Circulation dynamics of *Salmonella enterica* subsp. *enterica* ser. Gallinarum biovar Gallinarum in a poultry farm infested by *Dermanyssus gallinae*

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Running head: *Salmonella enterica* in *Dermanyssus gallinae*

Abstract

Dermanyssus gallinae (Mesostigmata: Dermanyssidae, De Geer, 1778) is an ectoparasite of poultry, suspected to play a role as a vector of *Salmonella enterica* subsp. *enterica* ser. Gallinarum. Despite the association between them was reported, the actual dynamics in field are not clear. Therefore, this study was aimed to verify the interactions among mites, pathogen and chickens. The study was carried out in an industrial poultry farm infested by *D. gallinae*, during an outbreak of fowl typhoid. The presence of *S.* Gallinarum in mites was assessed and quantified by seminested PCR and real-time PCR, respectively, in mites collected during two subsequent productive cycles and the sanitary break. The anti-group D *Salmonella* antibodies were quantified by ELISA. During the outbreak and the sanitary break, *S.* Gallinarum was constantly present in mites. In the second cycle, scattered positivity was observed, but hens did not exhibit signs of fowl typhoid, also because of the vaccination with BIO-VAC SGP695. Data strongly suggest that *D. gallinae* acts as reservoir of *S.* Gallinarum, thus allowing the pathogen to persist in farms. Furthermore, the study highlighted the interactions among *D. gallinae*, *S.* Gallinarum and hens to enhance the mite-mediated circulation of *S.* Gallinarum in an infested poultry farm.

Keywords: *Dermanyssus gallinae*, *Salmonella enterica* subsp. *enterica* ser. Gallinarum, Fowl typhoid, infection rate, BIO-VAC SGP695

Introduction

The poultry red mite *Dermanyssus gallinae* is a widely diffused ectoparasite of poultry, infesting about the 80% of laying hens farms in Europe, with prevalence rates ranging from 11 to 100% (George *et al.*, 2015; Sparagano *et al.*, 2009; Cencek, 2003). Infestations by *D. gallinae* have also been reported from China (Wang *et al.*, 2010), Japan (Sparagano *et al.*, 2009), Australia and Brazil (Roy & Buronfosse, 2011). The infestation by *D. gallinae* is a recognized cause of stress for hens, evidenced by increase in feed and water intake, cannibalistic behaviors, decrease in growth rate and even death of birds in heavier infestations (Sparagano *et al.*, 2014). It has a deep impact also in terms of morbidity, as it causes decrease in egg production and detriment to egg quality. The economic impact is further worsened by the costs of treatments and control measures (Sparagano *et al.*, 2009). Besides the direct effects of *D. gallinae* on poultry, many Authors hypothesizes that the mite is a potential vector for a number of pathogens (Valiente Moro *et al.*, 2005). In fact, it was found associated with viral and bacterial pathogenic agents such as equine encephalitis viruses, Newcastle disease virus, *Pasteurella multocida*, *Coxiella burnetii*, *Listeria monocytogenes* (Valiente Moro *et al.*, 2009), *Erysipelothrix rusiopathiae* (Chirico *et al.*, 2003) and *Chlamydia psittaci* (Circella *et al.*, 2011). *Dermanyssus gallinae* was also found to be a potential carrier of *Salmonella enterica*. Valiente Moro *et al.* (2007a) evidenced that mites could be infected by *S. enterica* subsp. *enterica* ser. Enteritidis (*S.* Enteritidis), and that chickens could, in turn, be infected by swallowing infected mites (Valiente Moro *et al.*, 2007b). Similarly, three decades ago, Zeman *et al.* (1982) reported that mites collected from infested poultry farms were positive to *S. enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (*S.* Gallinarum) and that the pathogen seemed capable to survive in mites for up to 4 months.

