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Factors Influencing the Persistence of *Salmonella* Infantis in Broiler Litter During Composting and Stabilization Processes and Following Soil Incorporation

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Broiler litter (BL), a by-product of broiler meat production, is frequently contaminated with *Salmonella* and other zoonotic pathogens. To ensure the safety of crop production chains and limit pathogen spread in the environment, a pre-treatment is desired before further agricultural utilization. The objective of this study was to characterize the effect of physico-chemical properties on *Salmonella* persistence in BL during composting and stabilization and following soil incorporation, toward optimization of the inactivation process. Thirty-six combinations of temperature (30, 40, 50, and 60°C), water content (40, 55, and 70%; w/w), and initial pH (6, 7, and 8.5) were employed in static lab vessels to study the persistence of *Salmonella enterica serovar* Infantis (*S.* Infantis; a multidrug-resistant strain) during incubation of artificially-inoculated BL. The effect of aeration was investigated in a composting simulator, with controlled heating and flow conditions. Temperature was found to be the main factor significantly influencing *Salmonella* decay rates, while water content and initial pH had a secondary level of influence with significant effects mainly at 30 and 40°C. Controlled simulations showed faster decay of *Salmonella* under anaerobic conditions at mesophilic temperatures (<45°C) and no effect of NH_3 emissions. Re-wetting the BL at mesophilic temperatures resulted in *Salmonella* burst, and led to a higher tolerance of the pathogen at increased temperatures. Based on the decay rates measured under all temperature, water content, and pH conditions, it was estimated that the time required to achieve a 7 $\log_{10}$ reduction in *Salmonella* concentration, ranges between 13.7–27.2, 6.5–15.6, 1.2–4.7, and 1.3–1.5 days for 30, 40, 50, and 60°C, respectively. Inactivation of BL indigenous microbial population by autoclaving or addition of antibiotics to which the *S.* Infantis is resistant, resulted in augmentation of *Salmonella* multiplication. This suggests the presence of microbial antagonists in the BL, which inhibit the growth of the pathogen. Finally,

Salmonella persisted over 90 days at 30°C in a Vertisol soil amended with inoculated BL, presumably due to reduced antagonistic activity compared to the BL alone. These findings are valuable for risk assessments and the formulation of guidelines for safe utilization of BL in agriculture.

Keywords: poultry litter, poultry manure, antagonistic microorganisms, thermal inactivation, pathogen elimination, zoonotic microorganisms, fresh produce contamination, field crops contamination

INTRODUCTION

The poultry sector is amongst the fastest growing agriculture-based meat production industries worldwide, due to the increasing demand for meat and egg products (Bolan et al., 2010). Broiler litter (BL) is a by-product of broiler meat production. It consists of a mixture of bedding (usually sawdust or shavings, rice hulls or straw), manure, and wasted feeds and feathers. BL can become a source of zoonotic pathogens such as *Salmonella* that are pathogenic to humans (Chinivasagam et al., 2010; Wilkinson et al., 2011; Gould et al., 2013). Often, *Salmonella* that are excreted from infected chickens, contaminate the litter and eventually, the poultry house environment and the entire flock (Jones et al., 1991; Bryan and Doyle, 1995; Corrier et al., 1999; Trampel et al., 2000). Thus, besides the immediate risk to public health through the consumption of contaminated eggs and broiler meat products, *Salmonella* can be transferred to the agricultural environment by contaminated litter.

BL is a valuable alternative fertilizer and soil additive that is used in conventional and organic farming. It has been proven in numerous studies to be an effective fertilizer for row crops, including corn, cotton, and soybean (Tewolde et al., 2013) and in some cases, it was shown to be more effective and valuable than synthetic fertilizers (Tewolde et al., 2011). A meta-analysis based on 116 studies showed positive effects of poultry litter compared to inorganic fertilizers regarding P and K plant uptake and other indicators of soil fertility (Lin et al., 2016). Yet, although BL may be contaminated by a variety of zoonotic pathogens, most farmers use it without processing or after partial stabilization by stockpiling (Ogejo and Collins, 2009; Wilkinson et al., 2011; Wiedemann, 2015). These common practices may facilitate pathogen spread in agricultural fields, which in turn may lead to crop contamination. Indeed, there are numerous reports on the contamination of fresh produce in the field due to soil contamination (Beuchat, 2002; Bell et al., 2015; Gu et al., 2018; Jechalke et al., 2019). In many such cases Salmonellosis outbreaks have been associated with consuming contaminated fresh produce, like tomatoes, cantaloupe, and leafy vegetables (Fatica and Schneider, 2011; Herman et al., 2015; Chaves et al., 2016). *Salmonella enterica serovar* Typhimurium (*S.* Typhimurium) was detected in soil up to 231 days after application of poultry and cattle manure composts that were artificially inoculated with the pathogen (Islam et al., 2004a,b). In these studies, *Salmonella* was also detected in vegetable crops grown in the tested soils, 203 and 84 days after seeding of carrot and radish, respectively (Islam et al., 2004a), or 231 and 63 days after seeding parsley and lettuce, respectively (Islam et al., 2004b). In spite of strict control measures and regulations, *Salmonella* outbreaks due to consumption of contaminated fresh produce are still a threat to public health.

Thermal processing is considered as a practical and effective approach for inactivating pathogens in BL or BL-based organic fertilizers prior to land application (Williams and Benson, 1978; Macklin et al., 2008; Wilkinson et al., 2011). Although thermal inactivation may occur partially by stockpiling the litter for some time before spreading, it cannot be effective as compared to controlled thermophilic composting. The first active stage of composting is an exothermic process governed by aerobic decomposition reactions. Typically, the efficiency of the composting process depends on proper aeration of the material (Sánchez et al., 2017; Alkoaik, 2019; Oazana et al., 2020), under which the degradation rates can be maximized. These conditions in turn yield high rates of heat emission and eventually lead to thermal inactivation of pathogens. Based on international regulations (USA and Canada; also adopted in Israel), to ensure effective pathogen elimination, all compost particles need to be exposed to a minimum temperature of 55°C for a period of at least 3 consecutive days (United States Environmental Protection Agency, 2003; Wichuk and McCartney, 2007). Yet, composting piles are often not extensively controlled, such that thermal inactivation is not efficient throughout the entire pile even after several turnings (i.e., temperatures do not reach a minimum of 55°C throughout the pile; Wilkinson et al., 2011; Avidov et al., 2017). Although thermophilic temperatures are reported in numerous composting studies, only average values are usually presented, while the spatial variability of the temperature in the pile is practically unknown. The situation is even more critical in static piles, where a minimal management regime is commonly applied (Avidov et al., 2019). Pathogens surviving the composting process or any phase of stabilization, may regrow during storage or following land application. Indeed, several studies have demonstrated the persistence of zoonotic pathogens in the finished compost at different levels of maturity and in compost-amended soils (Chen and Jiang, 2014; Reynnells et al., 2014; Hruby et al., 2018).

The combination of temperature with other environmental factors may have variable effects on *Salmonella* persistence in BL. Several studies have examined the effect of temperature and water content, generally showing that thermal susceptibility of the pathogen increases with increasing water content. At low water contents, desiccation may play a major role in pathogen inactivation. Yet, desiccation-adapted *Salmonella* spp. persisted longer in aged chicken litter compared to non-adapted cells (Chen et al., 2013). The increased persistence

and temperature-tolerance of *Salmonella* at low water content was evident in a study by Liu et al. (1969), who showed that *Salmonella* Senftenberg strain 775 W in meat and bone meal of 5% water content remained relatively stable at temperatures as high as 50°C. Heat inactivation of *Salmonella* spp. in fresh poultry compost was faster at 50% water content compared to 40%, both at 50 and 55°C (Singh et al., 2012). Yet, an opposite trend was shown by Wilkinson et al. (2011) who found that at lower temperatures (35 and 45°C) more effective reduction of *S.* Typhimurium in poultry litter occurred at 30% than at 65% water content. Other co-factors may also play a role in bacterial inactivation, such as the combined and intensified effect of drying and NH_3 emissions, shown by Himathongkham and Riemann (1999). Biological mechanisms, such as competition between indigenous microorganisms and pathogens (Wichuk and McCartney, 2007), and microbial antagonism (Millner et al., 1987; Erickson et al., 2010; Gurtler et al., 2018) may also affect pathogen inactivation.

Overall, beyond thermal inactivation, which is considered as the primary mechanism responsible for the inactivation of human pathogens in BL and other livestock manures, there is a lack of knowledge about the combined effect of different physico-chemical and biological factors. This study aimed at investigating various key factors and the interactions among them that influence the persistence of *Salmonella* in BL during composting and stabilization processes and following soil incorporation.

MATERIALS AND METHODS

Broiler Litter (BL) and Soil

BL

Selected physical and chemical properties of the BL and the soil used in this study are presented in **Tables 1A**,**B**. *Un-stabilized (fresh)* BL was collected throughout the experimental period from tunnel-ventilated broiler houses of several farms located at the Jezreel Valley, northern Israel: Moshav Barak, Moshav Beit She'arim, Moshav Balfouria, and Kibbutz Yifat. The farms use raising protocols of the main poultry cooperatives in Israel, while some of the variability shown in **Table 1** may represent spatial variation within any given poultry house and between different growing cycles at the same poultry house. In all cases, the BL was collected at the end of 6-weeks growing period. *Stabilized BL* was obtained from a static pile, representing a common practice in Israel. About 35 m^3 of BL from the poultry farm of Moshav Balfouria were stockpiled in the open yard without any further treatment for a period of 8 months. This pile was closely monitored within the first 2 months, showing that 27 and 23% of the stockpile volume were below 45°C during the first and second weeks, respectively. Later, about 30% of the stockpile volume remained below that temperature (based on *ca.* 53 sampling points monitored on a weekly basis in the first month and then biweekly in the second month). *Composted BL* was prepared using a polyethylene sleeve with forced aeration (Avidov et al., 2017, 2018). For that, about 35 m^3 of BL was pre-wetted to achieve a water content of *ca.* 50–55% (51.1 ± 1.71%), which is within the optimal range for composting (Christian et al., 2009; Zakarya et al., 2018), and then packed in a polyethylene sleeve that was sealed manually (Avidov et al., 2019). Controlled composting was processed for 56 days with blower settings of 2 min on and 30 min off. The entire compost material within the sleeve maintained thermophilic temperatures (>45°C) up to 69 and 66°C during the first and second weeks, respectively. Only *ca.* 2 and 4% of the volume was estimated to be below 45°C during the third and fourth weeks, respectively (based on *ca.* 48 sampling points monitored weekly in the first month and biweekly in the second month. Temperatures were measured using Type K thermocouples, constructed on 80 cm-long stainless steel rods; Elcon Ltd., Israel). The composted BL was left (stored) within the sleeve which was partly open for additional 6 months. Finally, the material of both the stockpile and the sleeve was sampled from 9 different locations each, unified and homogenized. All materials (un-stabilized, stabilized, or composted BL) were stored at 4°C until use, except for the experiments used to assess the potential of antagonistic indigenous populations against *Salmonella*, for which the un-stabilized BL was used without any storage. Before each experiment, the BL was acclimated for *ca.* 24 h at room temperature.

