DETECTION AND FATE OF SALMONELLAE IN BOVINE FECES

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DETECTION AND FATE OF SALMONELLAE IN BOVINE FECES

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ABSTRACT

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by

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High densities of domestic animals in human settings can result in potential human health concerns because many animals harbor and thus be vector for human pathogens. *Salmonella* sp. belong to a group of pathogens enter farming operations through infected feed, farm equipment, and trading infected animals. In the USA, one-third of all farming operations own animals that are carriers of *Salmonella* sp. *Salmonella* cause salmonellosis, a gastrointestinal disease mainly producing symptoms like diarrhea in mild cases, though death can occur in severe cases with immuno-compromised patients. Salmonellosis costs billions of dollars in medical care. *Salmonella* transmission to humans occurs from cross-contamination of meat or ready-to-eat products, or from vegetables watered or washed with contaminated water. Contamination of water is generally believed to occur through runoff from terrestrial sites, with feces from animals

carrying salmonellae being washed into the aquatic system. Since feces are meant to be the original source of contamination with salmonellae, I was interested to explore the presence of salmonellae in feces, with emphasis on cattle feces, and study the fate of salmonellae after defecation as a function of changes in environmental conditions in time. In this thesis, I am addressing three hypotheses:

1. Salmonellae can be detected regularly in fresh feces of cattle, though likely in low numbers and without noticeable impact on animal health
2. *Salmonella* populations persist in these feces more likely better under moist conditions, compared to dryer conditions (i.e. drying out in time)
3. Salmonellae can stay viable in feces over time, though numbers might be reduced to below the detection limit of molecular tools

We detected *Salmonella* in fresh cattle feces in densities of up to 10^6 cells [g feces {dry wt.}] $^{-1}$] using *in situ* hybridization, and were interested in their fate as a function of changes in environmental conditions in time. Population dynamics of indigenous populations of salmonellae as well as of an inoculated strain (10^7 cells [g feces {dry wt.}]$^{-1}$) were monitored daily for 10 days in treatments with or without water evaporation, and contrasted to changes in abundance of all bacteria. Reduction in abundance of bacteria was obtained in all treatments though more pronounced at lower water availability. Populations of salmonellae followed the same pattern, however, with higher reduction rates in time in all treatments. At day 10, populations of *Salmonella* were below the detection limit of the *in situ* hybridization technique, however, still present and alive. Isolates obtained at that time represented 2 strains of *Salmonella* as demonstrated by rep-PCR, with one indigenous strain being most prominently isolated

even from inoculated treatments. These results demonstrate that salmonellae remain viable for at least 10 days in cattle feces even though their abundance is reduced in time, with specific indigenous populations being highly competitive.

I. INTRODUCTION

Agriculture is a pillar of any modern economy providing food, commodities and wealth to sustain both the producing and the consuming societies. Besides the obvious food production coming from animal ranches, non-edible goods such as leather, wool, and feathers also form a significant portion of the overall output of the typical ranch. To provide both edible and non-edible items to the public, farms utilize many types of animals such as pigs, sheep, cattle, and horses. Cattle are often kept in large farming/ranching operations, in close proximity to people, water sources, and other cattle. An agricultural census performed by the United States Department of Agriculture (U.S.D.A.) in 2007 found that 963,669 agricultural operations included cattle and calves as inventory, with a total of 96,347,858 individuals [46]. Concentrated Animal Feeding Operations (C.A.F.O.) encompass 15% of cattle operations within the U.S.A. [14]. Animals in C.A.F.O.s are a source of zoonotic diseases transmittable to humans. Transmittable cattle diseases can be bacterial, parasitic, viral, or fungal, or more specifically be caused by *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, *Cryptosporidium parvum*, pseudocowpox virus, and *Giardia lamblia*.

Various mechanisms likely explain the presence of *Salmonella* on a ranch. *Salmonella* is carried by a variety of animals. Wildlife, rodents, birds, cats, dogs, and flies

living in close proximity to bovine or porcine farms with *Salmonella*-infected animals have been shown to be infected. Partially migratory or short-to-medium distance

migratory birds which ate infected insects or invertebrates, subsequently were at a higher risk of disease [43]. Contaminated feed products such as soybean meal and canola meal [54], cottonseed and fish meal [45] can cause infection in domesticated porcine and bovine herds. *Salmonella* are introduced into lifestock by trading [53], transmission by people, or using common equipment [26].

During a study performed by the USDA/APHIS in 5 farming operations in each of 21 states, salmonellae were recovered from fecal samples in 7% of dairy cows, with a total of 31% of farming operations having *Salmonella*-positive cows [47]. Carrier animals within a farm are sources of continued herd infection [27]. Infected cattle may excrete up to 10^8 colony forming units of *Salmonella* per gram of feces [23]. Cows seem to be most often infected by *Salmonella* Dublin, but can also carry other serovars including *S.* Typhimurium, and *S.* Enteriditis [23]. Others, however, found *S.* Typhimurium to be most prominent in cattle [53].

Non-typhoidal salmonellae, the most common, can cause four clinical syndromes in humans including diarrhea, invasive bacteremic illness, focal suppurative infection, and asymptomatic carriage in feces [33]. *Salmonella* pathogens cause enteric symptoms and fever [37]. Indicators of disease comprise nausea, vomiting, abdominal cramps and diarrhea. The symptoms of *Salmonella* infection are similar to those of many other illnesses, and may go unreported by people not seeking medical attention [32]. The possibility of sepsis or death increases with age, and depends on the whether the affected person is immuno-compromised [28, 33]. During the years 2001-2004 in Canada, 17,459 cases of salmonellosis were reported to the National Notifiable Diseases database. 13% of these required hospitalization, with 18 deaths being reported [40]. Due to a higher

overall population, Americans experienced non-typhoidal *Salmonella* spp. accounting for 41,930 laboratory confirmed foodborne illnesses between the years 2000-2008[41]. Mead et al. estimated that the annual number of cases of disease caused by non-typhoidal *Salmonella* species was over 1.4 million cases throughout the USA, with over 16,000 hospitalizations and almost 600 deaths [31]. The annual costs of medical care and lost productivity due to salmonellosis was estimated to be conservatively from $0.5 billion dollars [12] to 3 billion dollars [52].

Contamination of drinking water and food by *Salmonella* is a significant health concern. *Salmonella*-related illness is 94-95% foodborne [32, 41], and usually contracted through contaminated animal source foods such as meat, poultry, eggs or milk [52]. Produce can be directly contaminated by pathogens through applying raw manure for fertilizer on plants, or indirectly by irrigating unknowingly with a contaminated water source [22, 20]. Lactating dairy cows produce approximately 70 kg of feces per day, so once infected with *Salmonella*; use of their manure on farms can be a viable reservoir for infection [55]. Food products which are considered ready-to-eat such as sprouts, leafy vegetables and root crops provide an especially high contamination risk due to the lack of cooking [22]. Surface water sources can be contaminated by microbes occurring from farmyards in which fecal deposits are stored or freshly deposited. Farmyards will vary in their amount and kind of contamination depending on the type of farm, along with their proximity to water [9]. Bacteria can enter water systems in many fashions including through groundwater flow, tile drainage systems, surface runoff and direct discharges [24]. Sources of contamination in water, however, are often difficult to isolate due to dilution in the environmental samples [16].

Water distribution systems within the United States can spread waterborne diseases such as *Salmonella* if distribution systems are not chlorinated, or not fully intact. From December 1989 to January 1990 Cabool, Missouri, experienced 243 cases of infection with *Salmonella* Typhi, resulting in four deaths. This outbreak occurred due to large populations of beef and dairy farms contaminating groundwater surrounding Cabool, and the groundwater remaining un-chlorinated prior to distribution as drinking water. Cabool now chlorinates their water [14]. Another *Salmonella* outbreak occurred in Alamosa, Colorado, between March and April 2008. Animal feces contaminated the drinking water through cracks and holes in water infrastructure pipes and contaminating *Salmonella* stayed viable due to lack of chlorination. This waterborne outbreak resulted in 442 reported illnesses, and one death. Epidemiological estimates suggested that up to 1,300 people were actually ill, out of a total population of 8,900 [10]. Aging, or improperly maintained water systems can also be the reason for large *Salmonella* outbreaks within a community. The National Institute of Standards and Technology estimates that US water and fuel delivery infrastructure using metal and concrete pipes results in over 240,000 breaks per year [34]. The American Society of Civil Engineers estimates that to fix all drinking water infrastructure leaks, including treatments, would cost an estimated $11 billion dollars per year [49].

Escherichia coli and other fecal coliform bacteria have been studied extensively, whereas less attention and research has been directed towards finding the optimal environmental conditions for *Salmonella* populations. Strains have been found to persist in terrestrial environments for up to five years with the continued ability to contaminate almonds [36], fish meal and animal feed [51]. Cow pat water content, and temperature

were discovered in a New Zealand experiment to provide optimal growth conditions for reproduction or maintenance of salmonellae during winter and fall [42]. While higher nutrient broth temperatures have been shown to increase death rates of salmonellae [17], a 37^{o}C incubation temperature has been found to initially increase salmonellae counts within bovine manure, followed by decreases after day three of the experiment [55]. Both time and temperature have significant impacts on *Salmonella* survival rates within manure slurry, and a moist atmosphere was more conducive to growth [15].

To expand current research on optimum *Salmonella* survival conditions, we aimed to test the hypothesis that salmonellae maintain high population numbers in moist cow pat conditions. Thus, as the moisture decreases within a cow pat in time, *Salmonella* populations should be decreasing as well. Secondly, salmonellae stay viable within an environment in low enough numbers to allow poor laboratory detection, but still be dangerous and potentially pose a threat to human health. In our study, control bovine feces were subjected to significant moisture loss. *In situ* hybridization noted extremely low countable cells. Despite this, we were able to resuscitate *Salmonella* cells under optimal conditions and detect them using end-point PCR. Lastly, we aimed to determine whether salmonellae and all organisms become dormant under sub-optimal conditions, or if other populations of organisms such as Eukarya or Archaea become more prominent in abundance under these conditions.

In this thesis, I was addressing several objectives. First, I wanted to demonstrate *Salmonella* can be commonly found in low numbers within fresh bovine pats. *Salmonella*, when found in feces, often has little or no impact on the health of carrier animals. Secondly, I tried to analyze *Salmonella* populations within samples over a

period of ten days in room temperature (25^{o}C) under moist and non-moist conditions. Our preliminary studies have shown that populations are likely to decrease significantly in response to large moisture losses within bovine feces. Lastly, we planned to evaluate if *Salmonella* populations could stay viable in feces over time, despite cell counts lower than detection limits of molecular tools.

II. METHODS

January bovine feces sampling- Bovine feces were collected at Freeman Ranch, 2101 Freeman Ranch Road, San Marcos, TX 78666, on January, 2, 2011 at 10 am. Fresh, moist cow pats were collected within cow pens for ten samples. Pats were placed into individual Ziploc-style 1-gallon bags by gloved-hand, and brought back to the laboratory for further processing.

Inoculation preparation- Inoculum was prepared by transferring a colony of *Salmonella* strain 6B aseptically to 5 mL of sterile Luria-Bertani Broth (LB) in a sterile culture tube. The inoculated broth was incubated at 37^{o}C for 16 hours on a shaker to a density of approximately 10^{8} cells ml^{-1}. Cells in the 5 mL broth were centrifuged for 15 minutes at maximum speed in an Eppendorf centrifuge (Model 5702). Supernatant was discarded, and 25 ml sterile diH_2O added to the conical tube. *Salmonella* cells were re-suspended by vortexing at low speed, and centrifuged as before. Twelve mL of sterile diH_2O was added to each conical tube, and vortexed again to re-suspend cells in preparation for inoculation.

Cow pat preparation- A 10-g sample was taken from each cow pat, and placed in a 50 mL conical tube for inoculation. Inoculation was performed by adding 1 mL of suspended *Salmonella* strain 6B to each 10-g sample which was thoroughly mixed using a metal stirrer. 200 mg samples were taken from each 10-g sample pre- and

post-inoculation placed in a 2 mL cryotube and fixed with 0.5 mL of 4% PFA as described below (see *in situ* hybridization).