The latter finding has particular relevance for the poultry system, as *S.* Gallinarum is the etiological agent of a very impactful disease of poultry, the fowl typhoid (FT). It is an acute or chronic septicemic disease that usually affects adult chickens and other birds, such as turkeys, guinea fowls, quails, sparrows and parrots (Shivaprasad & Barrow, 2013; Barrow & Neto, 2011). It is usually

characterized by high morbidity, and acute and subacute mortality (OIE, 2012). Clinical signs of FT include reduction in eggs production, diarrhea, dehydration and anorexia (Christensen *et al.*, 1992). At necropsy, lesions are mainly evident in spleen and liver. The latter may present enlargement and white foci of 2-4 mm in diameter. In acute cases, spleen and kidneys appear enlarged and congested, too (Gast, 1997).

The heavy impact of FT on poultry system led the World Organisation for Animal Health (OIE) to include the disease in the list of notifiable diseases, and to adopt control measures for the importation of eggs and birds (OIE, 2016). The national and international control policies contributed to the eradication of the disease from commercial poultry farms in Western Europe, US, Canada, Australia and Japan (OIE, 2012).

However, fowl typhoid is still widely reported in Central and South America, Africa, China and the Indian subcontinent. Foci are also reported from Southern, Eastern and central Europe, other than from rural farms in the US and Europe (Shivaprasad & Barrow, 2013), underlining that the disease is a serious threat wherever control measures are not efficient and environmental conditions are favorable for the growth and spread of the pathogen (Barrow and Neto, 2011).

The disease control is further complicated by the different transmission routes of the pathogen, which may be transmitted both vertically and horizontally (Paiva *et al.*, 2009; Cox *et al.*, 1996), and it may also contaminate feed, water and litter (Shivaprasad & Barrow, 2013). However, to date very few data are available about the possibility of a vector-mediated transmission route, apart the cited study of Zeman *et al.* (1982).

In the light of these considerations, this study was aimed to evaluate the association between *D. gallinae* and *S.* Gallinarum in field, by investigating the association between the mites and the pathogen during an outbreak of FT in an industrial laying hen farm heavily infested by *D. gallinae*. Furthermore, the analyses were carried out during and between two consecutive production cycles, in order to verify whether *D. gallinae* has the potentiality to act as vector and reservoir of *S.* Gallinarum.

Materials and Methods

Description of the fowl typhoid outbreak

The study was carried out in a laying hen farm for production of eggs for consumption in Apulia (South Italy), naturally infested by mites, in a time period of 23 months. The flock consisted of 13,500 Lohmann Brown hens. At the beginning of the investigation, an outbreak of fowl typhoid had been being in progress for approximately 3 months (T_0). The FT diagnosis was confirmed by necropsy findings and isolation of *S.* Gallinarum.

At the same time, the farm was experiencing a massive infestation by *D. gallinae*, whose level was assessed according to the Cox's scale, reported by Mul *et al.* (2015).

During the FT outbreak, clusters of mites larger than 1 cm^2 were clearly visible in unprotected places and on the cages, corresponding to the higher level (IV) of the scale. The high degree of infestation contributed to worsen the health conditions of animals by causing severe anemia, too. Overall, an average mortality of 22% was recorded, and oviposition was about 25% less than expected. At the end of the 2nd month of the investigation (T_2), the flock was treated with colistin sulfate (Chemifarma, Forlì, Italy) in drinking water at a dosage of 5-6 mg (roughly corresponding to 100,000 IU) per kg of body weight per day. Following antibiotic treatment, the circulation of disease was reduced, with a significant reduction in mortality, but not completely eradicated. Obviously, the antibiotic treatment did not affect the level of *D. gallinae* infestation, which remained at level IV.

In these conditions, because of the severe deterioration of the flock, it was dismissed during the third week of the 8th month of investigation (T_8), and a sanitary break was established for 60 days. At the end of sanitary break, the mite infestation dropped to the level 0 of the Cox's scale as no mites were visible.

The new cycle started at the end of the 10^{th} month (T_{10}), when a group of 22,000 hens was housed. The new birds came from a *S.* Gallinarum free hatchery, which had no contacts with staff or equipment of destination farm.