Soil

A Vertisol-type soil was collected from 0 to 30 cm depth at the Newe Ya'ar Research Center, Jezreel Valley; northern Israel.

Physical and Chemical Analyses

Dry based aqueous extracts (1:9 w/w) of BL or soil samples were prepared with distilled water by shaking the suspension for 1 h at 200 RPM on a reciprocal shaker. The pH was analyzed directly in the suspension (LL-Ecotrode Plus WOC; Metrohm, Herisau, Switzerland), while the electrical conductivity (EC) was determined in the supernatant after centrifugation at 6,000 RPM for 20 min at 25°C (CyberScan CON 11, Eutech Instruments, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Total C and N were determined after grinding sub-samples (mixer mill MM 400, Retsch, Haan, Germany) by FlashSmart 2000 Elemental Analyzer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Bacterial Strain and Inoculum Preparation

We used a clinical isolate of *Salmonella enterica* serovar Infantis; a multi-drug resistant strain containing a mega plasmid (pESI) that carries several antibiotics resistance genes (Aviv et al., 2014; courtesy of Prof. Ohad Gal-Mor, Sheba Medical Center, Tel-Ha'shomer, Israel). The culture was stored in phosphate-buffered saline (PBS) containing 15% glycerol (Duchefa biochemie, Netherlands) at −80°C. All growing media were prepared according to the manufacturer's instructions. Before each experiment, about 100 μl of the stored culture (PBS with 15% glycerol) were transferred into 100 ml of nutrient broth (NB; Oxoid, Basingstokes, UK) with tetracycline (20 μg ml^{-1}) and incubated for 24–72 h at 37°C. The culture was then transferred into several sterile polypropylene (PP) tubes, and washed 3 times with 40 ml sterile PBS by centrifugation at 6,500 RPM for 10 min at 25°C. The final pellet was re-suspended in 40 ml PBS to achieve a final concentration of *ca.* 9 $\log_{10}$ colony forming units (CFU) ml^{-1}. To inoculate the BL, the suspension was added together with the amount of water needed to adjust the BL to the desired

TABLE 1A | Selected properties of broiler litter (BL) and soil.

BL type and source	Aim and experimental setup	Bulk density (kg l^{-1})	Aqueous extract (1:9)		Total (% dry-based)	
			pH	EC (dS m^{-1})	C	N
Un-stabilized BL* (Moshav Bark)	The combined effect of temperature, water content, and pH on the persistence of *S.* Infantis in BL during lab incubation (**Figures 1**, **2** and **Supplementary Figure 1** at 40, 50, and 60°C).	0.44	6.61 (±0.08)**	10.67 (±0.95)	41.80 (±0.75)	5.44 (±0.15)
	Persistence of *S.* Infantis under controlled aerobic and anaerobic conditions using lab-scale simulations (**Figures 3**, **4**).		7.37 (±0.08)			
Un-stabilized BL (Moshav Beit She'arim)	The combined effect of temperature, water content, and pH on the persistence of *S.* Infantis in BL during lab incubation (**Figures 1**, **2** and **Supplementary Figure 1** at 30°C).	0.50	6.67 (±0.1)	11.46 (±0.11)	41.11 (±1.33)	4.65 (±0.05)
	Effect of drying and re-wetting on the persistence of *S. Infantis* in BL at 30°C (**Figure 5**).					
Un-stabilized BL (Moshav Beit She'arim)	Heat inactivation of indigenous microbial populations in BL. *S.* Infantis was grown in autoclaved BL suspension at room temperature (**Figure 6A**).	0.50	6.89 (± 0.00)	7.77 (±0.11)	42.27 (±0.68)	3.69 (±0.07)
Composted BL (Moshav Balfouria)		0.51	8.03 (± 0.74)	13.13 (±3.81)	39.52 (±0.55)	4.04 (±0.05)
Un-stabilized BL (Kibbutz Yifat)	Heat inactivation of indigenous microbial populations in BL. *S.* Infantis was grown in autoclaved BL suspension at room temperature (**Figure 6A**–inset).	0.50	6.67 (± 0.10)	7.77 (±0.86)	43.31 (±0.98)	3.92 (±0.25)
Un-stabilized BL (Moshav Beit She'arim)	Heat inactivation of indigenous microbial populations in BL. Un-stabilized and stabilized BL were artificially contaminated with the pathogen and incubated under aerobic and anaerobic conditions at room temperature (**Figure 6B**).	0.50	6.89 (±0.00)	7.77 (±0.11)	42.27 (±0.66)	3.69 (±0.07)
Stabilized BL (Moshav Balfouria)		0.50	7.12 (± 0.40)	10.16 (±1.32)	39.05 (±0.62)	3.61 (±0.21)
Un-stabilized BL (Kibbutz Yifat)	Antibiotics-induced inactivation of indigenous microbial populations in BL. *S.* Infantis was grown at room temperature in BL suspension that was amended with a cocktail of antibiotics to which the pathogen is resistant (**Figure 7**).	0.50	6.67 (± 0.10)	7.77 (±0.86)	43.31 (±0.98)	3.92 (±0.25)
Un-stabilized BL (Moshav Beit She'arim)	Persistence of *S.* Infantis in mixtures of soil with un-stabilized or stabilized BL (**Figure 8**).	0.50	6.67 (±0.1)	11.46 (±0.11)	41.11 (±1.33)	4.65 (±0.05)
Stabilized BL (Moshav Balfouria)		0.50	7.12 (± 0.40)	10.16 (±1.32)	39.05 (±0.62)	3.61 (±0.21)

**All materials (un-stabilized, stabilized, or composted BL) were stored at 4°C until use, except for the experiments used to assess the potential of antagonistic indigenous populations against Salmonella, for which the un-stabilized BL was used without any storage.*

***Standard deviation of triplicate analyses.*

water content. The final concentration of *Salmonella* was *ca.* 7 $\log_{10}$ CFU g^{-1} dry matter.

Salmonella Enumeration

The initial stock prepared for each experiment was enumerated by serial dilution in sterile PBS and plating triplicate aliquots of 100 µl on XLD agar (Oxoid Basingstokes, UK) amended with tetracycline (20 µg ml^{-1}). The plates were incubated at 37°C for 48 h before counting. BL samples, before and following *Salmonella* inoculation, were analyzed by weighing 8 g of dry matter (based on a preliminary analysis of water content) and suspending it in a sterile stomacher bag after the addition of 80 ml PBS. The stomacher (STO-4, MRC, Israel) was operated at 10 pedals s^{-1} for 3 min, and then the suspension was transferred into a sterile PP tube and let to settle for *ca.* 5 min. A 1 ml sample was taken from the top suspension to prepare serial dilutions, from which 100 µl (or 200 µl in cases where we had to decrease the detection limit) were plated in triplicate plates, as described above. Typical black colonies were counted as presumptive *S.* Infantis. No black colonies were observed in un-inoculated BL samples. *The detection limit* was determined as 10–20 CFU g^{-1} dry matter. *Salmonella* enrichment was also performed to ensure complete bacterial inactivation. A 1 ml of the undiluted suspension was transferred into each of 5–10 tubes containing 9 ml of buffered peptone water (BPW, Oxoid, Basingstokes, UK) and tetracycline (20 µg ml^{-1}). The tubes were incubated for 24 h at 37°C. Each of the enrichment tubes (undiluted) was checked for positive or negative growth on XLD agar amended with tetracycline to check for the presence of typical *Salmonella* colonies. *The detection limit after enrichment* was determined as 1–2 CFU g^{-1} dry matter.

TABLE 1B | Vertisol soil.

pH	7.54 (±0.35)[a]
EC (dS m^{-1})	1.88 (±0.19)
Organic matter (%)	2.89 (±0.27)
Sand (%)	12.57 (±1.69)
Silt (%)	24.04 (±1.86)
Clay (%)	62.16 (±0.12)
$CaCO_3$ (%)	11.93 (±0.21)

[a] *Each value represents the average of 9 sub-samples taken from 0 to 30 cm depth; Newe Ya'ar Research Center, Jezreel Valley, Israel.*

Decay Rate Calculations

Exponential decay rates of *Salmonella* were calculated using Equation (1)

$$C(t) = C_0 e^{k(t)} \quad (1)$$

where *C(t)* is the concentration of *Salmonella* (CFU g^{-1} dry matter) at point in time *t* (d), C_0 is the initial concentration of *Salmonella*, and *k* is the first-order decay constant (d^{-1}). Decay rate constants were calculated using a linear curve fit between the natural log-transformed concentrations ($ln\ C/C_0$) and time. The number of data points to be included in the linear correlation of each dataset, was selected to provide the highest R^2, while excluding data points below the detection limit.

Laboratory Simulations of Temperature, Water Content, and pH Conditions

Thirty-six combinations of four temperatures (30, 40, 50, and 60°C), three water contents (40, 55, and 70%; w/w), and three initial pH (6, 7, and 8.5) were tested. For each combination, triplicate vessels were prepared with 200 g (dry based) of un-stabilized BL that was artificially inoculated with *S.* Infantis at a concentration of *ca.* 7 $\log_{10}$ CFU g^{-1} dry matter. The BL was first placed in a biohazard bag and adjusted to the desired pH by adding acid (1 M H_2SO_4) or base (1 M NaOH). The amount of required acid or base was pre-determined in preliminary titrations on parallel samples (at 55% water content). The pH-adjusted BL was thoroughly mixed by massaging the bag over several minutes and then inoculating it with *S.* Infantis, as described above, to achieve the desired initial concentration. The inoculated BL was thoroughly mixed by massaging the bag one more time and finally the bag was placed (loosely tied) in a 600 ml glass beaker. Aeration was not controlled in these experiments. The beakers were placed in an incubator (pre-verified over 4 days before each experiment) at the desired temperature (30, 40, 50, or 60°C) for 14 days, and then transferred to 30°C for additional 14 days of incubation to evaluate regrowth potential. The desired water content was adjusted during the incubation period, based on gravimetric analyses performed in a preliminary experiment on vessels with non-inoculated BL under identical conditions. Adjustments were made every sampling day during the first 2 weeks and every 3 days during the rest of the experiment. *Salmonella* enumerations were performed on sub-samples from each vessel, on days 0, 1, 3, 7, 14, and 28. A preliminary analysis showed that the percentage of inoculated *S.* Infantis that could be recovered from the BL after incubation for 24 h at 4°C was 75 and 71% on average, at 50 and 70% BL water content, respectively (data not shown).