July bovine feces sampling- All cow pats were collected at Freeman Ranch. Sampling occurred once within cow pens, but also around water troughs visited by free-roaming cattle. Feces were collected when their exterior looked fresh and notably moist. Seven cow pats were collected throughout pens to maintain independence in samples. Pats were placed into individual Ziploc-styled 1-gallon bags by gloved-hand, and brought back to the laboratory for further processing.

Inoculation preparation- Inoculum was prepared from a colony of *Salmonella* strain 34-2-2 obtained from cow feces. The colony were transferred aseptically to 100 mL sterile Luria-Bertani Broth (LB) in a 500 mL flask. Inoculated broth was incubated at 37^{o}C for 18 hours on a shaker to a density of approximately 10^{9} cells ml^{-1}. Turbid media was centrifuged in three 35 mL aliquots for 15 minutes at maximum speed in an Eppendorf centrifuge (Model 5702). Supernatant was discarded, and 25 ml sterile diH_2O added to each conical tube. *Salmonella* cells were re-suspended in 25 ml sterile diH_2O by vortexing, and again centrifuged as described above. 8 mL of sterile diH_2O was added to each conical tube, that were vortexed to re-suspend cells in preparation for inoculation.
Cow pat preparation- Samples were labeled 1 to 7 within the lab. From each sample, 300 grams were placed into an autoclave bag. This bag was then double-bagged, and closed firmly. Kneading was performed to the sample mixture for ten minutes to ensure adequate homogeneity. Conical tubes were numbered, 4 for each day, for a total of 11 days. 31 grams of homogenous sample were placed into each conical tube and vortexed to prevent air pockets. Of the samples, 22 became controls. Half the controls (11 total)

were left exposed to air, and the other half covered with parafilm. 1 mL of suspended *Salmonella* cells was inoculated into each of the remaining 22 samples, which were well mixed throughout with a metal stirrer for 10 minutes. The inoculated *Salmonella* was isolated from a farm near Aquarena Springs, within San Marcos, TX. Half these samples were covered with parafilm to prevent evaporation, while the other half were open and thus subjected to air and evaporation. Two experimental and two control samples for each day (parafilm and air exposure for all 4 treatments) were placed in a -80^{o}C freezer for ~30 min. Samples were then removed, and allowed to warm to 25^{o}C for 10 minutes. After 10 minutes elapsed, conical tubes had pressure circularly applied using a BrassCraft 1/8"-1 1/8" Screw Feed Tube Cutter. Pressure was placed near the 5 mL, 15 mL and 30 mL conical tube demarcations. Samples were then evacuated onto Petri dishes for weighing. Initial weights were taken of the whole sample. Samples were then further divided into top, middle and bottom portions for weighing. Once weighed, representative samples were placed into an 80^{o}C oven for 48 hours, and were re-weighed afterwards to obtain moisture content. Beginning on day 0, aliquots from each sample type (top, bottom, and middle) were taken and distributed as follows: 600 µL for freezer storage (placed in 200 µL aliquots in 1.5 mL Eppendorf tubes), and 200 µL for *in situ* hybridization (placed in a 2 mL Cryotube).

Sample preparation for end-point PCR- End-point PCR was performed on samples treated as follows: 100 µL sub-samples were transferred to a 2 mL cryotube containing 1 mL of Buffered Peptone Water [(BPW) (l^{-1}: 10 g peptone, 5 g NaCl, 9 g Na_2HPO_4, 1.5 g KH_2PO_4, pH 7.2)] and incubated at 37ºC. After 24 hours of incubation, 100 µL of these samples were transferred to a 2 mL cryotube containing 1mL of

Rappaport-Vassiliadis Enrichment Broth [(RVS) (l^{-1}: 4.5 g peptone (soymeal), 29 g $MgCl_2$ x 7 H_2O, 8 g NaCl, 0.4 g KH_2PO_4, 0.036 g malachite-green, pH 5.2)] and incubated at 37ºC for 48 hours. Sub-samples (100µL) of this semi-specific enrichment for salmonellae were transferred to new tubes with RVS, and salmonellae were enriched a second time as stated above. For PCR analyses, 100 µL samples of this second enrichment was transferred to a sterile 1.5 mL Eppendorf tube, and cells were pelleted by centrifugation for 2 minutes at 14,000 rpm. The cell pellet was washed with 500 µL of sterile diH_2O once, and subsequently lysed in 100 µL of 50 mM NaOH by incubation at 65ºC for 15 minutes with shaking. Lysed cells were kept at –20ºC until use.

Sample preparation for *in situ* hybridization- 200 µL of feces were mixed with 500 µL of 4% paraformaldehyde (PFA) and fixed for 16-24 hours at 4ºC. Samples were then centrifuged at 14,000 rpm for 2 minutes and the supernatant was discarded. The remaining pellets were washed twice with 500 µL of phosphate buffered saline [(PBS) (0.13 M NaCl, 7 mM $Na_2H_2PO_4$, pH 7.2)] and centrifuged for 2 minutes at 14,000 rpm and the supernatant discarded. Lastly, cell pellets were re-suspended in 300 µL of 50% ethanol in PBS. Samples were stored at –20ºC until further use.

Sample analyses- All analyses were performed in the Biology Department at Texas State University, San Marcos, TX. An Eppendorf Mastercycler model 22331 (manufactured in Hamburg, Germany) was used for all PCR testing, and a Nikon Eclipse 80i© microscope and Photometric Cool Snap ES$^{2©}$ camera were used to view and capture images for all *in situ* hybridization reactions.

End-point PCR- The presence of *Salmonella enterica* was analyzed. One microliter of lysate was used as template for PCR amplification with primers 139 ($^{5'}$GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 ($^{5'}$TCA TCG CAC CGT CAA AGG AAC C) [33 38 39] in a final volume of 50 µL containing 10 x PCR buffer (500 mM KCl, 25 mM $MgC1_2$, 200 mM Tris/HCI, pH 8.4, 0.1% Triton 100), 1 µL dNTPs (each 10 mM in 10 mM Tris/HCI, pH 7.5), 0.2 µL *Taq* polymerase (5 U μl^{-1}), and 1 µL of each primer (100 ng μL^{-1}). PCR amplification started with heat denaturation at 96°C for 1 minute followed by 35 rounds of temperature cycling performed in a Thermocycler with denaturation at 96°C for 30 seconds, primer annealing at 54°C for 30 seconds, and elongation at 72°C for 30 seconds [29]. Next was incubation at 72°C for 7 minutes [13]. Lysates of *Salmonella enterica* Typhimurium (ATCC 14028) as well as sterilized diH_2O was used as positive and negative controls, respectively. PCR products were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with GelRed Nucleic Acid Stain.

In situ hybridization- Samples were prepared for viewing by the following: a small amount (1-10 µL) of sample was added to an 8-well, 8 mm HTC Super Cured slide coated with gelatin, and air-dried at 42ºC for 15 minutes. Next, slides underwent 3 minutes dehydrations with 50%, 70%, and 95% ethanol, respectively, after which they air-dried for 10 minutes. For the detection of *Salmonella*, samples were hybridized in 9 µL of a hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) containing 10% formamide, to which 1 µL of Cy3-labeled oligonucleotide probe Sal3 (25 ng μL^{-1}), and 1 µl of a solution of DAPI (200 ng μl^{-1}) were added, at 42°C for 2 hours [56]. Probe Sal3 ($^{5'}$AAT CAC TTC ACC TAC GTG, *E. coli* position 1713-1730)

[35] binds to 23S rRNA of all *Salmonella enterica* subspecies tested so far (excepting only subspecies IIIa), but should not detect *S. bongori* [11]. For the detection of all Bacteria, samples were hybridized in 9 µL of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) with 30% formamide, 1 µL of Cy3-labeled oligonucleotide probe EUB338 (25 ng μL^{-1}), and 1 µl of a solution of DAPI at 44°C for 2 hours. Probe EUB338 ($^{5'}$GCT GCC TCC CGT AGG AGT, *E. coli* position 338-355) binds to the 16S rRNA of all Bacteria [1] except Plantomycetales and Verrucomicrobia [4].

Following hybridization, slides were briefly washed with diH_2O and placed into a 50 mL conical tube with buffer containing 20 mM Tris/HCl, pH 7.2, 10 mM EDTA, 0.01% SDS and 440 mM NaCl for 25 minutes at room temperature. Slides were briefly washed again with diH_2O and air-dried for 5 minutes. Slides were mounted with Citifluor AF1 solution (Citifluor Ltd., London, UK) and examined with a Nikon Eclipse 80*i* microscope, fitted for epifluorescence microscopy with a mercury lamp (X-Cite^{TM} 120; Nikon**)** and filter cubes UV-2E/C (Nikon; EX340-380, DM400, BA4435-485, for DAPI detection) and CY3 HYQ (Nikon; EX535/50, DM565, BA610/75, for Cy3 detection), respectively. Bacteria were counted at 1000 x magnification. Twenty fields, selected at random, covering an area of 0.01 mm^2 were examined from a sample distributed over eight circular areas of 53 mm^2 each. DAPI and Cy3 counts were obtained from the same image. Pictures were taken from these images using a cooled CCD camera (CoolSNAP *ES*^{2}; Photometrics, Tucson AZ), and Nikon's NIS Elements imaging software (Version 3).

Rep-PCR- Isolates positively identified as salmonellae by PCR were further characterized using rep-PCR to identify unique patterns and predominance of strains in samples after 10 days of incubation. Rep-PCR was performed as listed by Hahn, Gaertner et al. [13]. Two microliters of lysate was used as template for rep-PCR amplification with primer BoxA1R ($^{5'}$CTA CGG CAA GGC GAC GCT GAC G) [50] targeting the BOX element[30]. PCR was performed in a total volume of 25 mL containing 5 x Gitschier buffer [83 mM $(NH_4)_2SO_4$, 33.5 mM $MgCl_2$, 335 mM Tris/HCl, pH 8.8, 33.5 mM EDTA, 150 mM b-mercaptoethanol], 1.25 mL dNTPs (100 mM each, mixed 1 : 1 : 1 : 1), 2.5 mL dimethyl sulfoxide (DMSO), 0.2 mL BSA (20 mg mL^{-1}), 1.3 mL of Box primer (300 ng mL^{-1}), 0.4 mL *Taq* polymerase (5U mL^{-1}) [38] and 2 mL of lysate [7]. Initial denaturation occurred at 95^{o}C for 2 minutes, and 30 rounds of temperature cycling were then performed in a PTC-200 thermocycler. Cycles started with denaturation at 94^{o}C for 3 seconds and subsequently 92^{o}C for 30 seconds, followed by primer annealing at 50^{o}C for 1 minute, and finally elongation at 65^{o}C for 8 minutes. The 30 cycles were followed by a final incubation at 65^{o}C for 8 minutes [38, 7] . Rep-PCR products were initially analyzed by gel electrophoresis on 2% agarose gels in Tris-acetate-EDTA (TAE) buffer after staining with ethidium bromide (0.5 µL mL^{-1}), and finally bands analyzed using a Bioanalyzer (Agilent 7500). Representatives of the inoculated strain and a frequent strain were isolated and sent to the Texas Department of Health (Austin, TX) for serotyping using slide agglutination [18].

Statistical Methods- T-tests to calculate 95 % confidence intervals were used on January 2011 data using R. Within July data we assessed if there was a difference between days within individual treatments. Data was grouped by days 0-3, 4-6 and 7-10

for replication within treatments. Performed a 1-way ANOVA on $\log_{10}$ transformed data due to homoscedasticity. If data were significant using $\alpha=0.05$, performed a post-hoc analysis Fisher's LSD on any significant data.