Birds were vaccinated with BIO-VAC SGP695 (Fatro, Ozzano Emilia (Bo), Italy) in drinking water when pullets were 30, 60 and 110 days old, thus following the proper vaccination schedule. Before housing, bacteriological tests were carried out to exclude the presence of *S.* Gallinarum, as described below.

Upon housing, the mite population was still at level 0, but, four months after, at the 14^{th} month of investigation (T_{14}), it suddenly increased to the level II (mites visible in unprotected places). In the 15^{th} month (T_{15}), the infestation rapidly reached the level IV and remained stable for the following months.

In the 16^{th} month (T_{16}), the farmer and the veterinarian decided to administer a vaccine booster, because the increase in anti-*S.* Gallinarum antibodies level and positivity of mites to *S.* Gallinarum indicated a steady increase in the infection pressure. Again, BIO-VAC SGP695 vaccine was administered in drinking water.

Bacteriological tests to exclude the *S.* Gallinarum infection were also performed.

Bacteriological screening

The isolation of *S.* Gallinarum was carried out from liver and spleen of the hens that died during the outbreak. Since they resulted positive, and clinical and necropsy finding were fully consistent with the fowl typhoid diagnosis, no more organs were checked.

When animals were reared, samples of 150 g of feces were collected from the conveyor belt under the cages.

Liver, spleen and caecal tonsils from spontaneously died pullets were also collected and checked.

During the second production cycle, the screening was carried out from liver, spleen, caecal tonsils and ovary of birds that spontaneously died.

All bacteriological tests were performed according to the OIE guidelines (OIE, 2012).

Mite samples

The mites were collected by using corrugated cardboard traps (Nordenfors *et al.*, 1999) placed at different levels in 12 points of the sheds.

Samples were collected at T_1, T_2, T_5, T_7, T_8, T_9, T_{10}, T_{14}, T_{15}, T_{16}, T_{17}, T_{18}, T_{19}, T_{21} and T_{23} (Tab. 1). From T_{11} to T_{13}, traps were removed as usually but no PRM were collected probably because the shed was cleansed and sanitized during the sanitary break. The mites present in the traps that were collected at the same time were joined in a single group, from which aliquots of 100 mites were prepared. The number of pools from each sampling group depended on the dimension of the group. Namely, 3 aliquots per thousand mites in the sample were prepared. From each pool, three to five female mites were analyzed for species identification based on their morphological features (Di Palma *et al.*, 2012), according to the morphological keys of Varma (1993) and Baker (1999).

Environmental samples

Samples of feces were collected under the cages during the outbreak and the second production cycle contemporarily the trap collection. During the sanitary break, at T_{10}, samples of dust were collected from the cages, the floor and the conveyor belt, while no feces were retrieved. Environmental samples (about 150 g per samples of feces and 100 g per samples of dust) were collected with sterile equipment and stored in sterile plastic bags till DNA extraction.

DNA extraction

Unwashed mites of each aliquot were homogenized in sterile pestle and mortar, and total DNA was extracted by using the PureLink Genomic DNA kit (Thermo Scientific, Milan, Italy), following the manufacturer's protocol for mammalian tissues and mouse/rat lysate. The same protocol was used to

extract DNA from the environmental samples. Specifically, three to five aliquots of about 50 mg were picked up from each sample, and they were used for DNA extraction.

The recovered DNA, resuspended in 1 mM Tris, 0.1 mM EDTA pH 8, was then used for seminested PCR (snPCR) and real-time PCR (qPCR). The quantification of DNA solutions was achieved by measuring optical density at 260 nm with NanoDrop 1000 spectrophotometer (Thermo Scientific).

Seminested PCR

The extracted DNA was used to detect *S.* Gallinarum by the mean of a previously validated snPCR protocol (Pugliese *et al.*, 2011). The reactions were carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany). Each assay was performed in triplicate and a negative control with distilled water instead of DNA was added.

PCR results were analyzed by electrophoresis at 7.5 V/cm for 60 min in 1.5% agarose gel followed by staining with ethidium bromide 0.5 μg/ml. Stained agarose gels were exposed to UV light and image were digitalized by the mean of a GelDoc-It Imaging System (UVP, Upland, CA, US).

