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Salmonella spp. are affected by different levels of water activity in closed microcosms

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Controlling water activity (a_w) can significantly impact the growth of *Salmonella* in poultry litter and manure — a phenomenon that was studied quantitatively using two common serotypes of *Salmonella*. The quantitative effect of changes in levels of a_w on *Salmonella* populations was determined using inoculated, frosted glass rectangles placed in closed chambers (microcosms). Glass rectangles with known concentrations of *Salmonella enteritidis* and *S. brandenburg* were placed in microcosms maintained at an a_w level of 0.893 for 24 h at room temperature (RT) and then transferred to other microcosms maintained at the same temperature but with higher a_w levels (0.932 and 0.987). *Salmonella* populations on the slides were quantified at 4, 18, 24, and 48 h. Slightly elevated levels of a_w (<0.1, i.e., 10% equilibrium relative humidity) for 24 h resulted in a 100-fold increase in counts of *Salmonella*. The data also suggested that *in vitro* adaptation to dry environments may occur when the organisms are exposed to alternating levels of relatively high and low (0.987 and 0.893) levels of a_w. Any increased tolerance of *Salmonella* to reduced levels of a_w could be the result of physico-chemical changes in the organism due to selective environmental pressure, formation of a protective biofilm, and/or entry into a dormant state. Results from this study are compatible with those from previously reported on-farm surveys, reinforcing the contention that maintaining a_w below 0.85 in and around litter/manure surfaces in poultry or livestock bedding areas may be a critical factor in safe production of food. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 222–225.

Keywords: food safety; Hazard Analysis Critical Control Points (HACCP); microcosms; quantification; *Salmonella*; water activity

Introduction

Salmonella infections are estimated to be responsible for 300000–4000000 cases of gastroenteritis reported each year in the United States [4]. The cost of salmonellosis in 1995 was estimated to have been between US$350 million and US$1.5 billion [4]. In addition to the direct health implications for humans, *Salmonella* compromises the safety and marketability of animal food. It has been estimated that prevention of *Salmonella* in poultry litter could be translated into an improvement of US$6 million in the annual cost of broiler production on the Delmarva Peninsula on the U.S. East Coast [9].

Creating a hostile environment for the survival or multiplication of *Salmonella* in broiler houses may, therefore, be mutually beneficial to consumers and producers. As a consequence, practical means of ensuring this hostile environment must be identified and more thoroughly understood.

Previous studies have shown that the control of water parameters at the poultry production farm might constitute an effective and economical way of suppressing and controlling the growth and spread of *Salmonella* [1,2,5–7,10,12]. Opara *et al.* [12] found a significant positive correlation between *Salmonella*-positive drag swabs and elevated values of a_w for samples of broiler house litter. This same observation in a larger number of houses was reported by Carr *et al.* [2]. These studies established that a_w values of litter ranging from 0.99 to 0.90, 0.89 to 0.85, and 0.84 and below constituted high-, intermediate-, and low-risk water parameters of litter/manure for *Salmonella* contamination, respectively. The same relations were reported for farms producing table eggs [5].

Although most previous studies suggested that the probability of detecting *Salmonella* on a farm increased with elevated a_w levels in litter, these studies generally did not compare the actual numbers of the organism present in litter/manure samples with a_w values of the samples. Thus, the impact of a_w on multiplication and survival of *Salmonella* required further verification.

The purpose of this study was to (1) determine if the qualitative (positive/negative) findings in the previous farm studies could be confirmed by observing and measuring the growth of *Salmonella in vitro* after exposure to different levels of a_w, and (2) to test the response of *Salmonella* to alternating levels of a_w to verify that maintaining litter/manure in a state that is continuously better ventilated and uniformly drier [5] will depress the numbers of salmonellae in litter.

Materials and methods

Preparation of microcosms

Preparation of chamber humidities: A saturated solution of sodium chloride was prepared and stored at room temperature (RT) in a tightly sealed 500-ml screw cap bottle (Nalgene, Rochester, NY). Ten-milliliter aliquots of various ratios of saturated sodium chloride solution and distilled water were prepared. The various preparations were transferred to plastic dishes (3 cm diameter) and analyzed with a previously standardized Aqua Laboratory Model

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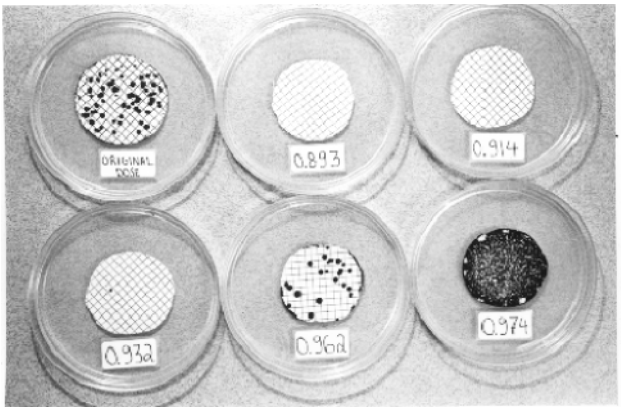

Figure 1 Membrane filter transfer (MFT) quantification of *S. brandenburg* (SB) populations on glass slide test surfaces initially inoculated with a 1:1 mixture of a broth culture of SB and a sterile fecal suspension. (a) Levels under MFT filters denote water activity levels present in the different microcosms evaluated.