Before each incubation series, the BL was checked for the presence of indigenous tetracycline-resistant *Salmonella* and was always found to be below the detection limit after enrichment. Triplicate non-inoculated vessels were incubated in each of the four temperatures and served as controls. In these samples, the BL was adjusted to a water content of 55% and initial pH 7 (the water content was adjusted using 10 ml of PBS plus the needed amount of deionized water and acid/base, as done for the inoculated samples). The aim of the control vessels was to negate any possible growth of indigenous *Salmonella* or cross-contamination between samples.

Controlled Simulations of Aerobic and Anaerobic Conditions

The fate of *S.* Infantis in un-stabilized BL under controlled aerobic and anaerobic conditions was evaluated using the Agricultural Research Organization Composting Simulator (ARO-CS) in which the temperature and aeration are controlled by a programmable logic controller (Oazana et al., 2018). The system includes six 9-liter reactors; each is mounted into a separate 80-liter bath and equipped with two temperature sensors: one thermocouple in each bath and one PT-100 in each reactor. The airflow was controlled by individual mass flow controllers (MFC) and transferred to the reactors through a humidifier mounted into the water bath, ensuring water-saturated inflow, and eliminating drying of the composting mixture. However, in cases of intense aerobic activity, during which it was difficult to restrain the heat evolved (Oazana et al., 2020), the air was transferred through a bypass to enable evaporative cooling.

Two simulation experiments were performed: In the first experiment the effect of aeration on the persistence of *S.* Infantis at 40, 50, and 60°C was evaluated during 38 days. Each of the six reactors was filled with 1.4 kg of un-stabilized BL (dry-based; 4.5 liters) that was adjusted to 40% water content, initial pH 7, and was artificially inoculated with *S.* Infantis at *ca.* 7 $\log_{10}$ CFU g^{-1} dry matter. Three of the reactors were maintained under aerobic conditions with a flow of 5 l min^{-1} (10 min on and 2 min off), and three reactors were kept under anaerobic conditions, without any aeration. The simulation was divided into three phases: (I) *mesophilic phase*: Temperature was maintained between 30 and 37°C for 9 days (aerobic reactors), or between 35 and 37°C for 7 days (anaerobic reactors). The humidifier bypass was used for the aerobic reactors to restrain the heat evolved during this phase. This in turn resulted in BL drying and required re-wetting, which delayed the move to phase II in 2 days. (II) *Heating phase*: Temperature was increased to 40, 50, and 60°C, under both aerobic and anaerobic conditions, one reactor for each temperature. These temperatures were maintained for 14 (aerobic) or 7 days (anaerobic). (III). *A second mesophilic phase*: The content of each reactor was transferred into a lab incubator for additional 14 days at 30°C, to explore regrowth potential.

The BL was sampled during the simulations on days 0, 3, 7, 9, 10, 11, 12, 14, 16, 23, and 37 (aerobic), and on days 0, 3, 7, 9, 10, 14, 28, and 37 (anaerobic) and analyzed for *Salmonella*, water content, and pH. Emission of NH_3 was measured on days 1, 3, 7, 9, 11, 16, and 23 (aerobic) or days 1, 3, 8, 10, and 14 (anaerobic). Air samples (*ca.* 25 liters) were collected in Nalophan^{TM} bags (polyethylene terephthalate; thickness 20 mm; Kalle GmbH, Wiesbaden, Germany) through a sampling port located on the cape of each reactor. Before sampling, the reactors were flushed for 20 min at 2.5 l min^{-1} of air (aerobic) or N_2 (99.999% purity) (anaerobic). NH_3 was analyzed in triplicates for each bag, using the spectrophotometric method of Willis et al. (1996) with slight modifications as described by Avidov et al. (2017).

In the second simulation experiment, the persistence of *Salmonella* in un-stabilized BL was evaluated during a more gradual increase of temperature under anaerobic conditions. Duplicate reactors were filled with 1.4 kg BL (dry-based; 4.7 liters) that was adjusted to 40% water content, initial pH 7, and artificially inoculated with *S.* Infantis, as described above. The reactors were heated to 45°C by increment steps of 1°C and a total heating time of 14 days. The BL was sampled on days 0, 1, 2, 3, 5, 7, 9, 11, and 14 and analyzed for *Salmonella* concentrations.

Modulation of Microbial Populations in BL

Two sets of experiments were conducted to check the possible involvement of antagonistic microorganisms in BL that inhibit the growth of *Salmonella* under mesophilic temperatures: (1) Heat inactivation of indigenous microbial populations of the BL. (2) Addition of antibiotics to which the *S.* Infantis strain is resistant. The effect of heat inactivation was explored both in liquid suspension and in the BL itself. *For BL suspension*—aliquots of 8 g (dry-based) of un-stabilized BL pre-adjusted to 60% water content, were placed in open 50 ml PP tubes and autoclaved for 1 h (Vertical pressure steam sterilizer LS-B50L-I, KWF, China; 121°C, 0.11 MPa). Autoclave performance was validated with 1 mL spore ampules (Crosstex, USA) placed within a BL sample of similar size. The autoclaved and non-autoclaved BL samples were inoculated with 100 μl of a *Salmonella* stock, and mixed by vortexing the tubes for a few seconds. The content of each tube was diluted 1:10 in PBS (mixing in a sterile stomacher bag, as described above), and 25 ml suspension were transferred into Erlenmeyer flasks containing 100 ml of sterile NB (a total volume of 125 ml). Control flasks (no BL suspension) contained 100 ml of sterile NB that was inoculated with 100 μl of the *Salmonella* stock. The initial concentration of *Salmonella* was *ca.* 2 log_{10} CFU ml^{-1} in EXP 1 (heat inactivation) and 4 log CFU ml^{-1} in EXP 2 (a second heat inactivation experiment and the addition of antibiotics). Multiplication of the pathogen was monitored over 24 h. *For the BL itself*—aliquots of 8 g (dry-based) of un-stabilized and stabilized BL were placed in 50 ml open PP tubes and autoclaved. Both autoclaved and non-autoclaved samples were inoculated with *Salmonella* as described above. The initial concentration of *Salmonella* in this experiment was *ca.* 8 log_{10} CFU g^{-1} dry matter. The tubes were placed inside a biological hood and incubated for 11 days at room temperature (20–25°C) under aerobic or anaerobic conditions. The tubes under aerobic conditions were left with untighten caps and those under anaerobic conditions were kept closed after replacing the headspace with N_2 (purging for 30 s). *Salmonella* was enumerated by the end of the incubation period.

The effect of antibiotics was explored in a liquid suspension of un-stabilized BL as described above (without autoclaving). The suspension was amended with a cocktail of antibiotics [tetracycline, 20 μg ml^{-1}; nitrofurantoin, 64 μg ml^{-1}; trimethoprim, 50 μg ml^{-1}; nalidixic acid, 20 μg ml^{-1}; sulfamethoxazole, 50 μg ml^{-1}; and rifampin, 100 μg ml^{-1} (Aviv et al., 2014); all from Sigma–Aldrich, St. Louis, MO; ≥97% purity]. Amended and un-amended NB medium were inoculated with the same *Salmonella* stock. The initial concentration of *Salmonella* in this experiment was *ca.* 4 log_{10} CFU ml^{-1} and multiplication of the pathogen was monitored over 120 h.

Persistence of *S.* Infantis in Soil-BL Mixtures

Batch of 1 kg soil was mixed with 5% (v/v) of un-stabilized or stabilized BL containing *S.* Infantis, at an initial concentration of *ca.* 6 log_{10} CFU g^{-1} dry matter of the final soil-BL mixture. The mixtures were brought to water field capacity of 30 or 70% (13.5 and 31.5% water content, respectively) and then divided into aliquots of 8 g (dry-based), which were transferred into 50 ml PP tubes and incubated at 30°C. Triplicate tubes were sacrificed for *Salmonella* counting on days 0, 4, 8, 11, 18, 31, 45, 60, 90, and 105. The content of each tube was suspended in PBS at 1:5 ratio, vortexed for 3 min, and *Salmonella* enumeration was performed as described above. Triplicate tubes containing non-inoculated soil-BL mixture at 70% water field capacity served as control on each sampling day, and were always found to be free of *Salmonella*.

Statistical Analyses

JMPIN software was used for all statistical analyses (SAS pro 14; SAS Institute Inc.). Mean values were compared by the Tukey-Kramer Honestly Significant Difference (HSD) test at $p \leq 0.05$. Analyses of CFU counts were performed using log-transformed values.

