63:128-148.

44. Celis-Estupiñan ALDP, Batista DFA, Cardozo MV, Secundo de Souza AI, Alves LBR, de Almeida AM, Barrow PA et al. Further investigations of fowl typhoid in Brazil. Avian Pathol 2017;46:416-425.

45. Tomley FM, Sparagano O. Spotlight on avian pathology: red mite, a serious emergent problem in layer hens. Avian Pathol 2018;47:533-535.

46. Thomas E, Chiquet M, Sander B, Zschiesche E, Flochlay AS. *Field efficacy and safety of fluralaner solution for administration in drinking water for the treatment of poultry red mite (Dermanyssus gallinae) infestations in commercial flocks in Europe*. Parasit Vectors. 2017;10:457.

Tables

**Table 1.** Incidence and effects of fowl typhoid within the groups of animals
The table presents the detection of *Salmonella enterica* subsp. *enterica* ser. Gallinarum in hens’ organs. The data includes the number of affected hens, deaths, and the presence of the bacteria in various organs: liver, spleen, ovary, and cecum. The groups are labeled as A, B, A+B, C, D, and C+D.

**Table 2.** Detection of *Salmonella enterica* subsp. *enterica* ser. Gallinarum in hens’ organs.

| Group | Affected hens | Deaths | Liver | Spleen | Ovary | Cecum |
|-------|---------------|--------|-------|--------|-------|-------|
| A     | 8             | 1      | 6     | 7      | 6     | 0     |
| B     | 7             | 5      | 8     | 8      | 2     | 3     |
| A+B   | 15            | 6      | 14    | 15     | 8     | 3     |
| C     | 7             | 7      | 8     | 8      | 7     | 7     |
| D     | 6             | 4      | 7     | 7      | 6     | 4     |
| C+D   | 13            | 11     | 15    | 15     | 13    | 11    |

**Positive organs**

| Group | Liver | Spleen | Ovary | Cecum |
|-------|-------|--------|-------|-------|
| A     | 6     | 7      | 6     | 0     |
| B     | 8     | 8      | 2     | 3     |
| A+B   | 14    | 15     | 8     | 3     |
| C     | 8     | 8      | 7     | 7     |
| D     | 7     | 7      | 6     | 4     |
| C+D   | 15    | 15     | 13    | 11    |

**Table 2.** Detection and quantification of *S. enterica* subsp. *enterica* ser. Gallinarum from mites.
| Mite aliquot | Isolator | Day of collection | Formaldehyde-washed | snPCR result | SG cells/mite |
|--------------|----------|-------------------|---------------------|--------------|--------------|
| AD9          | A        | D9                | No                  | +            | 64.50        |
| BD9          | B        | D9                | No                  | +            | 16.75        |
| AD10         | A        | D10               | No                  | +            | 1.42         |
| BD10         | B        | D10               | No                  | +            | ND³          |
| AD11         | A        | D11               | No                  | +            | 0.88         |
| BD11         | B        | D11               | No                  | -            | -            |
| AD36         | A        | D36               | No                  | +            | 28.71        |
| BD36         | B        | D36               | No                  | +            | 14.29        |
| AD36         | A        | D36               | No                  | +            | 28.71        |
| BD36         | B        | D36               | No                  | +            | 14.29        |
| CD36-1       | C        | D36               | No                  | +            | 389.27       |
| CD36-2       | C        | D36               | No                  | +            | 5.25         |
| CD36-3       | C        | D36               | No                  | +            | 65.71        |
| CD36-4       | C        | D36               | No                  | +            | 629.05       |
| DD36-1       | D        | D36               | No                  | +            | 69.11        |
| DD36-2       | D        | D36               | No                  | +            | 5.96         |
| DD36-3       | D        | D36               | No                  | +            | 16.50        |
| CD36-5       | C        | D36               | Yes                 | +            | 274.22       |
| CD36-6       | C        | D36               | Yes                 | +            | 6.05         |
| CD36-7       | C        | D36               | Yes                 | +            | 64.15        |
| CD36-8       | C        | D36               | Yes                 | +            | 958.73       |
| CD36-9       | C        | D36               | Yes                 | +            | 0.03         |
| CD36-10      | C        | D36               | Yes                 | +            | 16.99        |
| DD36-4       | D        | D36               | Yes                 | +            | ND³          |
| DD36-5       | D        | D36               | Yes                 | +            | ND³          |
| DD36-6       | D        | D36               | Yes                 | +            | 0.14         |
| DD36-7       | D        | D36               | Yes                 | +            | 10.34        |

SG: S. Gallinarum; +: SG detected; -: SG not detected;

³ ND: non detectable. A late amplification curve is observed in qPCR, with putative quantification < 0.01 SG cells/mite.

**Table 3. Entomological parameters**

| Parameter                           | Min   | Central value | Max   |
|-------------------------------------|-------|---------------|-------|
| Infection rate³                     | 13.72 | 27.93         | 55.21 |
| (maximum likelihood estimation)     |       |               |       |
| Entomological infection rate³       | 12.35 | 25.14         | 49.69 |
| Vectorial capacity³                 | 36.25 | 73.79         | 145.87|
a expressed as infected mites per thousand

b expressed as infected contacts per thousand.

c expressed as a pure number.

Figures
Figure 1

Gross lesions. a) Discoloration of comb and wattle. b) Greenish fecal residues (red circle). c) Hepatomegaly with hemorrhagic foci (blue arrow) and discoloration of lobes (red arrow). d) Pericarditis
(green circle), hepatomegaly with necrotic foci (purple arrow). e) Splenomegaly (azure circle). f) Misshapen or atretic ovaries (oophoritis) (blue oval).

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