Real-time PCR

Primers (RTSGf and RTSGr) and probe (RTSGp) were designed by using the Primer3 (Untergrasser *et al.*, 2012) software based on a previously detected (Pugliese *et al.*, 2011) distinctive regions of the *S.* Gallinarum chromosome (GenBank accession number HQ014664). All primer and probe sequences were checked for self-complementarity, hetero-complementarity and melting temperature according to the MIQE guidelines (Bustin *et al.*, 2009). The nucleotide sequences of the primers and their positions referred to the *S.* Gallinarum reference genome (RefSeq accession number NC_003197) are listed in Table 2.

The qPCR was performed by using ABI 7300 Real-Time PCR System (Applied Biosystems, Milan, Italy) in 96-well optical plate (BioRad, Milan, Italy). Amplification was carried out in 20 μL of

mixture containing 1X SsoFast™ Probes Supermix with ROX (BioRad), 100 nM of each primer (RTSGf and RTSGr, Sigma-Aldrich, Milan, Italy), 400 nM of labeled probe (RTSGp, Sigma Aldrich, Milan, Italy) and 1 μL of template. Non-template controls (NTC) were included in each run by adding sterile distilled water instead of DNA. Condition were as follows: one cycle at 95 °C for 5 minutes for *Taq* polymerase activation, followed by 45 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 65 °C for 30 seconds. Fluorescence was acquired during each extension step. Cycle threshold (C_T) and baseline were calculated automatically under default settings for the Sequence Detection Software version 1.2.3 (Applied Biosystems). Each pool and NTCs were amplified in triplicate in the same plate.

Seven serial dilutions (each in triplicate) of purified DNA from pure culture of *S.* Gallinarum were used to set up the standard curve. The initial concentration of the DNA solution was determined by using the NanoDrop 1000 and 1:50, 1:100, 1:500, 1:1,000, 1:5,000, 1:10,000, 1:50,000 serial dilution were prepared in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The calibration curve was generated by the Sequence Detection Software version 1.2.3.

qPCR data treatment

Total DNA concentration of each samples used in qPCR was normalized and the qPCR output was adjusted according to the normalization parameter. The normalized qPCR amount was converted in target copy number (TCN) considering the *S.* Gallinarum genome dimension and that the target locus was unique in the *S.* Gallinarum genome. Under the latter assumption, TCN were considered as measure of the *S.* Gallinarum cells.

Considering also that, per each sample, total DNA was extracted from 100 mites and eluted in 100 μL, and that 1 μl of DNA solution was used as template in qPCR, the amount of DNA used in qPCR has been treated as approximately coming from 1 mite. Therefore, data were expressed as number of *S.* Gallinarum cells per mite.

Infection rate

The maximum likelihood estimation (MLE) of *D. gallinae* infection rate (IR) was measured during the outbreak before (T_1-T_2) and after (T_5-T_8) the antibiotic treatment, during the sanitary break (T_9-T_{10}), at the beginning of the second production cycle (T_{14}-T_{15}), two months after the emergency vaccine booster (T_{16}-T_{17}), and at the end of the study (T_{21}-T_{23}). The MLE was calculated as:

$MLE = 1 - \left(1 - \frac{Y}{X}\right)^{1/m}$ (Gu *et al.*, 2003)

with *Y* the number of positive pools; *X* the number of pools; and *m* the pool size. The equation could be applied as the pools were equal in size.

Serological investigations

Venous blood was collected on T_1, T_7, T_9, T_{10}, T_{11}, T_{12}, T_{13}, T_{15}, T_{16}, T_{18}, T_{19}, T_{21} and T_{23}. Each time, about 1 mL of blood was collected from 15 randomly selected laying hens. The blood sampling at T_9 and T_{10} were carried out while animals were still in the pullet farm. The blood collection was performed by expert veterinarian personnel and no animals suffered because of this operation. The procedure was part of the diagnostic, prevention and monitoring procedures scheduled to contrast the FT outbreak and the other infectious diseases of poultry farms.

Serum was separated soon after collection and it was stored at -20 °C until testing.