RESULTS

The Combined Effect of Temperature, Water Content, and pH on the Persistence of *S.* Infantis in BL During Static Incubation

The persistence of *S.* Infantis under the 36 combinations of temperature, water content, and pH is presented in **Figures 1A–I**. Each figure represents the results of four temperatures at one selected water content and initial pH. The BL was incubated under the selected temperature (30, 40, 50, or 60°C) for 2 weeks (phase I), during which *Salmonella* count was reduced below the detection limit after enrichment (1–2 CFU g^{-1}

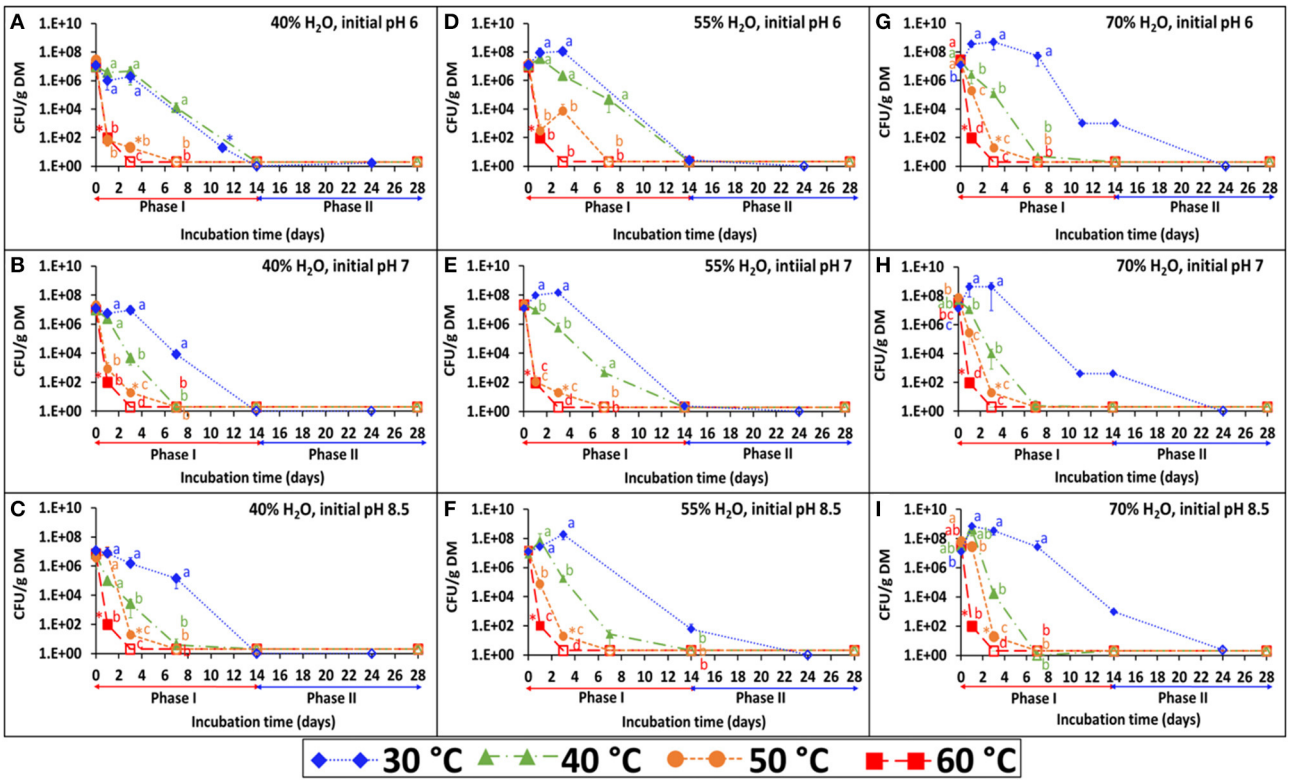

FIGURE 1 | Persistence of *S*. Infantis in broiler litter (BL) incubated at different temperatures (30, 40, 50, and 60°C), water content (40, 55, and 70%) and initial pH (6, 7, and 8.5). The different incubation temperatures were applied during phase I (weeks 1–2), while at phase II (weeks 3–4) the incubation temperature was 30°C. Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} dry matter); *values below the detection limit without enrichment (10–20 CFU g^{-1} dry matter). The figures are grouped according to water contents of 40% **(A–C)**, 55% **(D–F)**, and 70% **(G–I)**. DM, dry matter.

dry matter). Besides water content which was adjusted during incubation, the pH (**Figure 2**) and EC (**Supplementary Figure 1**) were dynamic and monitored in a parallel experiment of non-inoculated BL. Under lower temperatures (30 and 40°C), the pH typically increased during the first 2 weeks and stabilized at the range of 9–9.5. Different dynamics were observed under the higher temperatures (50 and 60°C), in which the pH tended to decrease during phase I and in certain cases to increase later during phase II. This effect of temperature on pH development was more evident at the lower water contents and the lower initial pH. Regarding the EC, a general increase was observed for all combinations during phase I, but it was more evident at a water content of 70% for which this increase was significantly higher in the lower (30 and 40°C) compared to the higher (50 and 60°C) temperatures.

The first-order decay constants in this experiment [$-k$ (day^{-1})], are presented in **Table 2**. Based on these values, the time needed to reduce *Salmonella* below the detection limit after enrichment (<1 CFU g^{-1} dry matter) was calculated for initial concentrations of 7 and 3 log CFU g^{-1} dry matter. Temperature was shown to be the main factor influencing *Salmonella* decay rates, while water content and initial pH were found to be of secondary level of influence with significant effects mainly at 30 and 40°C. Under all water content and pH conditions, it was estimated that the time required to achieve 7 log reduction in *Salmonella* concentration ranges between 13.7–27.2, 6.5–15.6, 1.2–4.7, and 1.3–1.5 days for 30, 40, 50, and 60°C, respectively. Thus, the effect of temperature was most evident in the transition from mesophilic to thermophilic conditions, whereas increasing the temperature from 40 to 50°C was associated with increased decay rates by a factor of 4.6. Water content had a significant effect at 30°C, with the highest decay rates at a water content of 40% at all initial pH values. In contrast, at 40°C, the highest decay rates were observed at a water content of 70% at all pH values, and also at a water content of 40% and initial pH 7 and 8.5 only. Such effects were still significant at 50°C, with the highest decay rates at water contents of 40 and 55% and initial pH 6 and 7. These effects were negligable at 60°C.

Regrowth of *Salmonella* was not observed in phase II in any of the combinations after transferring the vessels to 30°C, neither in the vessels which were initially incubated at 30°C. *Salmonella* counts in non-inoculated control samples were below the detection limit throughout the experiment, negating the possibility that indigenous *Salmonella* were present in these samples or that cross-contamination occurred between samples.

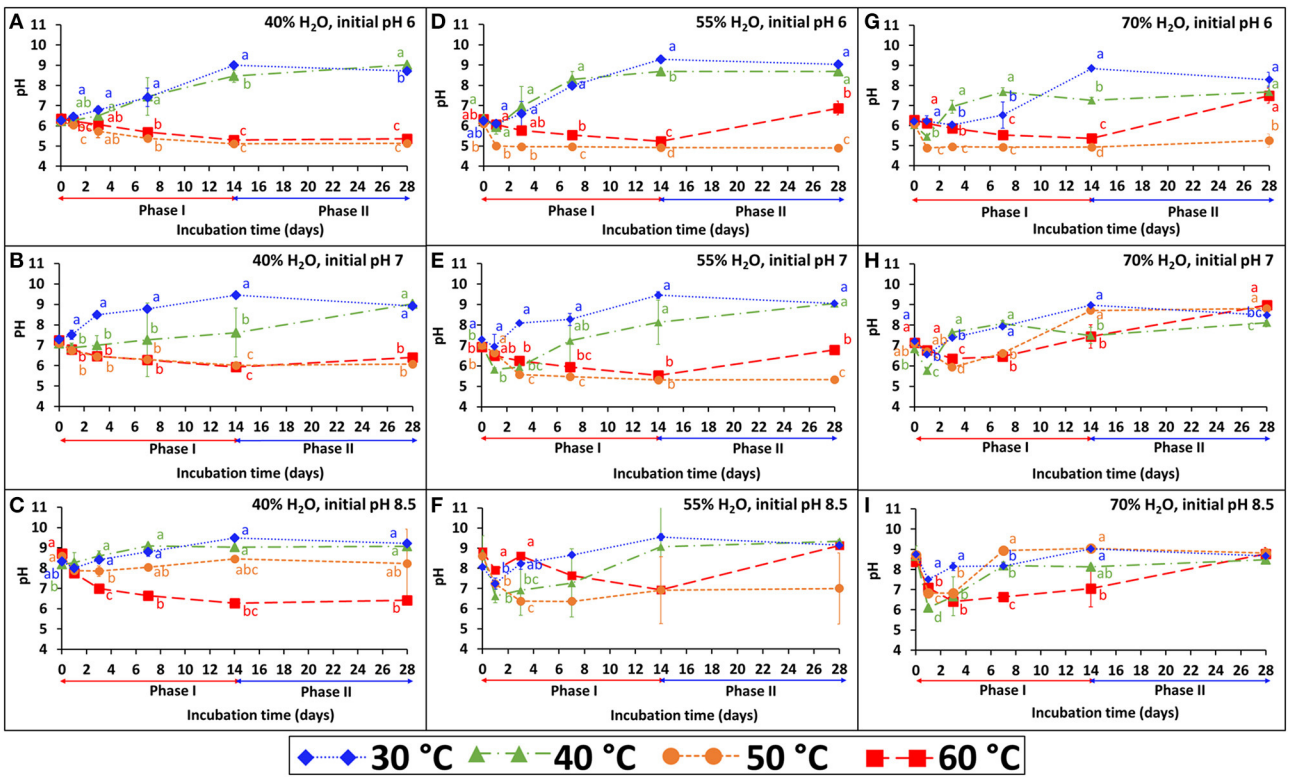

FIGURE 2 | pH dynamics during incubation of broiler litter (BL) at different temperatures (30, 40, 50, and 60°C), water content (40, 55, and 70%) and initial pH (6, 7, and 8.5). The different incubation temperatures were applied during phase I (weeks 1–2), while at phase II (weeks 3–4) the incubation temperature was 30°C. Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (Tukey-Kramer HSD; $P \leq 0.05$). The figures are grouped according to water contents of 40% **(A–C)**, 55% **(D–F)**, and 70% **(G–I)**.

Persistence of *S.* Infantis Under Controlled Aerobic and Anaerobic Conditions using Laboratory-Scale Simulations

The persistence of *S.* Infantis under controlled aerobic and anaerobic simulations of BL, pre-adjusted to a water content of 40% and initial pH 7, is demonstrated in **Figures 3A,B**. During phase I (mesophilic), *Salmonella* concentrations decreased substantially under both conditions, with 4–5 $\log_{10}$ reduction under aerobic conditions (**Figure 3A**) and 7 $\log_{10}$ reduction (below the detection limit) under anaerobic conditions (**Figure 3B**). During this phase, the water content decreased unintentionally in the aerobic reactors from 40 to 18.7% (**Figure 3A**–inset), since aeration was provided through a bypass instead of using the humidifier (see Materials and Methods) On day 7, the water content of the three aerobic reactors was re-adjusted to 40%, which in turn resulted in a 4–5 $\log_{10}$ increase of *Salmonella* concentration in one of the reactors; the one that was set to 50°C during phase II of the simulation. When phase II started (day 9), *Salmonella* concentrations increased even more in that reactor, but dropped below the detection limit after an additional 3 days. The effect of BL drying was also recognized at 60°C, where *Salmonella* showed a higher persistence and decreased below the detection limit only after 3 days, which is longer than expected based on the static vessels experiment (**Figure 1** and **Table 2**). Moreover, following 2 days at 40°C in phase II, *Salmonella* increased by 4 $\log_{10}$ and then steadily increased, reaching a concentration similar to the initial conditions of the simulation. Under these conditions, *Salmonella* was not reduced below the detection limit, even after 2 more weeks of incubation at 30°C (Phase III). In contrast to aerobic conditions, *Salmonella* did not persist under anaerobic conditions, and no growth was observed during phases II and III of the simulation (**Figure 3B**).