CX-1 Water Activity Automated Systems instrument (Decagon Devices, Pullman, WA). A standard curve was prepared relating the different saline solutions and their corresponding a_w values in closed chambers. As used in this paper, a_w is defined as the percent equilibrium relative humidity (%ERH) generated in equilibrium with the product sample in a closed system at a constant temperature [2]:

$$\%\mathrm{ERH} = 100a_w$$

Fecal suspension (FS) material: FS material was prepared by suspending 10 g of chicken manure in 100 ml of buffered peptone water (BPW) (Difco, Detroit, MI), vortexing the suspension for 2 min, and filtering the suspension through a gauze bag (Spiral Biotech, Bethesda, MD).

The filtrate was autoclaved for 15 min at 121°C. Sterile 1-ml aliquots were stored at −70°C in 1.5-ml Eppendorf microtubes. The FS was prepared to provide a potentially protective menstruum for the *Salmonella* inocula when used in the microcosms, creating suspensions that more closely simulated natural conditions.

Initial baseline count of Salmonella: Baseline counts were prepared by mixing 500 μl of *S. enteritidis* or *S. brandenburg* grown in 10 ml of BPW for 18–24 h at 20–22°C with 500 μl of thawed FS in a sterile 15-ml plastic centrifuge test tube (Fisher Scientific, Pittsburgh, PA). To determine the viable baseline cell population, the *Salmonella* suspension was serially diluted in BPW to 10^{-5}, and 0.1 ml was spread-plated onto tryptic soy agar (TSA) (Difco). Colonies were counted after incubation at 35°C for 24 h.

Preparation and evaluation of glass test surfaces: Microcosm test surfaces were prepared from glass microscope slides with frosted ends. The slides were scored with a glasscutter and divided equally in half along the horizontal axis to conserve space in microcosms. This yielded two identical halves, each 7.5 cm long and 1.25 cm wide. Thus, the resulting rectangular frosted end of the divided slide, with an area of 125 mm^2 (12.5×10 mm^2) was sterilized and then evenly inoculated with 10 μl of stock culture in FS.

This step was performed in a room with the relative humidity (RH) set at 80%. Using sterile tweezers, each slide was placed on a sterile pipet rack. After the initial inoculation and placement of all the slides, the rack was immediately transferred to a closed, pre-equilibrated chamber at an a_w level of 0.893 at RT and tightly sealed.

After exposure to an a_w of 0.893 for 18 h at RT, several slides were transferred immediately to separate microcosm test chambers pre-equilibrated with different saline suspensions to establish a_w levels of 0.932 and 0.987. The remaining slides were used for quantitative comparisons with populations at a_w levels of 0.932 and 0.987. The initial dose (the number of post-dry salmonellae per frosted glass surface) was determined by vigorously vortexing the inoculated slide in 10 ml of BPW for 15 s, serially diluting the suspension, and spread-plating onto TSA as described above. All remaining glass slides were handled similarly.

For the experiments using alternating levels of a_w, the slide rack containing all of the initially inoculated slides was transferred from the 0.893a_w to the 0.987a_w chamber after 24 h of exposure. Before each transfer, one slide was removed and vigorously rinsed in BPW, serially diluted, and plated as previously described. The transfer procedure was performed within a few seconds in a pre-humidified room at 80% RH.

Membrane filter transfer procedure

To compare the detectability of *Salmonella* at different levels of a_w in a single, initial experiment, 30-μl volumes from each rinse were added to 50 ml of BPW contained in the loading column of a 0.45-μm pore size membrane filter monitor (Biopath, Reno, NV) after rinsing the frosted glass slide preparations. Following previously described protocols [9], the rinse suspension was lightly hand-vortexed to increase dispersion of cells and then filtered through the monitor under a 20-mm Hg vacuum. The vacuum port at the base of the monitor was plugged, and the assembly was incubated overnight at 35°C for 12 h. After incubation and using a sterile scalpel blade and forceps, the sealed filter membrane containing microcolonies was removed by carefully cutting around the periphery of the membrane and transferring it, top-side up, onto the surface of a Miller–Mallinson (MM) agar plate [8]. The agar plate with the transferred filter was then incubated at 35°C for an additional 24 h, and the number of H_2S-positive (black) colonies on the filter surface was counted. All counts were calculated to