Indirect ELISA test was performed using the Chicken Salmonella Antibody Test Kit (BioChek, Reeuwijk, The Netherlands), designed to measure anti-group-D-Salmonella (AGDS) antibody in the chickens' serum. The assay was carried out according to the manufacturer's instructions. Data were analyzed by the BioChek II software version 2013.0.07.

Results

Serological data

During the active phase of the outbreak, before colistin sulfate treatment, high rates of mortality and morbidity were recorded and the mean AGDS antibody level was high, not less than 10^4 (Tab. 3, Fig. 1). Following antibiotic administration, mortality and morbidity decreased, but antibody count remained high.

Antibody level in sera collected in the pullet farm (T_9 and T_{10}) was expectedly high, within the order of magnitude of 10^3, as effect of the vaccination program applied to the new flock.

As expected, antibody levels remained quite constant during the first months after housing, with a slight and not significant average decrease. On T_{16}, AGDS level unexpectedly raised, despite not significantly, but this led the farmer and the veterinarian to perform the vaccine booster. Probably as a consequence, AGDS level raised on T_{18} and T_{19}, reaching a mean peak of about 8×10^3.

Salmonella Gallinarum infection rate of poultry red mites

Overall, 19 out of the 46 tested mite pools were found positive to *S.* Gallinarum by snPCR and the aggregate MLE of IR was 4.27 per 1,000 mites.

During the acute phase of the FT outbreak, MLE was 13.77 per 1,000, while, after the colistin treatment of the flock, it decreased to 6.91 per 1,000. Due to the sanitary break, *D. gallinae* population dropped off in the next months, but it was still possible to collect 2 samples at T_9 and T_{10}. Interestingly, out of the 6 pools from those samples, 1 was found positive to *S.* Gallinarum (MLE equal to 1.82 per 1,000 mites).

Despite the very low mite population density during the first months after the housing of the new flock, 2 of the 9 pools collected at T_{14} and T_{15}, when *D. gallinae* population boomed, were positive to *S.* Gallinarum, (MLE of 2.51 per 1,000 mites).

During the following two months, T_{16} and T_{17}, when mite population slightly decreased, 2 out of 5 pools were positive to *S.* Gallinarum, with a further increase of MLE to 5.10 per 1,000.

Conversely, the pools from the samples collected at T_{18} and T_{19} were negative, but mites returned to be positive at T_{21} and T_{23}, when 3 out of 4 pools were positive again, with the same MLE of IR

obtained during the most active phase of the disease in the first production cycle (13.77 per 1,000 mites).

Quantification of Salmonella Gallinarum in poultry red mites

The *S.* Gallinarum loads measured in mites are reported in Table 1. Consistently with snPCR, no appreciable amplification signals were recorded from the negative pools, while all but one positive pools returned a measurable amplification signal. The only exception was the snPCR-positive pool collected at T_9, probably because of a greater sensitivity of the snPCR if compared to qPCR.

During the acute stage of the outbreak, when the pathogen was widely circulating in the flock, mites harbored up to about 400 copies of the target gene, corresponding to about 400 cells of the pathogen (T_2).

Few weeks after the animals were treated with colistin sulfate, the quantity of *S.* Gallinarum per mite suddenly dropped to about 1-2 cells per mite, remaining stable during the last months of the first production cycle, when the disease was less active but mortality was still higher than expected for a healthy flock.

The snPCR-positive pool collected during the sanitary break did not return a readable amplification curve.

Six months following the housing of the new flock (T_{14}), mites were positive again to *S.* Gallinarum, but the mean measured quantity of the pathogen was very low (0.05 ± 0.05 cell/mite).

The pathogen load in *D. gallinae* slightly increased up to 0.41 ± 0.03 and 0.38 ± 0.01 at T_{16} and T_{17}, while 5 weeks after the vaccine booster no detectable quantity of *S.* Gallinarum was found out in PRM, thus confirming the lack of circulation of the pathogen in *D. gallinae*.