NH_3 monitoring (**Figures 3C,D**) revealed a major difference between aerobic and anaerobic conditions, with no clear effect of temperature (phase II). Concentrations peaked on day 3 under aerobic conditions (*ca.* 270 mg m^{-3}) while under anaerobic conditions they were mostly below 5 mg m^{-3}. The respective pH values (**Figures 3E,F**) also showed a clear difference between aerobic and anaerobic conditions. The pH fluctuated or slightly increased during aerobic simulation, while under anaerobic conditions a distinct reduction (from 6.5–7 to 5.5) was observed in phase I.

The fate of *S.* Infantis in BL (pre-adjusted to a water content of 40% and initial pH 7) under anaerobic conditions was further investigated in another simulation, during which the mesophilic temperatures increased more gradually from 28 to 42°C (**Figure 4**). *Salmonella* concentration decreased from

TABLE 2 | First-order decay rate constants (*–k;* day^{-1}) and the calculated time needed to reduce *S.* Infantis below the detection limit after enrichment, at initial concentrations of 7 and 3 log CFU g^{-1} dry matter.

Temperature (°C)	H_2O (%)	pH	$n^{\dagger}$	$R^{2\dagger\dagger}$	Decay rate constant, *–k* (day^{-1})	Significance of differences between *–k*-values (Tukey-Kramer HSD; $P \leq 0.05$)$^{++}$		Days required to reduce *S.* Infantis below the detection limit after enrichment (<1 CFU g^{-1} dry matter)	
						Analyzed for all 36 combinations together	Analyzed for each temperature separately	Initial concentration: 7 log CFU g^{-1} dry matter	Initial concentration: 3 log CFU g^{-1} dry matter
30	40	6	5	0.96	1.174	H	a	13.72 (13.64–13.81)*	5.88 (5.84–5.92)
		7	5	0.95	1.117	H	a	14.44 (13.90–15.00)	6.19 (5.96–6.43)
		8.5	5	0.93	1.097	H	a	14.47 (13.55–16.06)	6.33 (5.81–6.88)
	55	6	5	0.85	0.770	H	b	20.95 (20.64–21.27)	8.98 (8.84–9.11)
		7	5	0.84	0.771	H	b	20.91 (20.43–21.39)	8.96 (8.76–9.17)
		8.5	5	0.89	0.717	H	bc	22.49 (21.90–23.09)	9.64 (9.39–9.90)
	70	6	7	0.77	0.636	H	cd	25.36 (25.22–25.50)	10.87 (10.81–10.93)
		7	6	0.85	0.705	H	bc	22.86 (22.73–22.99)	9.80 (9.74–9.85)
		8.5	6	0.78	0.593	H	d	27.18 (26.45–27.93)	11.65 (11.34–11.97)
40	40	6	5	0.94	1.036	H	c	15.58 (14.88–16.30)	6.68 (6.38–6.98)
		7	4	0.98	2.242	GH	a	7.20 (6.80–7.62)	3.09 (2.91–3.27)
		8.5	4	0.96	2.297	FGH	a	7.05 (6.47–7.67)	3.02 (2.77–3.29)
	55	6	5	0.91	1.055	H	c	15.31 (14.42–16.25)	6.56 (6.18–6.96)
		7	4	0.97	1.606	GC	b	10.38 (8.27–12.74)	4.45 (3.55–5.46)
		8.5	4	0.89	1.664	GC	b	9.72 (9.06–10.41)	4.16 (3.88–4.46)
	70	6	4	0.98	2.176	GC	a	7.41 (7.14–7.70)	3.18 (3.06–3.30)
		7	4	0.98	2.496	DEFGH	a	6.47 (6.09–6.87)	2.77 (2.61–2.94)
		8.5	4	0.89	2.381	EFGH	a	6.78 (6.49–7.07)	2.90 (2.78–3.03)
50	40	6	2	1.00	13.004	A	a	1.24 (1.15–1.35)	0.53 (0.49–0.58)
		7	2	1.00	12.286	AB	a	1.35 (1.09–1.65)	0.58 (0.47–0.71)
		8.5	3	0.80	3.617	CDEFG	b	4.74 (3.43–6.43)	2.03 (1.47–2.73)
	55	6	2	1.00	11.563	AB	a	1.41 (1.23–1.61)	0.60 (0.53–0.69)
		7	2	1.00	13.092	A	a	1.25 (1.09–1.42)	0.53 (0.47–0.61)
		8.5	3	0.99	4.571	CDE	b	3.53 (3.46–3.60)	1.51 (1.48–1.54)
	70	6	3	1.00	4.595	CD	b	3.51 (3.45–3.57)	1.50 (1.48–1.53)
		7	3	0.98	5.132	C	b	3.15 (3.00–3.29)	1.35 (1.29–1.41)
		8.5	3	0.87	4.486	CDEF	b	3.61 (3.34–3.89)	1.55 (1.43–1.67)
60	40	6	2	1.00	11.33	AB	bc	1.42 (1.38–1.46)	0.61 (0.59–0.63)
		7	2	1.00	11.38	AB	abc	1.42 (1.34–1.51)	0.61 (0.57–0.65)
		8.5	2	1.00	10.78	B	c	1.50 (1.37–1.64)	0.64 (0.59–0.70)
	55	6	2	1.00	11.87	AB	abc	1.36 (1.33–1.38)	0.58 (0.57–0.59)
		7	2	1.00	12.19	AB	ab	1.32 (1.27–1.38)	0.57 (0.54–0.59)
		8.5	2	1.00	11.86	AB	abc	1.36 (1.32–1.40)	0.58 (0.57–0.60)
	70	6	2	1.00	12.15	AB	ab	1.33 (1.30–1.36)	0.57 (0.56–0.58)
		7	2	1.00	12.45	AB	ab	1.29 (1.27–1.32)	0.55 (0.54–0.56)
		8.5	2	1.00	12.71	AB	a	1.27 (1.25–1.29)	0.54 (0.54–0.55)

$n^{\dagger}$, number of time points included in the linear correlation. For each set of data (including all replicate samples in each condition), the exact number of time points used for the linear correlation was selected to provide the highest R^2 (excluding data points below the detection limits).

$R^{2\dagger\dagger}$, the average linear regression coefficient of triplicate set of data (based on $n^{\dagger}$ data points).

**Minimum and maximum values calculated for triplicate –k data.*

$^{++}$Letters denote significant differences between treatments (Tukey-Kramer HSD; $P < 0.05$).

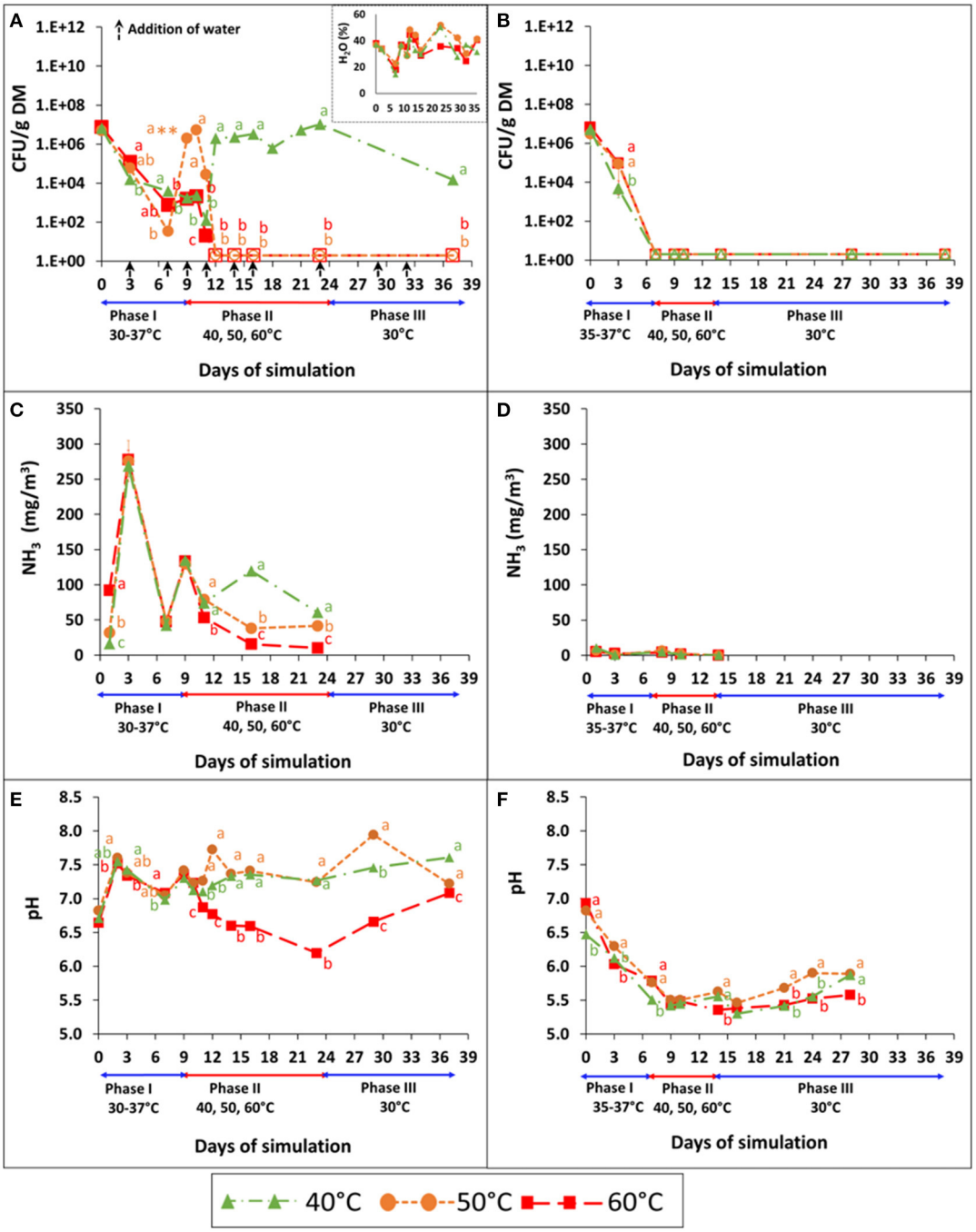

FIGURE 3 | Persistence of *S.* Infantis **(A,B)**, emissions of NH_3 **(C,D)**, and pH dynamics **(E,F)** in broiler litter (BL), during controlled aerobic and anaerobic simulations performed in six reactors (one reactor for each treatment). The water content was monitored and adjusted under aerobic conditions (**A**–inset). Mesophilic temperatures were maintained between 30 and 37°C in phase I and then increased to 40, 50, or 60°C in phase II until *Salmonella* was reduced below the detection limit. Finally, in phase III, the contents of each of the reactors were transferred to 30°C for 2 more weeks. The airflow was constant (5 l min^{-1}) during the simulations of aerobic conditions. Error bars represent the standard deviations of triplicate analyses performed on a unified sample collected from each reactor. Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} dry matter); **a value above plate countability. Letters denote significant differences between treatments at time intervals (Tukey-Kramer HSD; $P \leq 0.05$). DM, dry matter.