III. RESULTS

January detection of salmonellae- Using Fluorescent *in situ* hybridization, prior to inoculation, 10 cow feces samples presented an average of 3.73×10^8 of DAPI-stained cells (Fig. 1). Immediately after post-inoculation to a density of approximately 10^8 cells ml^{-1}, the mean number of DAPI-stained cells increased to 5.55×10^8. Via a one sample t-test, 95% confidence intervals were calculated, and many samples were found to not be within the 95% confidence interval. Pre-inoculation, (upper = 4.4×10^8, lower boundary =3.2×10^8), 8 samples were not within the 95% confidence interval. Post-inoculation, 6 cow pat sample DAPI counts were not within the 95% confidence interval (upper boundary=7.0×10^8, lower boundary =4.1×10^8).

Initially, as samples dried out at 25^{o}C over a period of 3 days, the number of DAPI-stained cells decreased (Fig. 2). On day 1, the average number of DAPI-stained cells was 4.25×10^8. By day 2, the mean number of DAPI cells increased slightly to 4.77×10^8. Lastly, by day 3, DAPI-stained cells decreased to 3.65×10^8. Testing for 95% confidence intervals using a one-sample t-test, many samples were not within the upper or lower boundaries. Day 1 post-inoculation had 6 samples above or under the confidence interval limits calculated (upper=4.7×10^8 and lower 3.8×10^8). Day 2 post-inoculation

had 3 samples outside the 95% confidence intervals (upper = 5.9 x 10^8 and lower = 3.7 x 10^8). Similarly, day 3 had 7 samples not within the 95% confidence interval (upper 3.9 x 10^8 and lower =3.4 x 10^8).

Bacterial and *Salmonella* counts-Via *in situ* hybridization , the percentage of bacteria detected by probe EUB338 remained stable in overall percentages as cow pats dried out (Fig. 3). Initially bacterial averages were 4.72 x 10^8, and accounted for 85% of the total population of organisms within samples (i.e. all DAPI-stained cells). On day 2, bacterial counts were 4.32 x 10^8, and comprised 90% of the total population of organisms. By day 3, bacterial populations were 3.10 x 10^8 cells, and represented 73% of the overall population. Post-inoculation, mean numbers of *salmonella* populations were 3.37% (Fig. 4) of the total organismal population within cow pats. At day 1 the numbers had lowered greatly, and *Salmonella* represented 1.36% of the overall population. Days 2 and 3 were similar to each other in *Salmonella* counts, with *Salmonella* populations signifying 0.65% of the total organism within cow feces. Eukarya and Archaea probes were also used, but insignificant numbers noted within these samples.

July Results-Fluorescent *in-situ* hybridization for *Salmonella* and DAPI-counts- A One-way ANOVA was performed using R version 2.11.1 on $\log_{10}$ transformed grouped samples to create replication within samplings. Data was transformed due to homoscedasticity. This also limited any extremes in confidence intervals. Significant results were then tested using a Fisher's LSD post-hoc analysis to identify where differences occurred with R package agricolae. Upon placement in the fume hood on day 5, samples without parafilm protection dried out quickly with moisture reduction from 81% to 57%. Performing *in situ* hybridization (Fig. 5), unprotected samples experienced

a rapid decline in the number of salmonellae. Natural top cow pat samples demonstrated a significant decrease of *Salmonella* counts (Fig. 6 A) ($F_{2,8}$=12.3; P<0.01). Days 4-6 had significantly higher *Salmonella* averages than days 0-3 or 7-10 in natural samples (Table 1). Also, inoculated feces protected by parafilm had a significant reduction in salmonellae ($F_{2,8}$=13.6; P<0.01), but initially maintained *Salmonella* percentages >1% for the first 5 samplings. Reductions occurred significantly throughout days 0-3, 4-6 and 7-10. Inoculated samples which desiccated (Fig. 6 D), and natural samples without water reduction (Fig. 6 C) did not change significantly. DAPI-stained cells (Fig. 7) demonstrated a significant decrease in all organism numbers over a period of 11 days. Naturally drying samples (A) ($F_{2,8}$=12.4; P<0.01) showed a significant decrease in microorganisms near the end of sampling days. Inoculated samples (B) ($F_{2,8}$=12.6; P<0.01) without parafilm also showed remarkable declines after 4 days of exposure to air (Table 7). In both non-inoculated (C) ($F_{2,8}$=9.1; P<0.01) and inoculated (D) ($F_{2,8}$=30.9; P<0.01) samples without water loss, all day groupings show a statistically significant diminution of the number of organisms (Table 1).

Middle samples with evaporation (Fig. 7) demonstrated a large amount of water loss (26%) once placed in the vent hood at day 5, similar to top samples. Parafilm-covered samples exhibited minimal water loss. Both dry (A) and wet natural feces (B) had an insignificant loss of *Salmonella* cells throughout the 11 day period. Inoculated feces without parafilm had a significant *Salmonella* loss ($F_{2,8}$=6.2; P=0.02) between each group of days (Table 2). Inoculated samples (D), despite having continuous moisture, did decline significantly ($F_{2,8}$=26.1; P<0.01) in *Salmonella* numbers. Inoculated feces with parafilm maintained *Salmonella* populations >1% for the initial 4 days (Table 8). Within

middle samples, DAPI-stained cells (Fig. 8) were noted to decrease significantly in natural feces exposed to air ($F_{2,8}$=7.9; P=0.01), and inoculated feces with air contact ($F_{2,8}$=11.3; P<0.01) decreased in similar proportions over days. Natural samples with continual moisture content throughout also showed slightly significant decreases in DAPI-stained cells ($F_{2,8}$=7.7; P=0.01). Inoculated samples with continual moisture showed no significant decrease in overall organism content.

Within the bottom layer (Fig. 9), water loss was minimal between exposed and covered samples. Samples lost 3% total of their moisture between days 0 to day 10. Non-inoculated feces (A and C) had stable *Salmonella* counts throughout the experiment, and no significant *Salmonella* diminution was found in sampling days (Table 3). Within inoculated feces, both uncovered (B) ($F_{2,8}$=8.1; P=0.01) and covered (D)(($F_{2,8}$=14.9; P<0.01) samples decreased gradually and in a similar fashion over the incubation time. Both inoculated and treatments maintained *Salmonella* population percentages of >1 % throughout most sampling days (Table 9). DAPI-counts were shown to be stably insignificant (Fig. 10) within the bottom layer, despite treatment type, or inoculation status.

Bacterial and DAPI-counts- To test overall bacterial diminution and significance, a One-way ANOVA was performed using R, version 2.11.1 using $\log_{10}$ transformed grouped samples. For replication purposes, sample counts were grouped into days 0-3, 4-6 and 7-10. If samples were statistically significant, a post-hoc Fisher's LSD then was used to test where significance occurred, using an additional R package agricolae. Upon examination of bacterial counts within top samples, bacterial counts decreased in all of the treatments (Fig. 11). Despite this decrease in bacterial counts, only natural parafilm

protected samples (C) differed significantly ($F_{2,8}$=8.6; P=0.01) among days (Table 4). DAPI-stained cells for natural and inoculated desiccated samples decreased similarly over the 11 day period (Fig. 12). Both significantly decreased in organism numbers when exposed to air (A) ($F_{2,8}$=10.6; P<0.01) and (B) ($F_{2,8}$=4.7; P=0.04) over the study period (Table 4). Natural and inoculated samples did not decrease in the number of organisms at a significant rate over the study time (C and D). Average percentages of bacterial cells by treatment in comparison with 100% DAPI-stained cells demonstrate that overall percentages of bacterial cells remain at approximately 60-70% for desiccated samples (Fig. 13); bacterial averages are initially between 50-60% , then decrease to 40-50% detection of overall organisms (Table 7).

Middle bacterial counts (Fig. 14) showed a large decrease over time in all treatment types. Natural feces without parafilm had a significant difference ($F_{2,8}$=41.6; P<0.001) between days (Table 5). Inoculated feces with air exposure also had a substantial diminution ($F_{2,8}$=15.3; P<0.01) in bacterial counts between all experimental days. Natural feces with parafilm ($F_{2,8}$=8.1; P=0.01) and inoculated feces with parafilm ($F_{2,8}$=7.5; P=0.01) also show a large difference in cell counts between experimental days. DAPI-counts within the middle region also had large decreases in overall organism counts including natural feces (Fig. 15) with air exposure ($F_{2,8}$=18.7; P<0.001), with significant decreases occurring throughout the trial (Table 5). Inoculated feces with air exposure also show a large decrease in microorganisms ($F_{2,8}$=14.4; P<0.01) between all days. DAPI-counts also decreased largely in natural samples with parafilm ($F_{2,8}$=17.0; P<0.01), and the inoculated treatment without moisture loss ($F_{2,8}$=9.4; P<0.01). Middle bacterial counts show similar detection percentages initially between 60-70% (Fig. 16).

Samples covered with parafilm are shown to have the largest decreases to approximately 20-30% of the total microorganisms in the sample (Table 8).

Bacterial counts in samples from the bottom of the incubation tubes showed differences only in natural and inoculated samples with air exposure (Fig. 17). Bottom counts with naturally occurring *Salmonella* decreased significantly ($F_{2,8}$=10.3; P<0.01), as did inoculated samples without parafilm ($F_{2,8}$=6.6; P=0.02). Bacterial counts steadily decreased (Table 6) throughout the experiment. DAPI-stained cells (Fig. 18) also had a large change in organism counts in both natural ($F_{2,8}$=4.4; P=0.05) and inoculated feces ($F_{2,8}$=18.9; P<0.001) without parafilm. All other samples were insignificant in their loss of microorganisms. Bottom bacterial counts show detection percentages initially between 60-70% (Fig. 19). Desiccated samples then have a 40% detection rate of bacterial cells in comparison to 100% of DAPI-stained cells. Overall, in moist samples there is a minimal percentage of detection loss (Table 9).

End-Point PCR and rep-PCR- PCR-based detection occurred in the final samples prior to enrichment in the wet natural, and dry inoculated samples at 100% (Table 4). Wet inoculated samples had a detection rate of 80% (Table 4). Natural samples exposed to air had 0% detection using End-point PCR prior to enrichment. Once enrichment occurred, 42% of naturally dried samples were identified as *Salmonella*-positive by PCR, and 90% of inoculated dried samples were detected as *Salmonella* positive. Naturally wet feces had a 92% detection rate for *Salmonella* after enrichment, and inoculated wet feces had a 68% detection rate (Table 4). Positive isolates from samples isolated 2 strains of *Salmonella* via rep-PCR. Most predominant was Strain 1, with 128 isolations of this strain including both natural and inoculated samples. Strain 1 had a similar base pair

analysis via Bioanalyzer to our inoculated strain 34-2-2. Strain 2 which included 26 natural and inoculated isolates. To confirm unique strains, unique samples were then compared using the Bioanalyzer to verify the 2 unique *Salmonella* strains (Fig. 21).

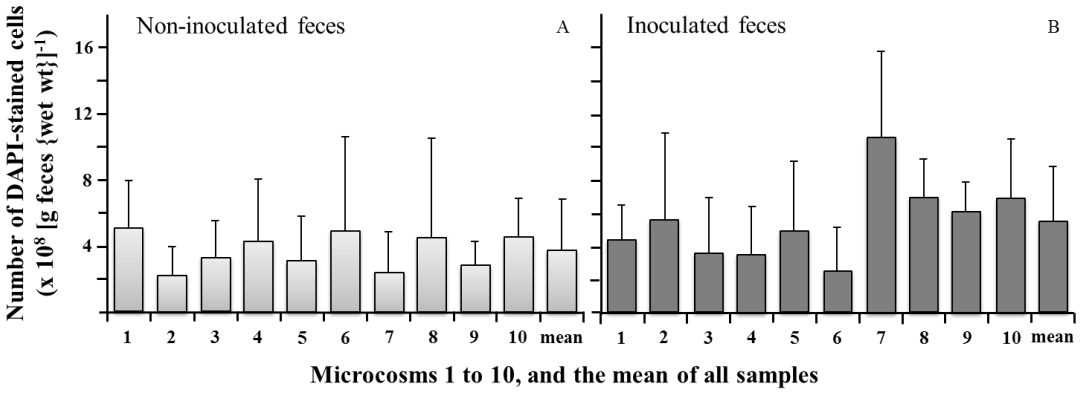

FIGURE 1- DAPI-stained cell averages of 10 individual samples of cow feces prior to inoculation (A) and post-inoculation (B). Mean indicates the average of all 10 samples for the sample day indicated.