However, the pathogen was still detected in mites collected at T_{21} and T_{23}, with an even higher quantity of *S.* Gallinarum per mite, estimated in 1.11 ± 0.10 and 4.39 ± 0.47 cell/mite, respectively.

Salmonella Gallinarum in environmental samples

293 Aliquots of feces collected during the outbreak at T_1, T_2 and T_8 were positive to *S.* Gallinarum,
294 while those collected at T_5 and T_7 resulted negative. Neither the aliquots of dust samples collected
295 during sanitary break, nor aliquots of feces collected during the second cycle returned positivity to
296 the pathogen.
297
298 **Discussion**
299 The herein presented results evidenced the association between *D. gallinae* and *S.* Gallinarum in
300 field, as both infection rate and extent of mite contamination appeared to be directly related to the
301 circulation of *S.* Gallinarum in the flock.
302 During the more active phase of the outbreak, mites showed the highest values of IR (MLE of 13.77
303 infected mites per 1,000), and the pathogen load rose up to 400 cells/mite.
304 The treatment with colistin sulfate, which was effective in reducing the severity of the outbreak,
305 was followed by a sudden drop of the pathogen load from 400 to about 1-2 cell/mite. On the other
306 hand, IR exhibited only a slight reduction, from 13.77 to 6.91 per 1,000 mites, suggesting that the
307 number of infected mites only moderately decreased, but infected mites harbored very few pathogen
308 cells.
309 Interestingly, mites continued to be infected even during the sanitary break, as one pool of mites
310 resulted positive by snPCR, which is sensitive enough to detect up to 1-4 copies of target DNA
311 (Pugliese *et al.*, 2011). This finding extends the early laboratory findings of the persistence of *S.*
312 Gallinarum infection of *D. gallinae* (Zeman *et al.*, 1982) also in field. Unfortunately, the amount of
313 *S.* Gallinarum DNA in the mites collected during the sanitary break was probably under the
314 detection limit of qPCR, and therefore it was not quantifiable. However, such a low level of
315 contamination is consistent with the direct relation between extent of circulation of *S.* Gallinarum
316 among animals, and the amount of pathogen carried by mites.
317 The gathered data further confirmed such hypothesis. During the entire second production cycle,
318 hens (vaccinated) did not exhibited signs of FT, but mites collected since T_{15}, five months after the

housing of birds, were positive again to *S.* Gallinarum. Intriguingly, the positivity was observed when the mite population density rose. In fact, the estimated IR remained low (2.51/1,000), and it is predictable that this negatively affected the probability to select pools with infected mites. In this case, the low IR corresponded to a very low pathogen load (0.05 cells per mite).

The increase in the *S.* Gallinarum load recorded in the following month seems to suggest that, in presence of animals, the pathogen had a better chance to proliferate, also in mites. Noteworthily, at T_{16} the pathogen load per mite increased together with the AGDS level in chicken sera.

The crucial role of the chickens in the circulation of *S.* Gallinarum in mites was confirmed by the sudden drop of the mite IR when the AGDS antibody level grew up following the supplementary vaccine booster. Consequently, when the antibody level physiologically returned to basal values, mites were found positive again to the pathogen, with high value of IR and relatively high amount of harbored cells. A possible explanation is that higher antibody level might somehow impair the overall circulation of the pathogen, thus preventing, or at least reducing, the contamination level of mites.

It should be underlined that the vaccination of chicks conferred an effective protection to the animals, confirmed by the high level of AGDS antibodies. This prevented the reoccurrence of FT, even though mites continued to be infected. Furthermore, the vaccine was effective in preventing the disease also when animals were exposed to a high infectious pressure, as it occurred after the vaccine booster.

On aggregate, these findings contribute to shed light on the possible role played by *D. gallinae* in the transmission of *S.* Gallinarum. This investigation, along with the pioneering investigation by Zeman *et al.* (1982) showed that the mites are prone to be a stable reservoir of infection. Despite specific studies are needed to confirm the mite-mediated transmission of *S.* Gallinarum from mite to chickens, our findings strengthen the hypothesis that the pathogen might reach the hens, starting an amplification loop that can lead it to extensively circulate in the flock, if proper conditions are encountered. The low IR measured after the acute phase of the outbreak does not seem to be a limit

for that cycle. When infestation level is middle to high, up to 50,000 mites may infest a chicken per day. Therefore, each bird may come in contact with more than 50 infected mites per day. This exposes the animals to a high risk, especially whether microbial load is high or if immune protection is not effective.