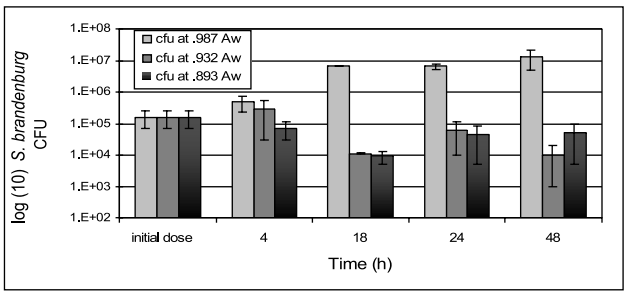

Figure 2 Growth response of *S. brandenburg* at an initial dose of 1.55×10^5 on glass slides after 4, 18, 24, and 48 h of exposure to microcosms with three different levels of a_w. *Salmonella* counts represent averages from two experiments. (a) Error bars represent colony-forming units of *Salmonella* obtained for each experiment and their distribution from the average count.

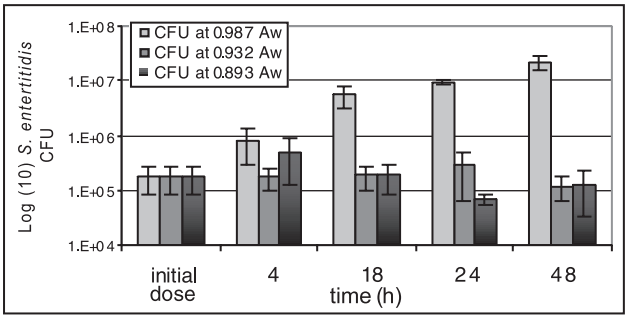

Figure 3 Growth response of *S. enteritidis* at an initial dose of 1.76×10^5 after 4, 18, 24, and 48 h of exposure to three different levels of a_w. *Salmonella* counts represent averages from two experiments. (a) Error bars represent colony-forming units of *Salmonella* obtained for each experiment and their distribution from the average count.

determine the number of colony-forming units (cfu, 10 ml^{-1}) of slide rinse (Figure 1).

Results and discussion

There was a remarkable effect on numbers of *Salmonella* after exposure to various levels of a_w (Figure 1). The filter membrane MM agar plate labeled original dose (top left-hand corner) shows the relative levels of viable colony-forming units of *S. brandenburg* on each slide before exposure to other levels of a_w. Compared with the initial dose, all slides, with the exception of the slide incubated at an a_w of 0.974, underwent significant reductions in the number of detectable salmonellae. For glass slides exposed in microcosms with levels of a_w of 0.962 and 0.932, the decrease in counts suggested that values of a_w in the 0.96–0.93 range did not promote growth of *S. brandenburg* within 24 h. Similarly, the complete lack of colonies on filters incubated at an a_w of 0.893 and 0.914 suggested that these ranges of a_w suppress the number of detectable *Salmonella*. However, populations of *Salmonella* on the slide preparation exposed to an a_w of 0.974 dramatically increased from the initial (original) concentration. This was shown by the almost completely blackened filter membrane in the bottom right-hand corner of Figure 1.

The lack of growth of *S. brandenburg* on the filter membranes at an a_w of 0.914 and 0.893 may not necessarily signify the complete absence of culturable cells, as salmonellae may have been detected had larger volumes of the slide rinse been filtered. However, similar results were obtained when using the serial dilution and direct plate counting technique on TSA.

The growth response of *S. brandenburg* and *S. enteritidis*, respectively, was measured over time at three different levels of a_w: 0.987, 0.932, 0.893 (Figures 2 and 3). Populations of both species increased approximately two orders of magnitude after 48 h of exposure to an a_w of 0.987. Conversely, populations of both species decreased after 48 h of exposure to an a_w of 0.893 and 0.932 (1.5×10^5 cfu initial dose to 1.0×10^4 and 5.2×10^4 cfu for *S. brandenburg* and 2.2×10^5 cfu initial dose to 1.4×10^5 and 8.8×10^4 cfu for *S. enteritidis* at a_w of 0.932 and 0.893, respectively). Growth of *S. brandenburg* was not apparent at an a_w of 0.932, whereas significant growth at an a_w of 0.932 was observed for *S. enteritidis* at 24 and 48 h (data not shown). Further studies are necessary to establish whether *S. enteritidis* is more osmotolerant than other salmonellae and thus capable of surviving and multiplying at lower levels of a_w.