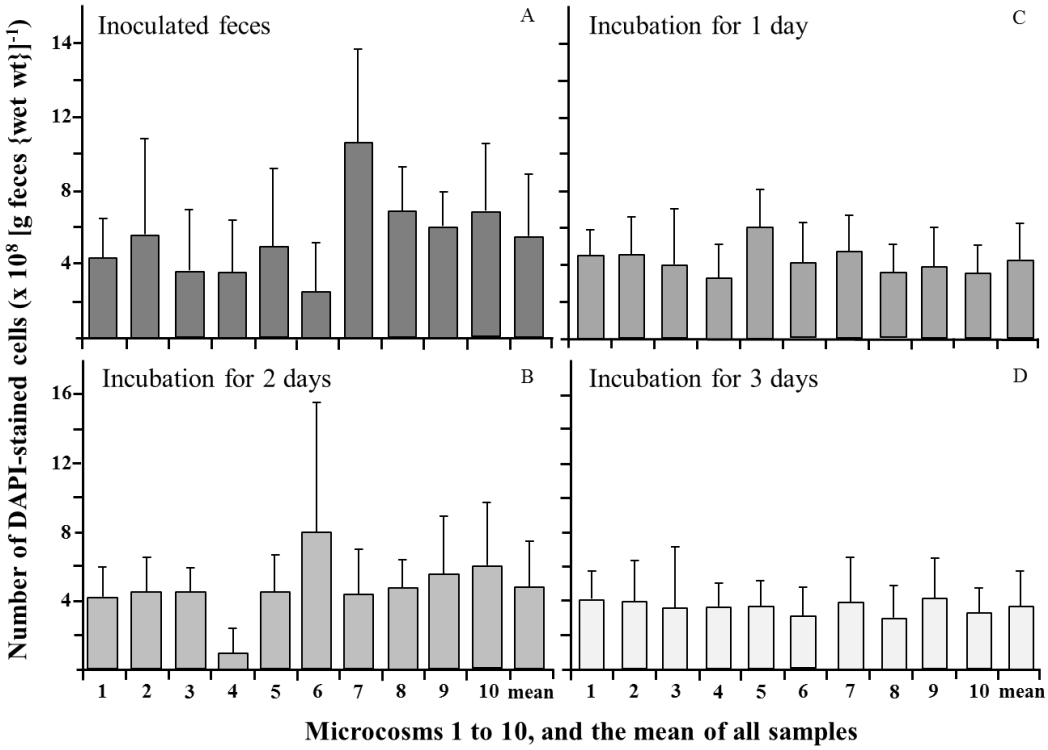

FIGURE 2- DAPI-stained cell averages of 10 individual samples and their mean of cow feces immediately post-inoculation day 0 (A), post-inoculation day 1 (B), post-inoculation day 2 (C), and post-inoculation day 3(D).

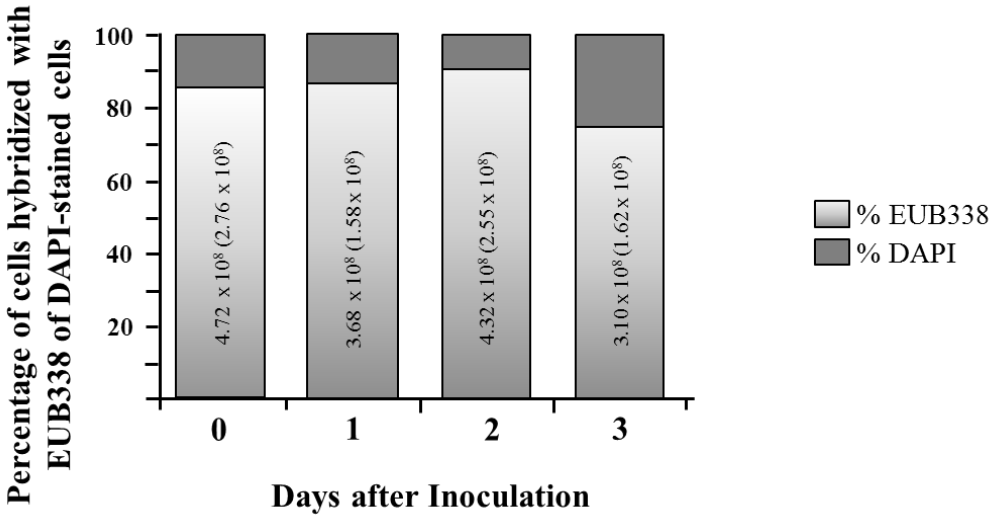

FIGURE 3- The percentage of hybridized bacterial cells after the indicated days of inoculation using probe EUB338 in comparison to a 100% of DAPI-stained cells. Numbers indicated within the bars are average cell counts on that day. Numbers indicated within parentheses are standard deviations.

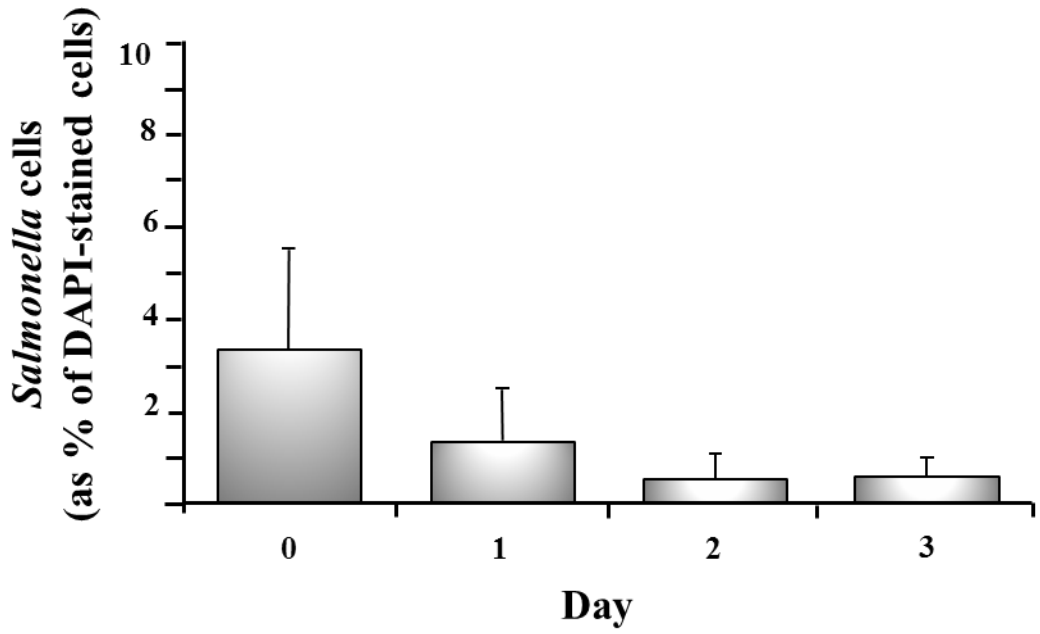

FIGURE 4- Percentages of hybridized *Salmonella* cells counted after the indicated days of inoculation using probe Sal3 as % of DAPI-stained cells.

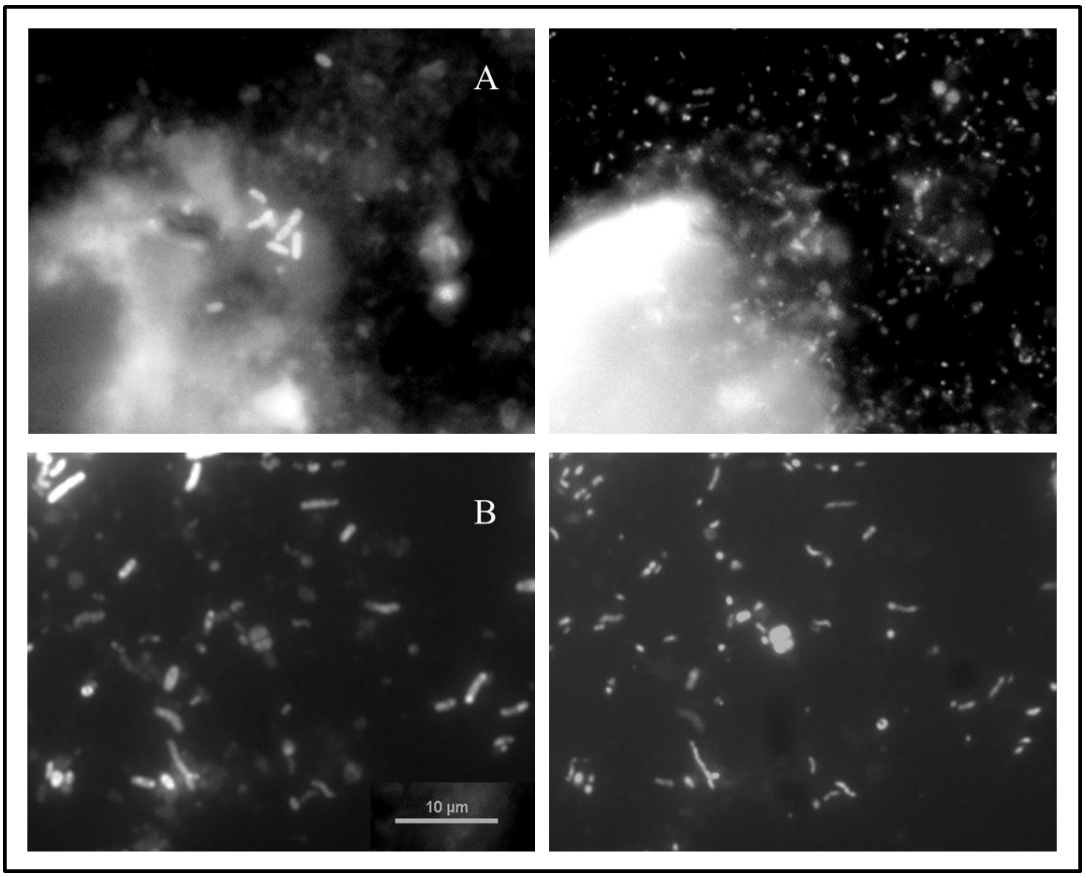

FIGURE 5- Detection of microorganisms in cow pats after DAPI-staining (right panel) and *in situ* hybridization with 16S or 23S rRNA-targeted, Cy3-labeled probes Sal 3 detecting *Salmonella* (A), or EUB338 detecting all bacteria (B).