7.5 log_{10} to <10 CFU g^{-1} dry matter (below detection limit) within 11 days, during which the temperature increased to 38°C only. In a similar manner to the other simulations under controlled anaerobic conditions (**Figure 3F**), the pH decreased from 7.04 (±0.1) to 6.09 (±0.04) over 14 days (data not shown).

Effect of Re-wetting on the Persistence of *S.* Infantis in BL

In addition to the effect of drying and re-wetting shown in the aerobic simulations (**Figure 3**), this effect was tested at 30°C in the static vessels for all water contents at pH 7 (**Figures 1B,E,H**) and for 40% water content only, also at pH 6 and 8.5 (**Figures 1A,C**). Regardless of the initial pH, adjusting the water content to 70% in BL that was previously incubated at 40 and 55% water content, resulted in a burst of *Salmonella* growth by 5–6 $\log_{10}$ CFU g^{-1} dry matter within the next 14 days (**Figure 5**–inset). During this period, no changes in *Salmonella* concentrations were observed in the BL samples which were initially incubated at a water content of 70%.

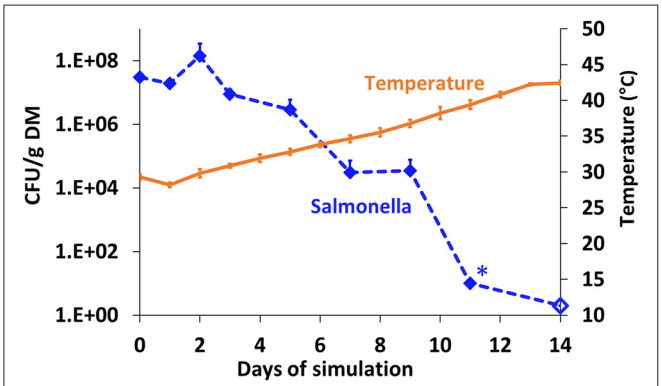

FIGURE 4 | Persistence of *S.* Infantis in broiler litter (BL) during controlled anaerobic simulation in duplicate reactors maintained at mesophilic temperatures. The BL was pre-adjusted to a water content of 40% and initial pH 7, and the temperature increased to 42°C over a period of 14 days. Error bars represent the standard deviations of two reactors. Empty symbols indicate a value below the detection limit after enrichment (1–2 CFU g^{-1} dry matter); *values below the detection limit without enrichment (10–20 CFU g^{-1} dry matter). DM, dry matter.

Potential Antagonistic Activity Against *S.* Infantis in BL

In suspensions containing autoclaved BL, *Salmonella* multiplied at a similar rate as in control medium without BL. From *ca.* 2 $\log_{10}$ CFU ml^{-1} it increased to 9.6 $\log_{10}$ CFU ml^{-1} within 20 h. On the other hand, *Salmonella* growth was completely inhibited in medium containing non-autoclaved BL (**Figure 6A**). These findings were repeated in another experiment using autoclaved BL from another farm (**Figure 6A**–inset). The effect of autoclaving was also observed in the BL itself (20–25°C; 60% water content). The concentration of *Salmonella* in the autoclaved BL increased from *ca.* 8 $\log_{10}$ to 9–10 $\log_{10}$ CFU g^{-1} dry matter within 11 days, both under aerobic and anaerobic conditions. On the other hand, *Salmonella* decreased to 3–6 log CFU g^{-1} dry matter in the non-autoclaved samples, with significantly more reduction under aerobic conditions for the un-stabilized BL (**Figure 6B**).

The effect of BL amendment with antibiotics on the persistence of *Salmonella* is presented in **Figure 7**. In the un-amended treatment (no antibiotics), *Salmonella* multiplication was inhibited within 24 h, while no such inhibition occurred in the antibiotics-amended BL suspension and in the control media with or without antibiotics (no BL). After 24 h

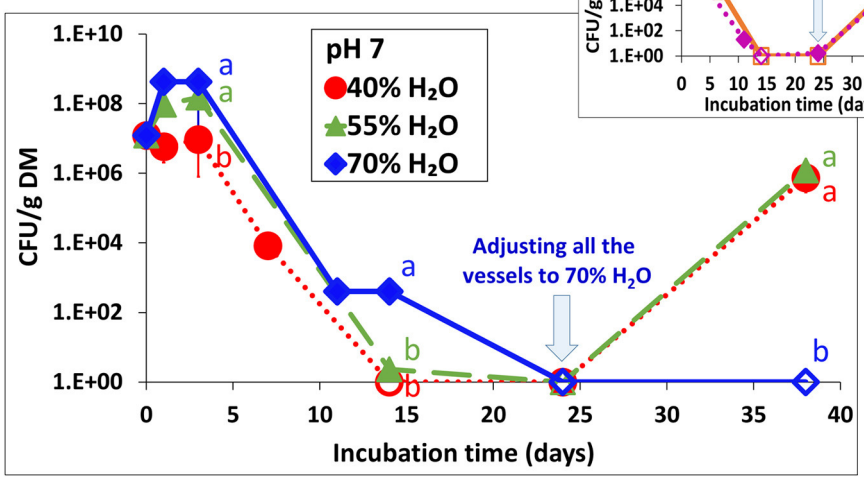

FIGURE 5 | Persistence of *S.* Infantis in broiler litter (BL) following re-wetting of samples which were incubated for 24 days at 30°C and various water contents (as shown in **Figure 1**). The vessels were incubated for additional 14 days following adjustment of water content to 70% in BL that was initially incubated at 40 and 55% water content (initial pH 7) and in the BL that was initially incubated at 40% water content also for initial pH of 6 and 8.5 (inset). Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} dry matter). DM, dry matter.

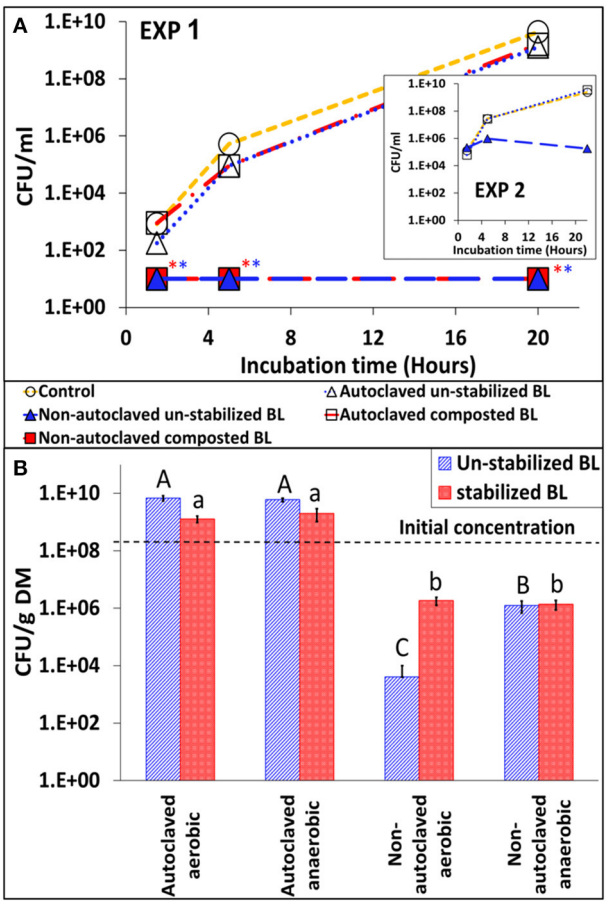

FIGURE 6 | The efect of heat inactivation of indigenous microbial populations in broiler litter (BL) on *S.* Infantis multiplication and persistance. **(A)** Growth of *Salmonella* in nutrient broth suspension of autoclaved or non-autoclaved BL (un-stabilized and composted BL; initial *Salmonella* concentration *ca.* 2 log CFU ml^{-1}). The inset figure shows the results of a similar experiment conducted with BL from another farm and at higher initial *Salmonella* concentration (*ca.* 4 log CFU ml^{-1}). *Values below the detection limit without enrichment (10 CFU ml^{-1}). **(B)** Persistence of *Salmonella* in the un-stabilized and stabilized BL itself, which were artificially contaminated with the pathogen and incubated for 11 days. Error bars represent the standard deviations of triplicate tubes. Letters denote significant differences between treatments by the end of the incubation period (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). DM, dry matter.

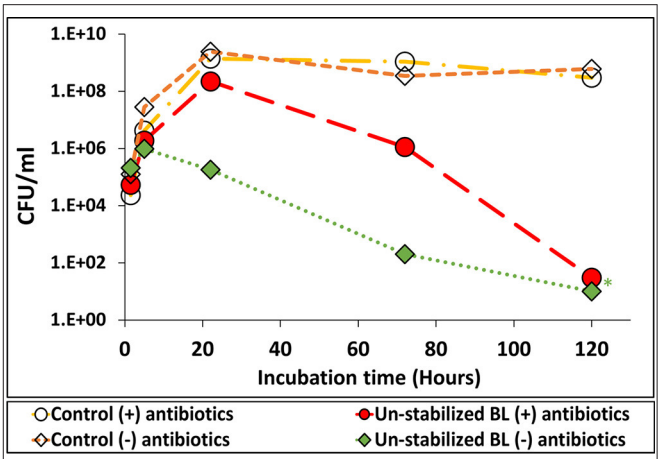

FIGURE 7 | The efect of antibiotics-induced inactivation of indigenous microbial populations in broiler litter (BL) on *S.* Infantis multiplication. Growth of *Salmonella* in nutrient broth suspension of un-stabilized BL, which was amended or not amended with a cocktail of antibiotics to which the pathogen is resistant. *Values below the detection limit without enrichment (10 CFU ml^{-1}).