Additionally, the close association with *D. gallinae* could be one of the reasons why antibiotic treatments may fail in completely eradicating *S.* Gallinarum in a poultry farm. We observed that the administration of colistin sulfate reduced the severity of the outbreak, but it did not eradicate the focus, despite it was effective against the pathogen strain. The association of *S.* Gallinarum with PRM might prevent the pathogen to come in contact with antimicrobials, therefore contributing to its survival in an infected farm.

Apart the role of *D. gallinae* as a reservoir of *S.* Gallinarum, it is still unclear if PRM could actually act as a vector of disease. The vector capability of an arthropod relies on several factors, whose the most important is the ability to transmit the pathogen from one organism to another (Reisen, 2002). To our knowledge, these are the first data about IR of *D. gallinae*, and this may be a starting point to calculate other parameters such as the entomological inoculum rate and the vectorial capacity.

In conclusion, this research contributes to add pieces of knowledge about the role of *D. gallinae* as reservoir, and maybe as a carrier, in the transmission of FT. Furthermore, it strongly suggests the contribution of *D. gallinae* to the persistence of *S.* Gallinarum in a poultry farm even between two consecutive production cycles. Such contribution had often been suspected, but, to our knowledge, there was no scientific evidence of it.

In the investigated case, the shed was washed with warm water and soap solution and then disinfected and disinfested by using the Virkon S (Medi-Nova, Reggio Emilia, Italy) and the Byemite (Bayer Italia, Milan, Italy), respectively, during the sanitary break. Such procedures reduced the circulation of mites and, consequently, of the pathogen, but they failed in completely removing those agents, as evidenced by the positive mite pool collected during the sanitary break, and the reoccurrence of *S.* Gallinarum in mites during the second cycle.

However, it should be clear that the eradication of pests in a complex environment, like the poultry farms, is very complicated. The employment of enriched cages, introduced by European Union on animal welfare grounds (EU Council Directive 1999/74/EC) does not help the farmers, as this system offer a great number of niches to protect mites during the sanitisation procedures (Koziatek & Sokół, 2015). This, in turn, could represent a significant risk for the human and animal welfare, because, the high infestation rate may contribute to the persistence and widespread of the pathogens in the farms, still making more difficult the eradication of the diseases.

In this scenario, the new strategy for the control of this pest will play a crucial role to reduce the presence of this pest in the livestock, to improve the welfare of the birds, and to limit the spread of important diseases harmful for both animal and human.

The appropriate application of preventive measure, especially vaccinations, might contribute to further reduce the risk of new outbreak in livestock, by breaking the chain which links hosts, pests and pathogens. In particular, in this case the herd immunity seems to be crucial, as few individuals without a proper protection could trigger the increase of pathogen load also in mites, and therefore considerably increasing the infectious pressure on the other animals. In the investigated case, the further vaccination with BIO-VAC SGP 695 was effective in boosting the protection to a level high enough to prevent the onset of the disease and, consequently, the increase of pathogen load in mites.

Finally, it is not negligible the importance of an integrated management of both infestation and infection. The finding of high pathogen load in mites could lead the farmers to adopt specific early measures targeted to prevent the onset of disease foci or even outbreaks.

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485 **Table 1.** Course of the fowl typhoid outbreak in the farm, and results of snPCR and qPCR for the detection and quantification of *S.* Gallinarum in
486 the poultry red mites.