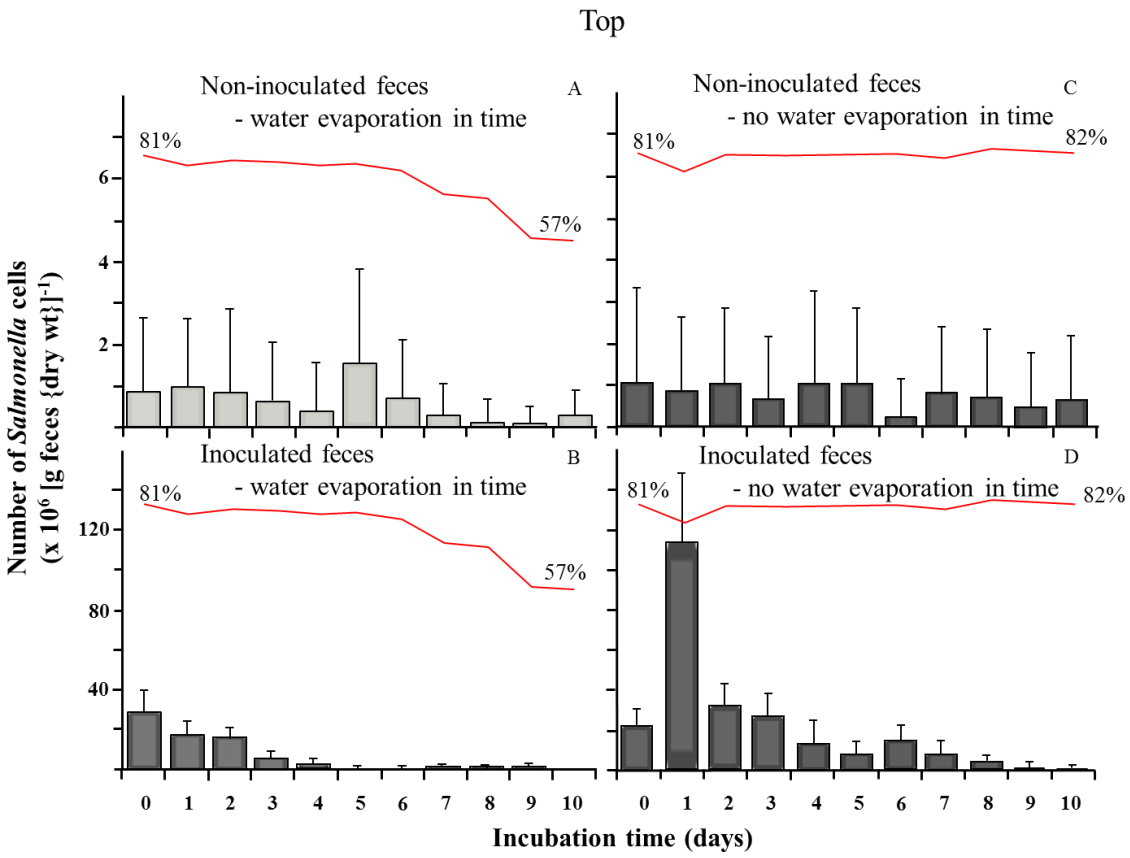

FIGURE 6- Average number of counted *Salmonella* hybridized cells using Sal3 probe and *in situ* hybridization. Sampling occurred from the top section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

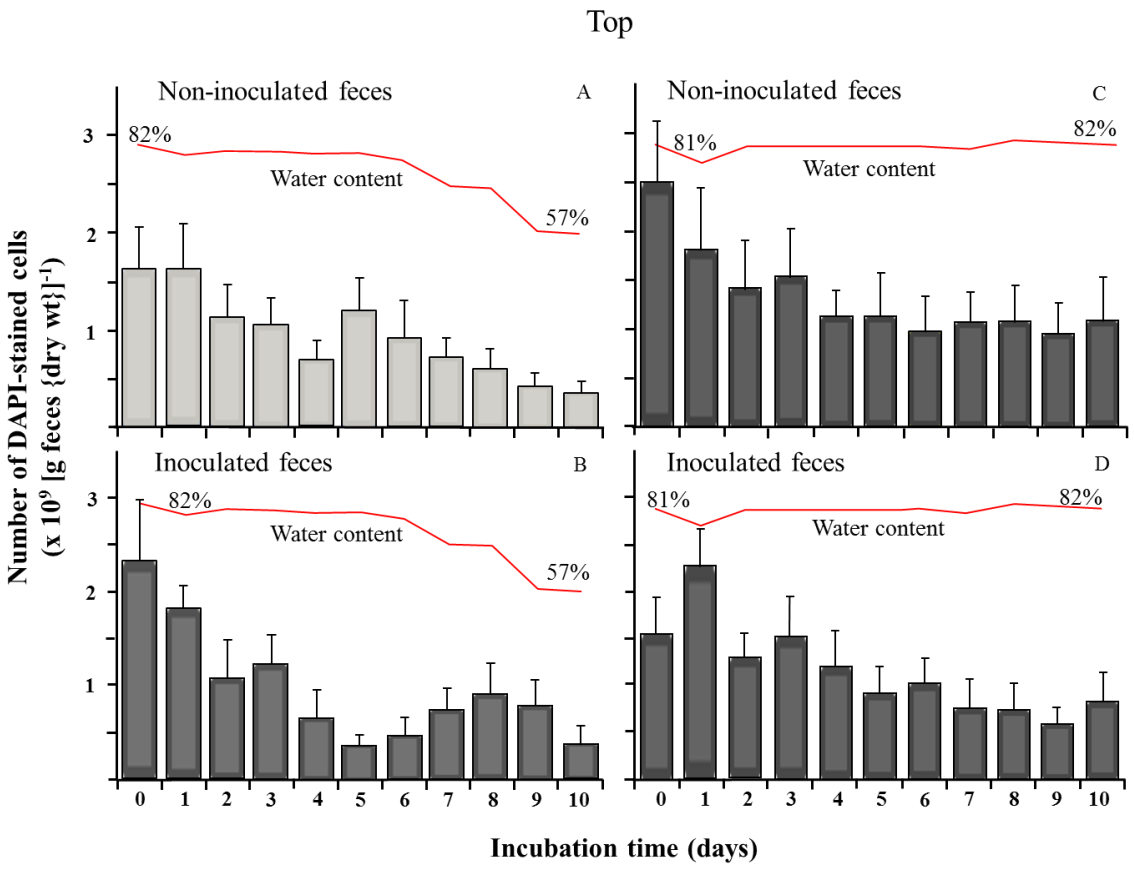

FIGURE 7- Average number of counted DAPI-stained cells using DAPI staining and *in situ* hybridization. Sampling occurred from the top section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

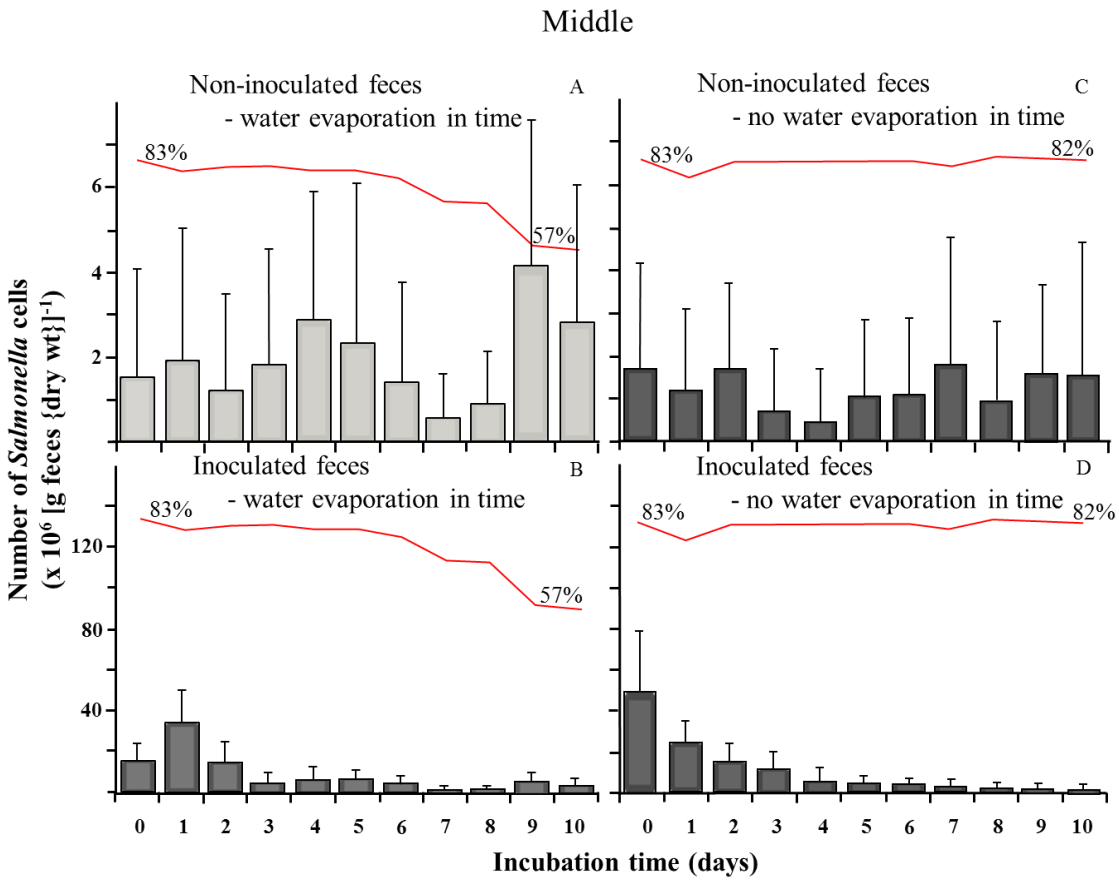

FIGURE 8- Average number of counted *Salmonella* hybridized cells using Sal3 probe and *in situ* hybridization. Sampling occurred from the middle section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

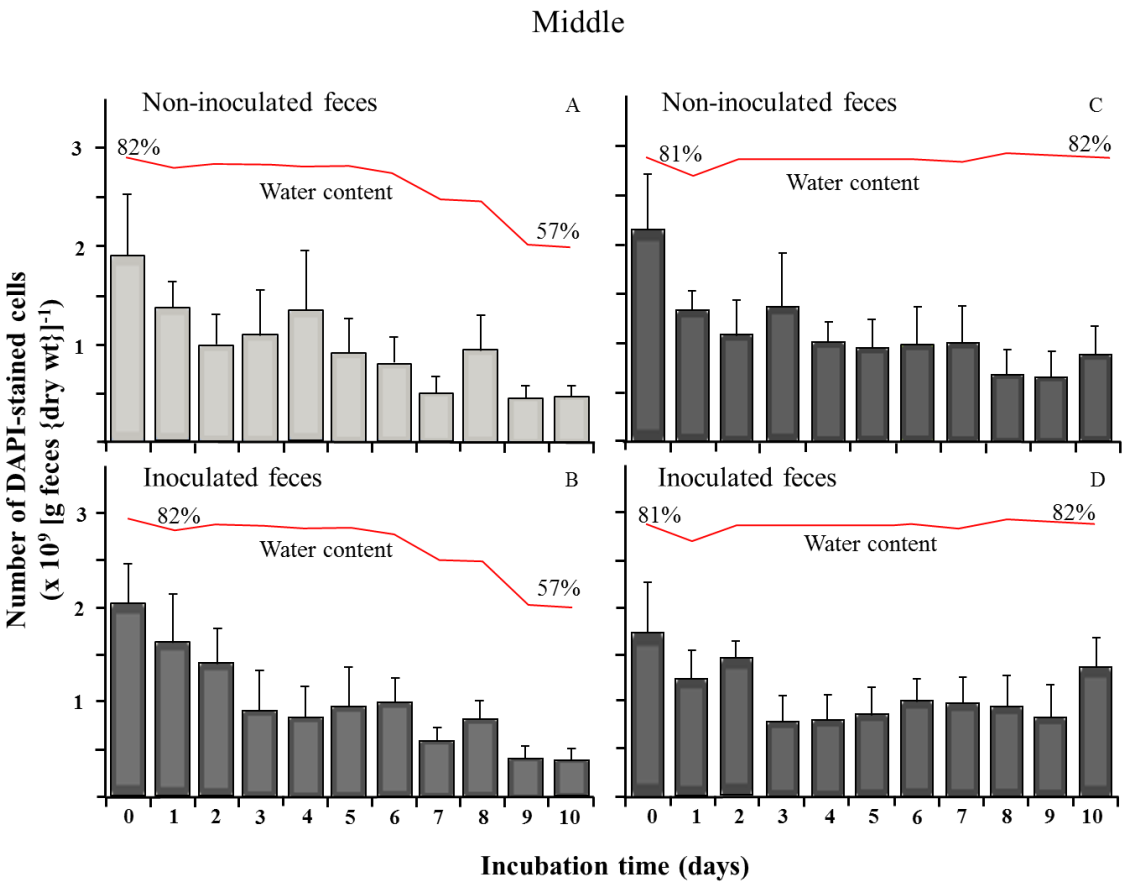

FIGURE 9- Average number of counted DAPI-stained cells using DAPI staining and *in situ* hybridization. Sampling occurred from the middle section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