Salmonella concentration declined also in the antibiotics-amended BL (compared to the maximum value reached in the controls), yet the decline rate was lower than in the un-amended treatment.

Persistence of *S.* Infantis in BL-amended Soil

Persistence of *Salmonella* in soil amended with artificially-inoculated un-stabilized or stabilized BL is shown in **Figure 8**. Within 60 days, *Salmonella* concentration reduced by 2 $\log_{10}$ in the soil with stabilized BL and by 3–4 $\log_{10}$ in the soil with un-stabilized BL. *Salmonella* reduction was substantially slower at the higher water content (70 vs. 30% of field water capacity). After 90 days, the relatively dry soil samples (either with un-stabilized or stabilized BL) were also adjusted to 70% of water field capacity, which resulted in *Salmonella* increase by 2–3 $\log_{10}$ CFU g^{-1} dry matter, 15 days later. Control samples, without addition of *S.* Infantis, were free of *Salmonella* throughout the experiment.

DISCUSSION

The persistence of *Salmonella* in poultry litter has been the subject of several studies that mainly focused on thermal inactivation (Williams and Benson, 1978; Wilkinson et al., 2011; Kim et al., 2012; Singh et al., 2012; Chen et al., 2013; Biswas et al., 2019). Most of them also demonstrated the effect of water content (Wilkinson et al., 2011; Kim et al., 2012; Singh et al., 2012; Chen et al., 2013), whereas the combined effect of multiple factors remained less understood. Beyond temperature, this study presents a more comprehensive investigation of various co-factors affecting the persistence of *S.* Infantis, an emerging pathogen associated with the poultry industry worldwide (Hindermann et al., 2017). These factors include water content, pH, drying and re-wetting, aerobic vs. anaerobic conditions, and potential antagonistic activity. The water content range was selected as being relevant for composting processes (Christian et al., 2009; Zakarya et al., 2018) and the range of initial pH was selected as being relevant for a spectrum of BL properties (Gordillo and Cabrera, 1997; Wood et al., 1999; Ekinci et al., 2000; Lopez-Mosquera et al., 2008). For practical reasons, four sources of BL were used in this comprehensive experimental scheme. Evidently, different sources may add additional factors to the already complex interactions among the co-factors examined in this study.

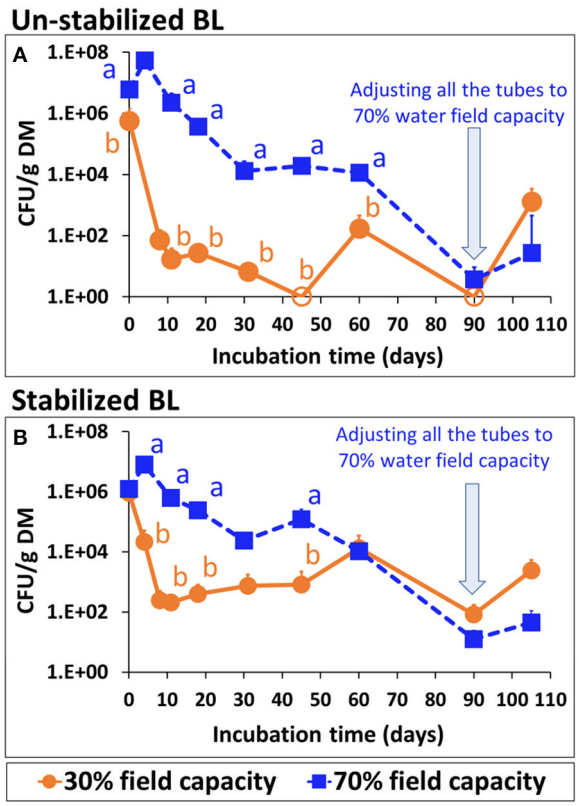

FIGURE 8 | Persistence of *S.* Infantis in a vertisol soil amended with 5% (v/v) of un-stabilized or stabilized broiler liter (BL), adjusted to 30 or 70% water field capacity. **(A)** Un-stabilized BL. **(B)** Stabilized BL. Letters denote significant differences between treatments at time intervals (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). Error bars represent the standard deviations of triplicate tubes. Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} DM). DM, dry matter.

Yet, these BL sources are from poultry growers in the same geographical region that use raising protocols of the biggest poultry cooperatives in Israel. Some of the variability shown in **Table 1** regarding the main BL properties, may be related to the spatial variability within the poultry house, as well as the time of BL storage before each of the experiments which may vary as well.

Overall, the results indicate the significant role of several co-factors under mesophilic temperatures, in which *Salmonella* may persist longer, rather than under thermophilic temperatures (above 50°C in the present study) in which thermal inactivation is the main effective mechanism. The primary role of temperature is evident from the series of BL incubation experiments, in which 36 combinations of temperatures, water contents, and pH were tested in lab vessels (**Figure 1**). A 7 $\log_{10}$ reduction of *Salmonella* was achieved within 6.5–27.2 days at 30–40°C and within 1.2–4.7 days at 50–60°C (**Table 2**). Although the bags inside the vessels were loosely tied, we could not ensure aerobic conditions throughout the entire material. However, this setup is highly relevant for real scenarios since it is practically impossible to avoid anaerobic pockets during composting, especially in the core of windrow piles (Poulsen, 2011; Stegenta et al., 2019) and it is certainly relevant for static uncontrolled litter piles. Under thermophilic temperatures, the results of Singh et al. (2012) on poultry litter-based compost, are generally in agreement with the range of decay rates found in the present study. Thermal inactivation is anticipated to remain the primary factor affecting the persistence of *Salmonella* during stabilization and composting of BL. Yet, since most of the BL worldwide is still applied without controlled processing (Ogejo and Collins, 2009; Wilkinson et al., 2011; Wiedemann, 2015), the fate of *Salmonella* under mesophilic temperatures has more practical implications. Moreover, even composting may not ensure thermophilic conditions throughout the pile. In their study on poultry litter composting, Wilkinson et al. (2011) showed that only *ca.* 35–40% of the pile's cross-sectional area was exposed to temperatures higher than 55°C during the first 2 weeks. Similarly, in their study on composting of municipal sewage sludge in open piles, Stegenta et al. (2019) estimated that 69% of the pile volume did not reach a temperature of 60°C within 7–8 weeks. Also, Isobaev et al. (2014) showed that 24% of the temperature probes that were introduced randomly into a covered aerated static pile of biosolids, did not meet sanitation conditions within 4 weeks (55°C for 3 days). Such studies emphasize that composting treatment may not ensure exposure of all particles to thermophilic temperatures and durations that are needed for pathogen elimination.

A longer persistence, yet consistent decay of *Salmonella* in BL under mesophilic temperatures (a non-thermal inactivation), has been more scarcely reported. Previous studies suggested that thermal sensitivity of microorganisms increases with increasing water content (de Bertoldi, 1988; Wilkinson et al., 2011), but at a lower temperature, desiccation may play a greater role in pathogen inactivation (Wilkinson et al., 2011). After 24 h under mesophilic temperature (35°C), Wilkinson et al. (2011) found higher persistence of *S.* Typhimurium in poultry litter at a water content of 65% compared to 30%. The present study indicates that under mesophilic temperatures and favoring water contents, *Salmonella* may initially multiply in BL following inoculation (**Figures 1**, **5**), as well as in BL-amended soil mixtures (**Figure 8**; 70% field water capacity), and then decay under all conditions. While the role of water content and initial pH was evident, there was no consistent effect with regards to these two factors. At 30°C, *Salmonella* decay rate was significantly higher at a lower water content. Likewise, *Salmonella* decayed substantially faster at 30 vs. 70% of water field capacity in soil-BL mixtures incubated at 30°C (**Figure 8**). In contrast, at 40°C, the effects of water content as well as initial pH were less consistent.

The varying effect of water content and pH and the dynamics of pH and EC during incubation (**Figure 2** and **Supplementary Figure 1**), further emphasize the complication of predicting the role of co-factors that may synergistically affect *Salmonella* persistence. It may affect *Salmonella* directly by modifying the physico-chemical conditions favored by the pathogens, or indirectly by stimulating or inhibiting the

activity of antagonistic populations. For example, high water content (70% in this study) may be associated with reduced air-filled porosity and the presence of anaerobic conditions. This, in turn, may affect the composition and density of antagonistic populations on one hand (biotic factor) and NH_3 emissions on the other hand (abiotic factor). Nevertheless, although the release of NH_3 was proposed as a factor that may inactivate *Salmonella*, the controlled simulations of the present study do not support this hypothesis. *Salmonella* decayed faster under anaerobic conditions (**Figures 3A,B**), during which NH_3 emissions were negligible compared to those obtained under aerobic conditions (**Figures 3C,D**). The lower NH_3 emissions under anaerobic conditions are expected due to the production of organic acids (Beffa et al., 1996; Naikwade et al., 2011), as evident also from the pH dynamics (**Figures 3E,F**). Thus, based on our findings, although aerobic processing of BL resulted in high levels of NH_3, it was not a major factor in *Salmonella* inactivation under mesophilic temperatures. Notably, NH_3 was shown to be effective as a process treatment designed to reduce enteric pathogens in livestock manure (Gurtler et al., 2018). Yet, this practice is based on the addition of high concentrations of liquid ammonia (Himathongkham and Riemann, 1999; Ottoson et al., 2008; Bolton et al., 2012) and not relied on the naturally associated NH_3 emissions during manure stockpiling. Other studies also suggested the involvement of NH_3 emission as a factor in the inactivation process (Kim et al., 2012; Chen et al., 2013, 2015). In these studies, inactivation was faster in more active samples (like fresh vs. aged manure), which also emitted more NH_3; however, the factor of ammonia emission was not isolated.