Month	Production stage (main events)	Mite infestation level*	snPCR-positive mite pools, *n*	snPCR-tested mite pools, *n*	Infection rate, maximum likelihood estimation	*S.* Gallinarum load, mean CFU/mite ± SD
1 (T_1)	Production cycle 1 (acute phase before antibiotic treatment)	IV	3	4	13.77	109.57 ± 13.53
2 (T_2)		IV	3	4		402.04 ±76.11
5 (T_5)	Production cycle 1 (following antibiotic treatment)	IV	1	2	6.91	0.72 ± 0.04
7 (T_7)		IV	2	4		1.72 ± 0.92
8 (T_8)		IV	2	4		1.20 ± 0.72
9 (T_9)	Sanitary break	0	1	4	1.82	ND
10 (T_{10})		0	0	2		ND
11 (T_{11})	Production cycle 2 (housing at the end of October 2013)	0			MNC	
12 (T_{12})		0			MNC	
13 (T_{13})		0			MNC	
14 (T_{14})		II	0	2	2.51	ND
15 (T_{15})		IV	2	7		0.05 ± 0.05
16 (T_{16})	Production cycle 2 (emergency vaccine recall at the beginning of April)	IV	1	3	5.10	0.41 ± 0.03
17 (T_{17})		IV	1	2		0.38 ± 0.01
18 (T_{18})	Production cycle 2 (following emergency vaccine recall)	IV	0	2	-	ND
19 (T_{19})		IV	0	2		ND
21 (T_{21})	Production cycle 2	IV	1	2	13.77	1.11 ± 0.10
23 (T_{23})		IV	2	2		4.39 0.47

487 * Cox's scale reported in Mul et al. (2015)
488 SD: Standard deviation; MNC: Mites not collected; ND: *S.* Gallinarum not detected in qPCR.

Table 2. Primers and probe for the identification and quantification of *S.* Gallinarum by qPCR

Oligomer name	Nucleotide sequence, 5'-3'	Size of amplicon, bp	Position[†]
RTSGf	CCGATATGAGGGATGTAC	144	334859 - 334876
RTSGr	AGGTCGTAATGAGTCAAA		335002 - 334985
RTSGp	FAM-ACATCGTAATTCATGCACTACCACCAT-BHQ1	-	334978 - 334952

FAM: 6-carboxylfluorescein; BHQ1 Black Hole Quencher® 1.

[†]Referred to the *S.* Gallinarum chromosome reference sequence RefSeq NC_003197.

492 **Table 3.** Anti-group-D-Salmonella antibody values, measured during the fowl typhoid outbreak and
493 the next production cycle.

Month	Production stage (main events)	Anti-group-D-Salmonella antibodies, mean ± SD
1 (T_1)	Production cycle 1 (acute phase before antibiotic treatment)	1.07E+04 ± 3.89E+03
2 (T_2)		BNC
5 (T_5)	Production cycle 1 (following antibiotic treatment)	BNC
7 (T_7)		1.05E+04 ± 1.54E+03
8 (T_8)		BNC
9 (T_9)	Sanitary break (birds in the pullet farm)	6.58E+03 ± 1.59E+03*
10 (T_{10})		4.70E+03 ± 1.44E+03*
11 (T_{11})	Production cycle 2 (housing at the end of October 2013)	6.60E+03 ± 1.20E+03
12 (T_{12})		6.20E+03 ± 1.41E+03
13 (T_{13})		6.09E+03 ± 1.45E+03
14 (T_{14})		BNC
15 (T_{15})		5.29E+03 1.79E+03
16 (T_{16})	Production cycle 2 (emergency vaccine recall at the beginning of April)	6.16E+03 ± 1.54E+03
17 (T_{17})		BNC
18 (T_{18})	Production cycle 2 (following emergency vaccine recall)	8,19E+03 ± 1.88E+03
19 (T_{19})		8.21E+03 ± 1.58E+03
21 (T_{21})	Production cycle 2	3.18E+03 ± 6.87E+02
23 (T_{23})		3,14E+03 ± 7.51E+02

494 * Blood collected in the pullet farm before housing.
495 SD: Standard deviation; BNC: Blood not collected.

Figure legend

Figure 1. Trends of the *S.* Gallinarum load per 100 mites (line) and of anti-group D *Salmonella* antibodies in the chickens' sera (histograms) during the investigation period.