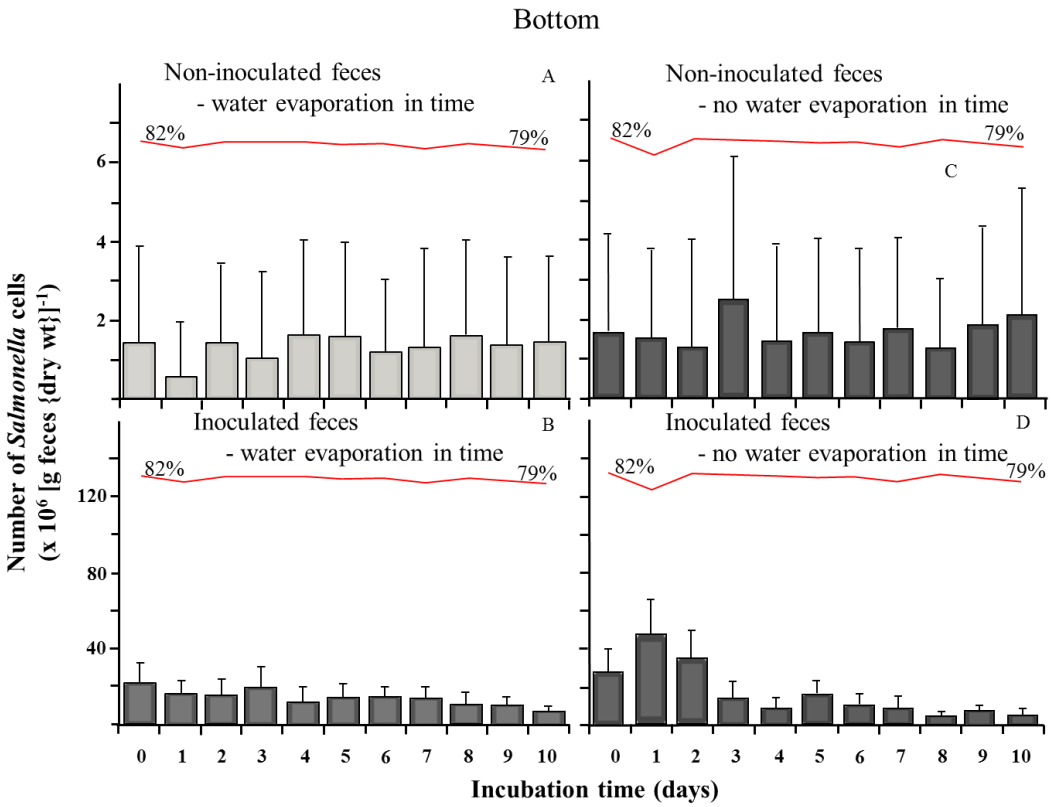

FIGURE 10- Average number of counted *Salmonella* hybridized cells using Sal3 probe and *in situ* hybridization. Sampling occurred from the bottom section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

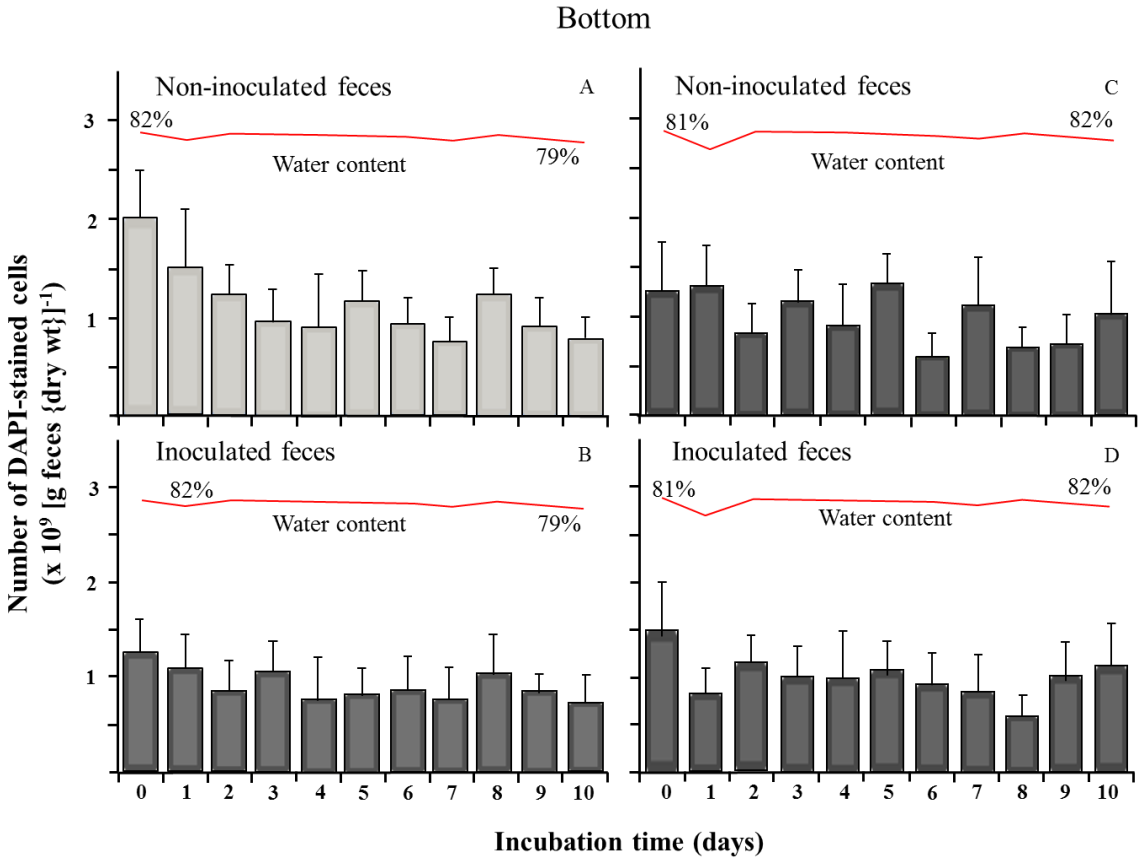

FIGURE 11- Average number of counted DAPI-stained cells using DAPI staining and *in situ* hybridization. Sampling occurred from the bottom section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

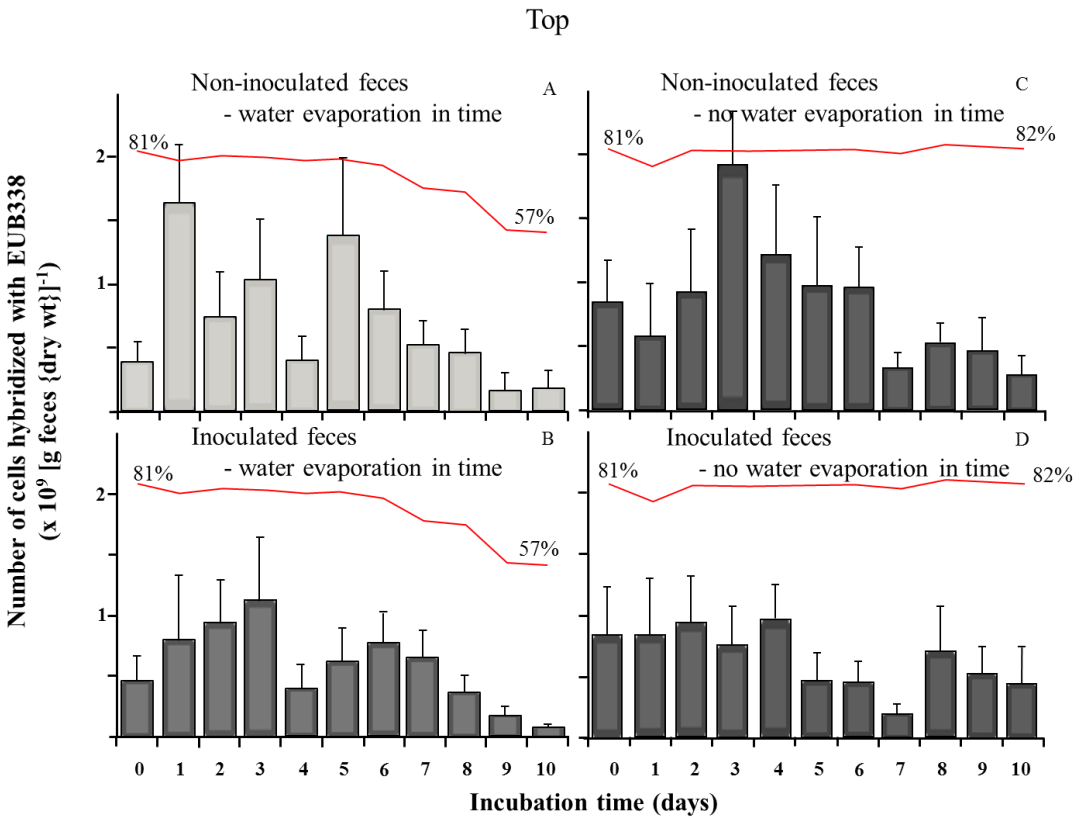

FIGURE 12- Average number of counted bacterial hybridized cells using EUB338 probe and *in situ* hybridization. Sampling occurred from the top section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

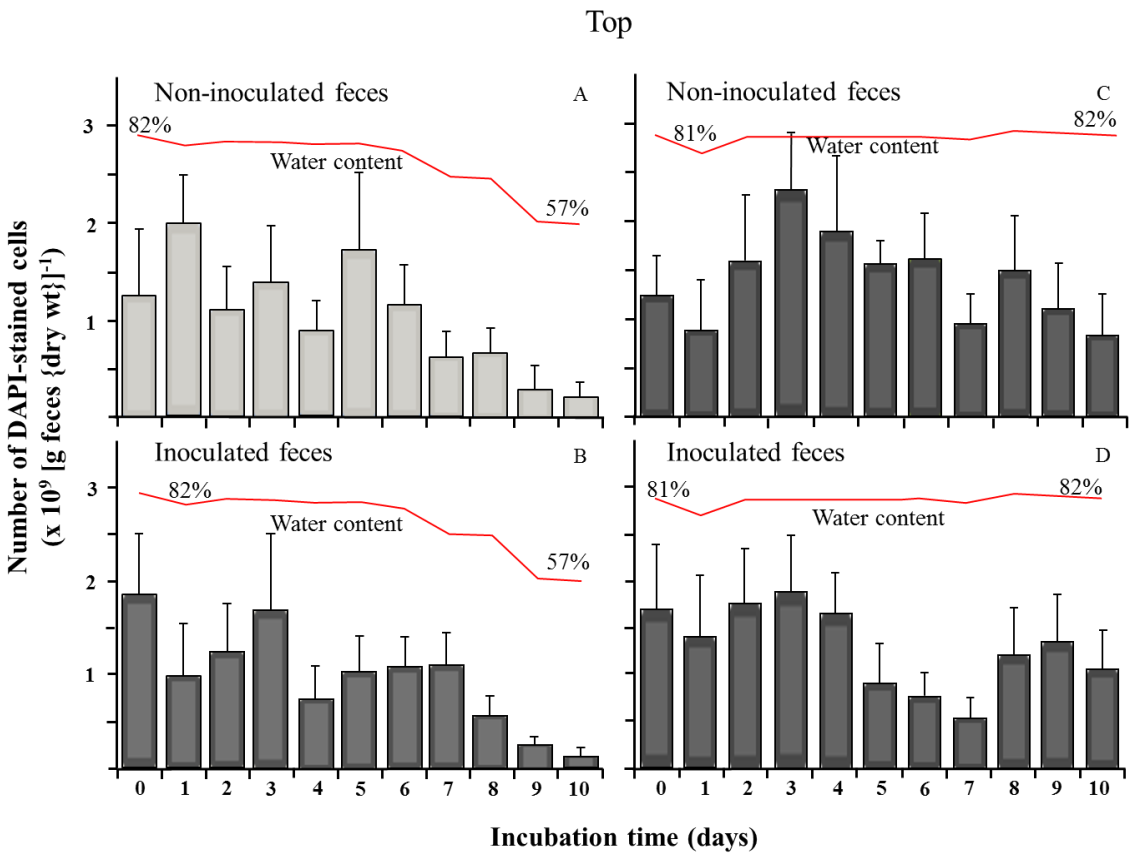

FIGURE 13- Average number of counted DAPI-stained cells using DAPI staining and *in situ* hybridization. Sampling occurred from the top section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