The effect of BL drying and re-wetting on *Salmonella* persistence was evident from three different experiments. First, during composting simulations under aerobic conditions, the unintentional drying (from 40 to 14–23% water content) and the following re-wetting, resulted in rapid multiplication of *Salmonella* with 4 and 5 $\log_{10}$ increase in bacterial counts, at 40 and 50°C, respectively. This burst of growth was associated with increased thermal tolerance of the pathogen and a longer persistence. At 40°C *Salmonella* persisted more than 30 days following re-wetting (**Figure 3A**) compared to only 6–16 days in the static vessels experiment under equivalent temperature and water content (**Figure 1** and **Table 2**). A second observation was obtained from the static vessels incubated at 30°C and water contents of 40 and 55%. *Salmonella* was reduced below the detection limit after enrichment; yet, re-wetting the BL to 70% water content, resulted in *Salmonella* increase by 5–6 $\log_{10}$ CFU within 14 days, irrespective to the initial pH (**Figure 5**). In the case of 55% water content, which is considered a suitable environment for the pathogen, the effect of water addition on *Salmonella* multiplication may suggest that the pathogen was dehydrated locally during the first phase of incubation. Finally, a third observation was obtained in soil-BL mixtures, in which the increase of field water capacity from 30 to 70% resulted in *Salmonella* multiplication within the following 15 days (**Figure 8**).

Our findings regarding the increased tolerance of *Salmonella* to high temperatures (50–60°C), are concurrent with previous studies. Increased tolerance of *Salmonella* to stress due to desiccation was investigated in pure culture (Gruzdev et al., 2011, 2012) as well as in poultry litter (Chen et al., 2013). In the study of Chen et al. (2013), a 5 $\log_{10}$ CFU reduction of desiccation-adapted cells in aged chicken litter of 20% moisture, required >6, >6, 4–5, and 3–4 h, compared to 1.5–2, 1–1.5, 0.5–1, and <0.5 h, for the non-adapted cells, at 70, 75, 80, and 85°C, respectively. Gruzdev et al. (2011) found that desiccated cells demonstrated high tolerance to a 1-h exposure to dry heat, with no substantial change in their viable counts at 60°C compared to their initial pre-challenge count and 1.5- and 3.1 $\log_{10}$ reductions at 80 and 100°C, respectively. In contrast, non-desiccated cells were highly susceptible to heat, with as much as a 3 $\log_{10}$ CFU reduction at 60°C and an 8 $\log_{10}$ reduction (below the detection limit) following 1-h incubation at 80 and 100°C. A reasonable mechanism to the increased tolerance of desiccated bacteria to high temperatures, is that very low water content in bacterial cells can inhibit or diminish the protein denaturation induced by high-temperature heating through vibration of water molecules to break S-S and hydrogen bonds of intracellular proteins. Thus, desiccation prevents the bacteria from denaturation of the membrane proteins and preserving their integrity even during exposure to a very high temperature (Earnshaw et al., 1995; Archer et al., 1998; Hiramatsu et al., 2005).

The increased thermal tolerance following re-wetting as observed in the present study has important implications. BL may dry before soil application and then be re-wetted by rain or field irrigation. The BL may also dry before composting, or any short phase of thermal stabilization, which may increase the chance of ineffective thermal inactivation. The increased stress-tolerance of desiccated cells may also be meaningful with regards to salinity. Due to organic matter oxidation (volatilization) and increased ash content, the salinity of BL increases during composting as shown in **Supplementary Figure 1**. Gruzdev et al. (2011) showed that desiccated *Salmonella* cells were able to maintain their viability in 1 to 5% bile salts and 0.1–0.5 M NaCl, while the number of the non-desiccated cells continuously declined in a dose-dependent manner. The increased tolerance to pH changes may also play a role, as the pH dynamics can vary substantially during BL processing (**Figure 2**). In this case, Gruzdev et al. (2012) showed that the survival of dehydrated *Salmonella* was maximal at pH 8.0 and decreased at lower or higher pH values, while that of the non-dehydrated cells was higher at the various pH values.

Out of the co-factors investigated in this study, the involvement of *Salmonella* antagonistic microorganisms (SAM) seems to play a major role in *Salmonella* inactivation by non-thermal mechanisms under mesophilic temperatures. Three different experiments provided indirect evidence that SAM activity was the primary mechanism for non-thermal inactivation of *S.* Infantis. Heat inactivation of the indigenous microbial populations of BL (**Figure 6**) or the addition of antibiotics to which the *S.* Infantis is resistant (**Figure 7**) resulted in augmentation of *Salmonella* multiplication, suggesting that raw BL contains microbial antagonists, susceptible to heat and

antibiotics, which inhibit the growth of *Salmonella*. In the case of antibiotics addition, this effect was lessened in the following days, presumably due to degradation of the antibiotics. Notably, the potential effect of SAM inactivation was shown both in liquid suspensions of BL (**Figures 6A**, **7**) and under aerobic and anaerobic conditions in the BL itself (**Figure 6B**), using different BL sources and degree of stabilization. Under anaerobic conditions, *Salmonella* may be inactivated also by the presence of volatile fatty acids (VFAs) that are produced during anaerobic digestion (Kunte et al., 1998; Salsali et al., 2006; Jiang et al., 2018). Yet, as shown in **Figure 6B**, *Salmonella* persisted longer in the non-autoclaved un-stabilized BL under anaerobic than under aerobic conditions, such that the effect of VFAs in this case seems unlikely. This varying magnitude of the potential effect of SAM can be related to the different experimental systems tested along this study but also due to the use of different BL sources and storage time. As suggested by Bucher et al. (2020), multiple factors and particularly physico-chemical variables are associated with litter microbiome succession. Yet, the authors emphasized the critical role of litter moisture and pH on bacterial diversity, which were pre-adjusted in all experiments of the present study. Moreover, heat inactivation by means of autoclaving was shown to facilitate the later proliferation of some heat-resistant bacterial populations in soil (Baker et al., 2020) and compost (Kim et al., 2011); yet the diversity of microbial populations after autoclaving is expected to be much lower before reaching a new equilibrium (Baker et al., 2020). Thus, although the water content and initial pH were pre-adjusted before each experiment, we cannot assess potential differences in *Salmonella* antagonistic populations. Moreover, we could not obtain specific information from the broiler growers about the possible use of antibiotics during the growing period, and we cannot assess the long-term impact of such use on BL microbiome.

The effect of antagonistic microorganisms on the fate of pathogens during composting of livestock manure and biosolids was suggested previously, based on a small number of observations (Millner et al., 1987; Sidhu et al., 2001; Jiang et al., 2002; Szala and Paluszak, 2008; Wilkinson et al., 2011; Weinberg et al., 2014). Similarly to the present study, Sidhu et al. (2001) showed the effect of sterilization of biosolids compost, in which *S.* Typhimurium multiplied rapidly as compared to non-sterilized compost in which *Salmonella* was suppressed. Likewise, Jiang et al. (2002) showed a longer persistence of *E. coli* in manure-soil mixtures, in which the soil was sterilized, compared to a mixture of non-sterilized soil. Following *Salmonella* decay in the present study, either by physico-chemical or presumably biological mechanisms, no regrowth was observed under mesophilic temperatures, besides those cases of BL re-wetting. These findings support the possibility that re-wetting is a major process inducing *Salmonella* regrowth. Then, if a *Salmonella*-contaminated BL or any BL-based organic fertilizer is applied, the pathogen may survive in the soil for several months, presumably due to a reduced antagonistic activity in the soil environment. Furthermore, multiplication of *Salmonella* may also be augmented upon soil wetting. Evidently, the fate of *Salmonella* will be further governed by horizontal and vertical transport mechanisms related to various soil properties, crop root systems, agricultural practice, and rainfall (Mawdsley et al., 1995; Islam et al., 2004a), as well as to the dynamics of SAM due to the same factors. Finally, the initial *Salmonella* concentrations used along this study (*ca.* 7 $\log_{10}$ g^{-1} dry mater) and the calculations regarding the number of days required to reduce *S.* Infantis below the detection limit, are relevant to real scenarios. *Salmonella* concentrations in the range of 3–5 $\log_{10}$ g^{-1} litter have been reported in the literature (Chinivasagam et al., 2009, 2010; Brooks et al., 2010); moreover, as shown in the present study, *Salmonella* may multiply in the litter or following soil application, and increase by several orders of magnitude.

CONCLUSIONS

Thermal inactivation is the primary mechanism of *S.* Infantis elimination in BL. Thus, composting or any thermal processing is expected to minimize the risk of *Salmonella* contamination upon soil application. In contrast, under mesophilic temperatures, other co-factors may play a significant role, including water content, pH, drying and re-wetting, aerobic *vs.* anaerobic conditions, and the presence of SAM. Although *Salmonella* persistence may be reduced at lower BL water content under mesophilic conditions, desiccation and re-wetting is critical, and increases the risk of *Salmonella* transfer from the poultry environment to soil and crops. Following soil application, *Salmonella* may persist for several months due to reduced antagonistic activity compared to the BL alone and retain its ability to multiply upon soil re-wetting. Desiccation and re-wetting of BL not only lead to augmentation of *Salmonella* multiplication, but may also increase its tolerance to thermophilic temperatures. The role of SAM under mesophilic temperatures is meaningful both under aerobic and anaerobic conditions with no clear effect of NH_3 emissions. These effects, at varying magnitudes, are expected to appear in BL from different sources, both un-stabilized, as well as stabilized or composted BL in which *Salmonella* persisted after treatment. Future studies are needed to elucidate the mode of action of SAM in BL and soil. Such studies may ultimately assist with selecting the BL-processing conditions under which SAM activity is most effective or by modulation of the BL/soil microbiome toward augmentation of SAM activity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

RA: conceptualization, methodology, data analysis, and writing original draft. VV and AL: conceptualization and methodology. IS: methodology and resources. AH: methodology. SS: conceptualization and reviewing. YC: supervision and reviewing. YL: supervision, conceptualization, writing, reviewing, and

editing. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.645721/full#supplementary-material

Supplementary Figure 1 | Electrical conductivity (EC) dynamics during incubation of broiler litter (BL) at different temperatures (30, 40, 50, and 60°C), water content (40, 55, and 70%) and initial pH (6, 7, and 8.5). The different incubation temperatures were applied during phase I (weeks 1–2), while at phase II (weeks 3–4) the incubation temperature was 30°C. Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (Tukey-Kramer HSD; $P \leq 0.05$). The figures are grouped according to water contents of 40% **(A–C)**, 55% **(D–F)**, and 70% **(G–I)**.

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