There is a potential for major *Salmonella* outgrowths in environments with a favorable a_w (Figures 2 and 3). Such a situation would be highly undesirable because of an increase in the likelihood of product contamination before the product leaves the farm [6], which would lead to an increased incidence of contaminated carcasses at the processing plant. The decrease in counts on slides in the $0.893a_w$ microcosm reflects the advantage of controlling the humidity of litter/manure on farms producing meats or eggs. Interventions to suppress the multiplication of *Salmonella* may have other benefits because other enteric pathogens may also be suppressed with attendant-improved flock performance.

The effects of alternating levels of a_w on the growth of *S. brandenburg* and *S. enteritidis* were apparent (Table 1). Populations of both species increased when the a_w was high (0.987) and dropped when the a_w was low (0.893). With each reversal in a_w, the relative change in colony-forming unit counts per slide was less dramatic, suggesting that alternating regimens of

Table 1 Counts of *S. enteritidis* and *S. brandenburg* on frosted glass surfaces exposed to alternating water activity (a_w)

Serotype tested	Comparative counts of colony-forming units (cfu)[a]					
	Initial dose		Subsequent 24-h alternating a_w regimens			
	Pre-dry	Post-dry[b]	First reversal		Second reversal	
			0.987	0.893	0.987	0.893
S. brandenburg[c]	5.5×10^5	6.9×10^4	5.3×10^6	2.8×10^5	1.2×10^7	9.2×10^6
S. brandenburg[c]	4.9×10^5	2.4×10^5	1.3×10^7	3.4×10^6	4.9×10^7	TNTC[d]
S. enteritidis[e]	4.4×10^5	8.3×10^4	1.06×10^7	4.05×10^6	9.3×10^6	7.3×10^6

[a]Numbers represent colony-forming units of *Salmonella* in 10 μl of the baseline dose and in 10 μl of the rinse prepared from identically inoculated glass surfaces.
[b]Drying regimen was exposure to an a_w of 0.893 for 24 h. Numbers were determined from serial dilutions plated on TSA.
[c]Two separate single experiments were conducted with this serotype.
[d]TNTC: too numerous to count. After additional 144 h at an a_w of 0.893, 1.04×10^6 cells were recovered.
[e]A single experiment was conducted with this serotype.

a_w may have contributed to the selection of more osmotolerant salmonellae (Table 1).

Recent work by Mattick *et al.* [11] suggests that *Salmonella* enterica serovar *enteritidis* PT4 and *Salmonella* enterica serovar *typhimurium* DT104 survive low levels of a_w by transitioning through a filamentous stage. They have shown that these filaments formed when grown in TSA and nutrient broth (NB) at a_w ranging from 0.92 to 0.95. These filaments divided when the organisms were transferred to broth without added salt. However, we have shown that the strains used in this study form filaments when grown in TSB at a level of a_w of 0.95 but not at 0.92. Furthermore, the strains suspended in poultry feces did not form filaments on glass slides after exposure for 6 days to levels of a_w at 0.893, 0.932, 0.964, and 0.987. Hence, we do not think that filament formation with subsequent division was responsible for the population fluctuation seen in the experiments using reversal of a_w (Table 1). Rather, these findings suggest the probability that salmonellae were entering a viable non-culturable state (VBNC) [13]. Evidence for the ability of *S. typhimurium* DT104 to enter the VBNC state has been compiled in our laboratory recently (to be submitted).

Conclusion

This quantitative study extends previous *in vitro* [3] and field observations that increased levels of a_w in the litter were associated with a higher rate of recovery of *Salmonella* in the pre-harvest production environment [1,2,12]. This study also corroborates reports indicating that levels of a_w above 0.90 may pose an increased risk for *Salmonella* on farm premises by showing that, *in vitro*, these organisms multiply under elevated levels of a_w.

Levels of a_w should be regarded as a critical control point in suppressing populations of *Salmonella* and the risks on the pre-harvest end of the food safety continuum. Attention may need to be directed towards a change in ventilation practices to ensure that air of low humidity (less than 85% RH) is uniformly circulated directly over and close to surfaces of litter/manure. Moderate drying of the litter, with the implementation of such other microbial barriers as litter acidification and addition of natural antimicrobials and humectants, may synergistically establish bedding environments hostile to *Salmonella* and possibly other enteric pathogens [10].

Further studies to quantify populations of *Salmonella* in the production environment should reinforce the importance of elevated surface a_w of litter/manure. Such quantitative data might also stimulate efforts to identify those locations in poultry and livestock bedding where specific physico-chemical parameters may promote the growth of *Salmonella*. Once identified, these areas or bedding conditions can be targeted for specific remediation.

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