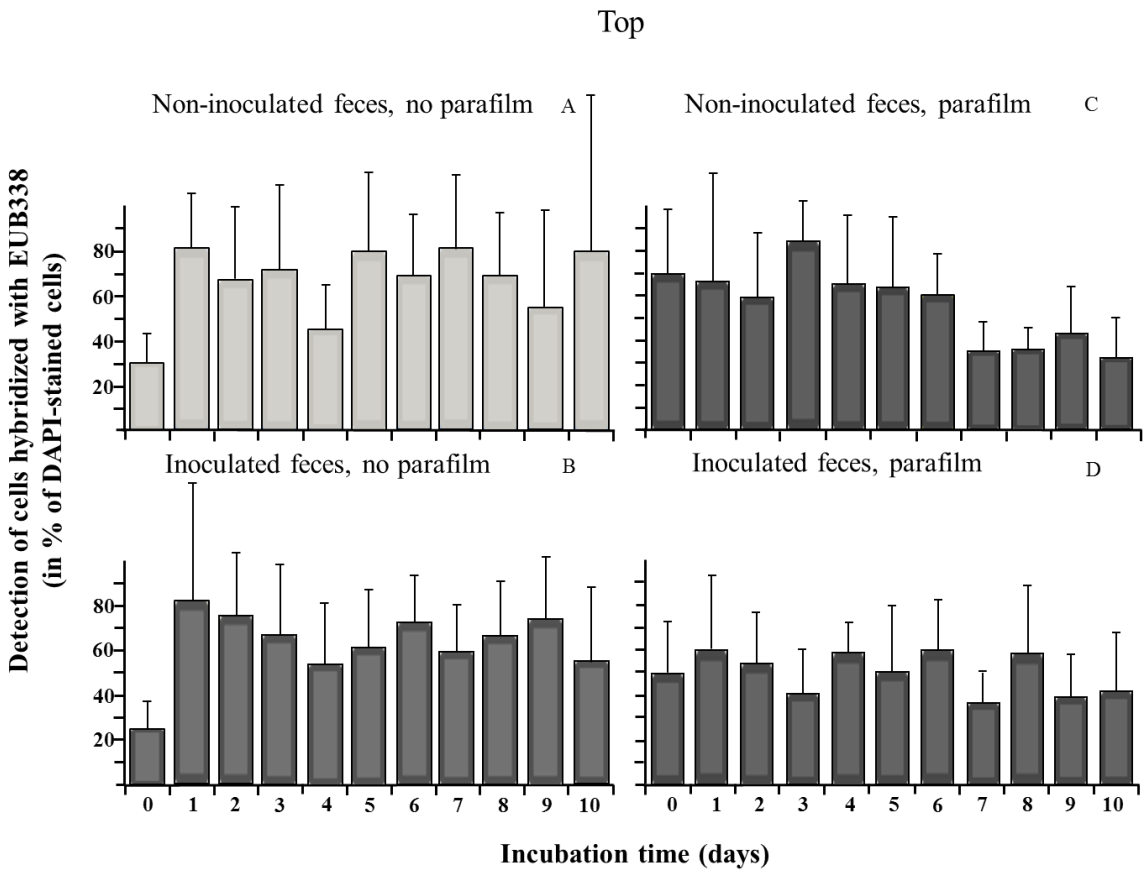

FIGURE 14- The percentage of hybridized bacterial cells in the top portion of artificial cow pats after the indicated days of treatment using probe EUB338 in comparison to 100% of DAPI-stained cells. Calculated standard deviations are indicated above each bar.

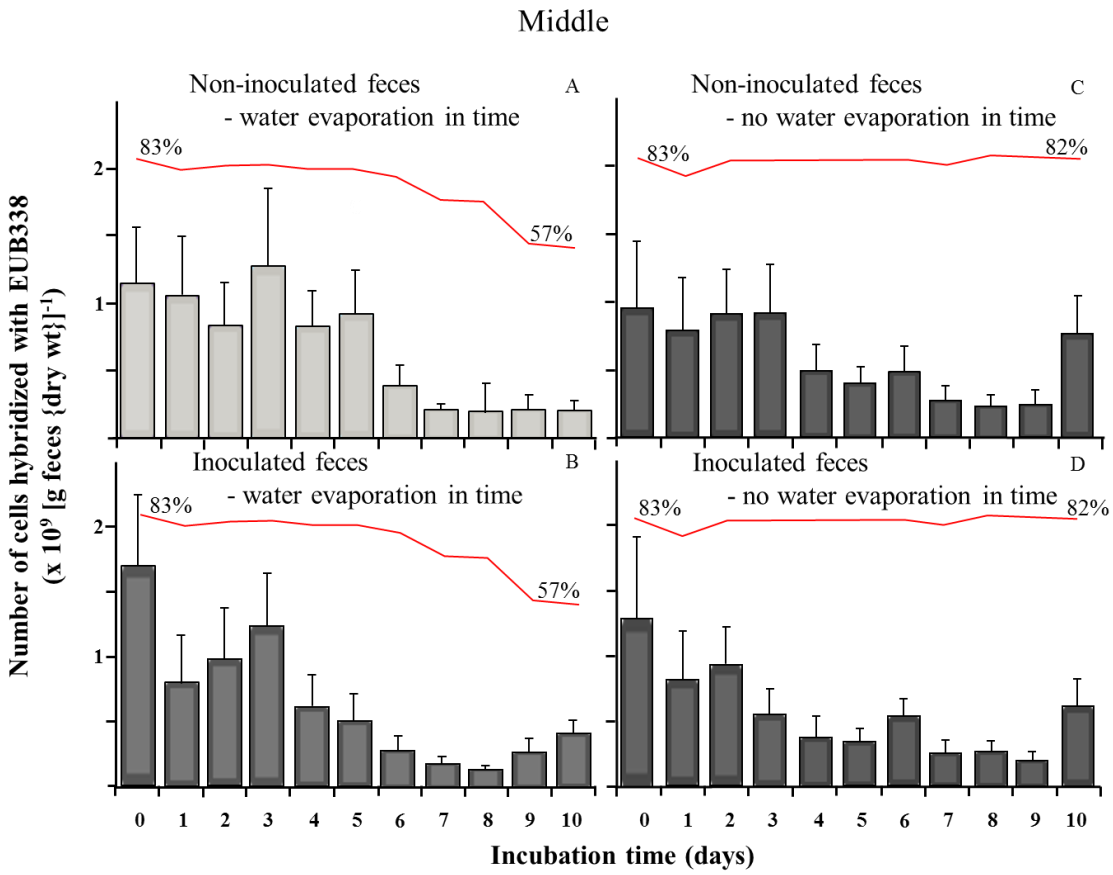

FIGURE 15- Average number of counted bacterial hybridized cells using EUB338 probe and *in situ* hybridization. Sampling occurred from the middle section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

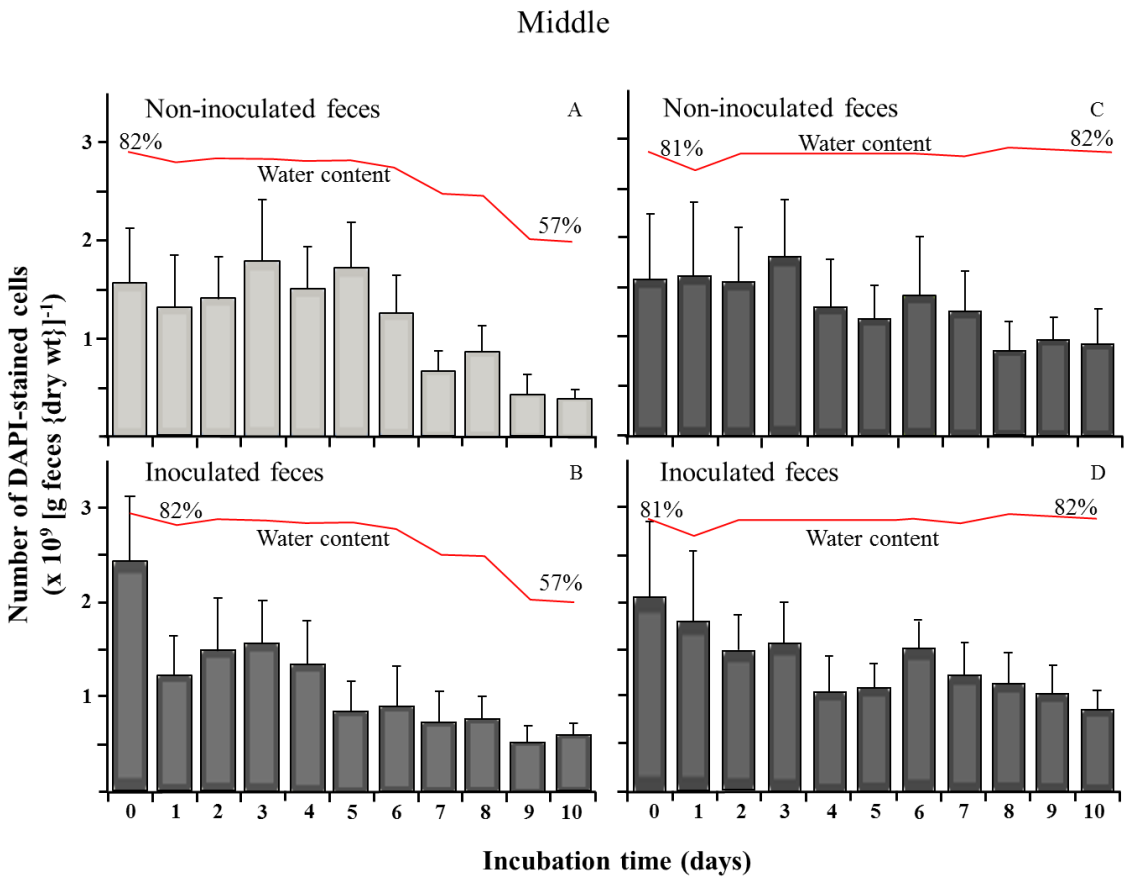

FIGURE 16- Average number of counted DAPI-stained cells using DAPI staining and *in situ* hybridization. Sampling occurred from the middle section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

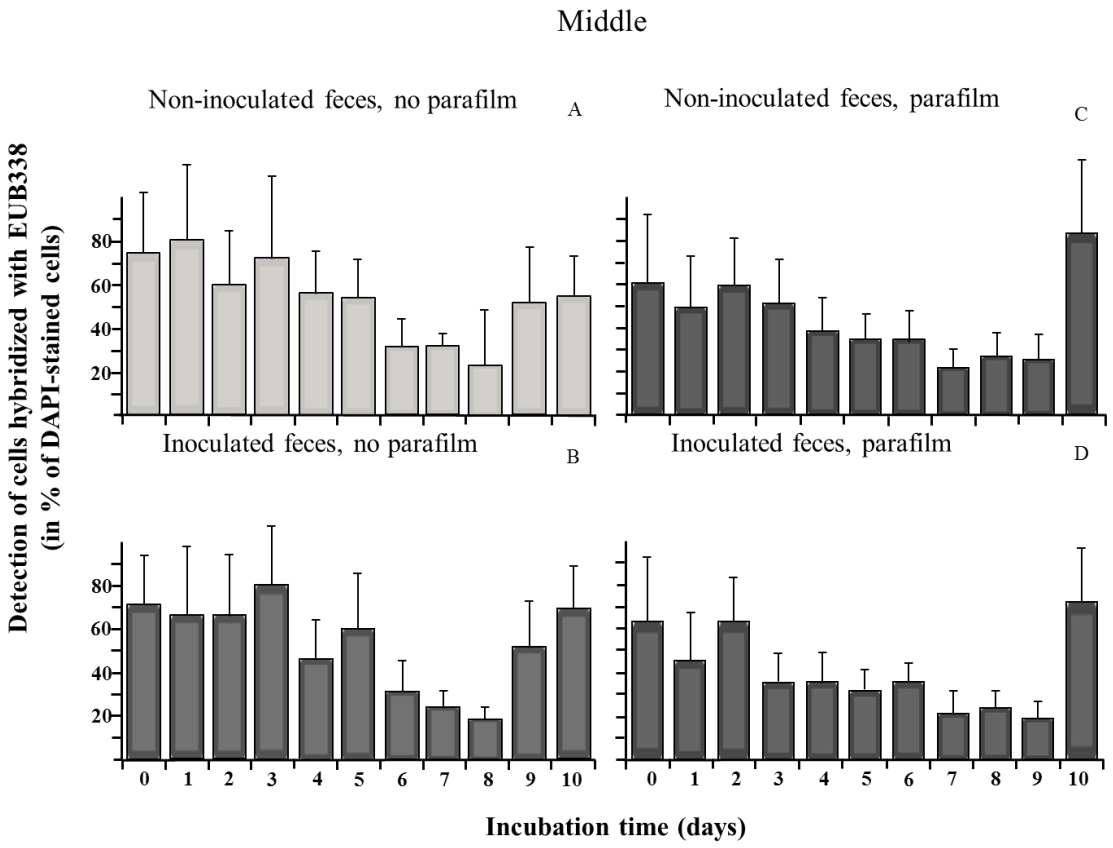

FIGURE 17- The percentage of hybridized bacterial cells in the middle portion of artificial cow pats after the indicated days of treatment using probe EUB338 in comparison to 100% of DAPI-stained cells. Calculated standard deviations are indicated above each bar.

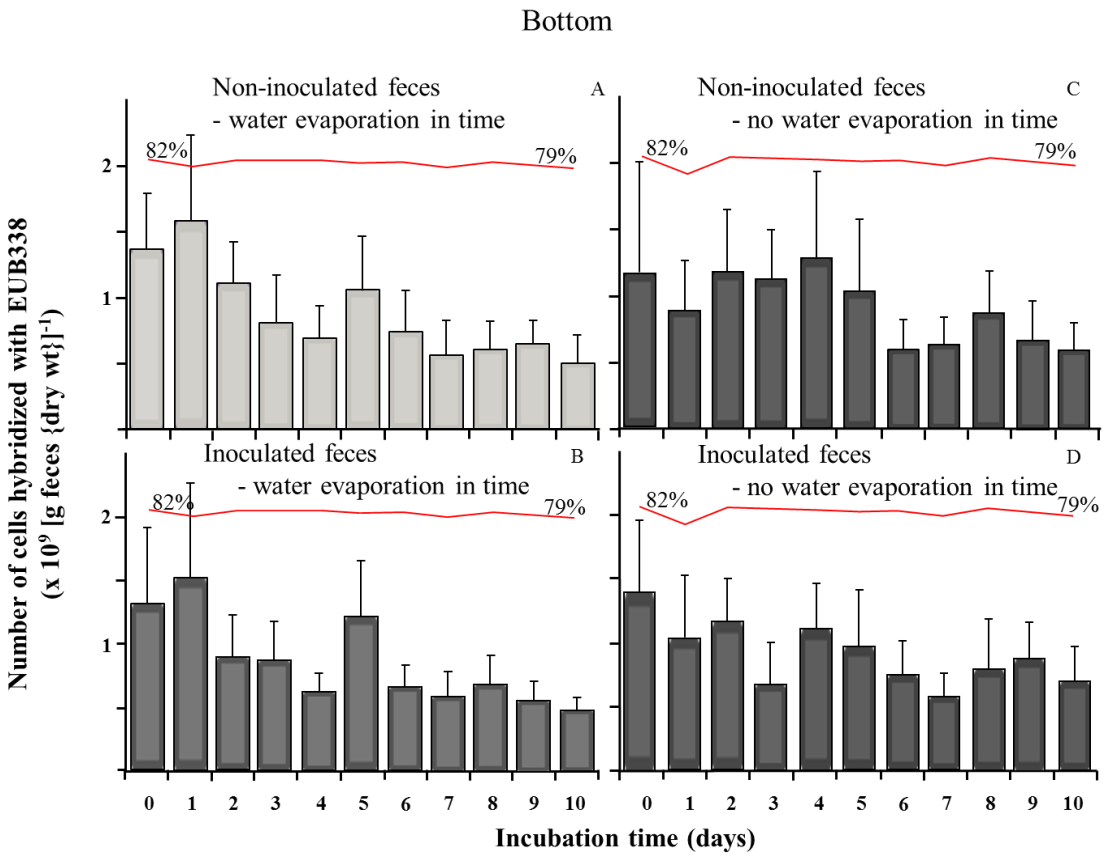

FIGURE 18- Average number of counted bacterial hybridized cells using EUB338 probe and *in situ* hybridization. Sampling occurred from the bottom section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

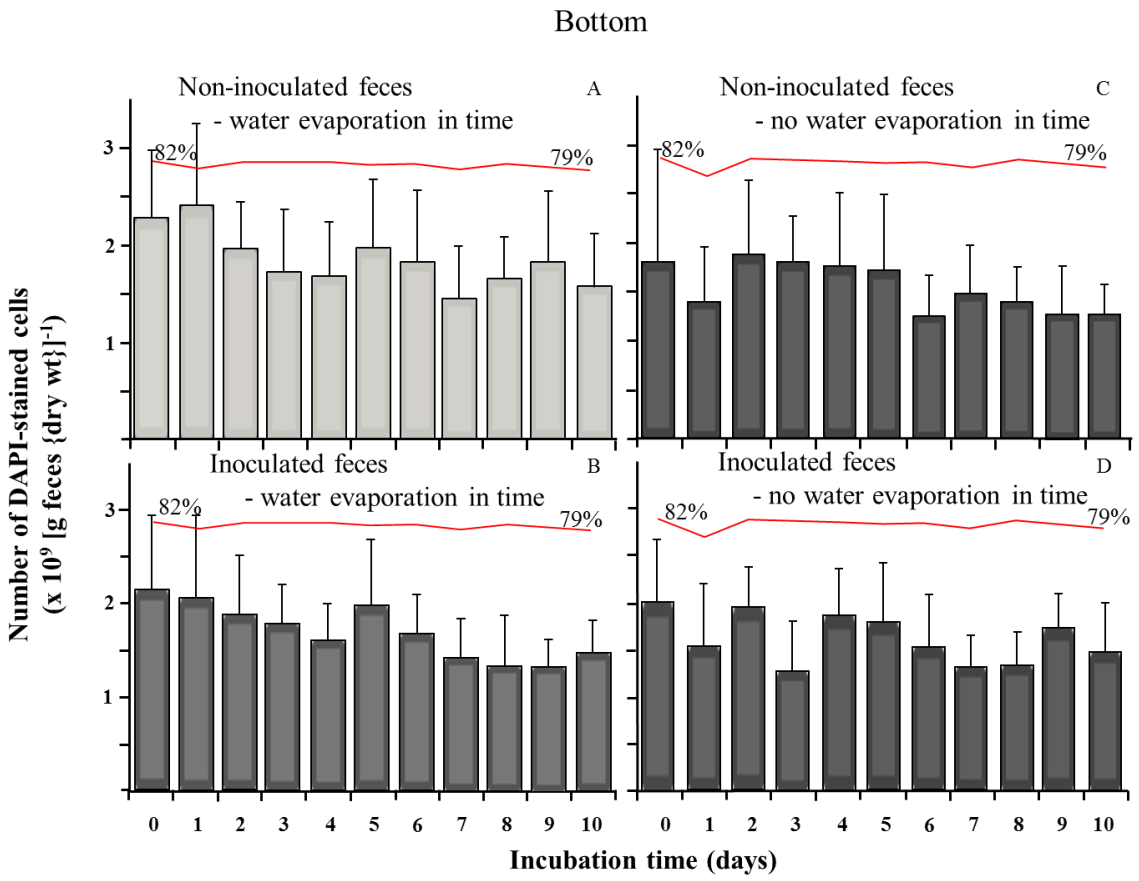

FIGURE 19- Average number of counted DAPI-stained cells using DAPI staining and *in situ* hybridization. Sampling occurred from the bottom section of artificial cow pats on a daily basis for 11 days. Averages were taken from 20 trials and respective standard deviations shown. Water content of cow pats is indicated by the red line and percentages indicating initial water content and final water content.

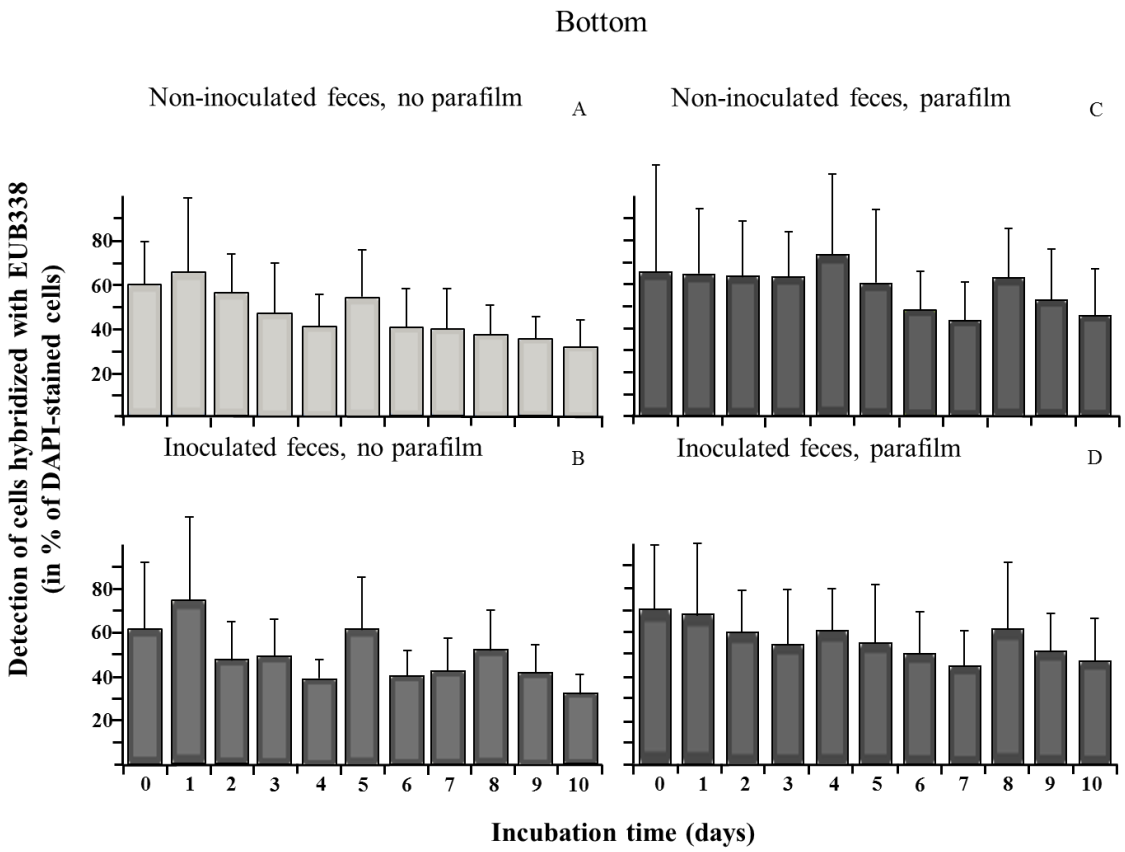

FIGURE 20- The percentage of hybridized bacterial cells in the bottom portion of artificial cow pats after the indicated days of treatment using probe EUB338 in comparison to 100% of DAPI-stained cells. Calculated standard deviations are indicated above each bar.

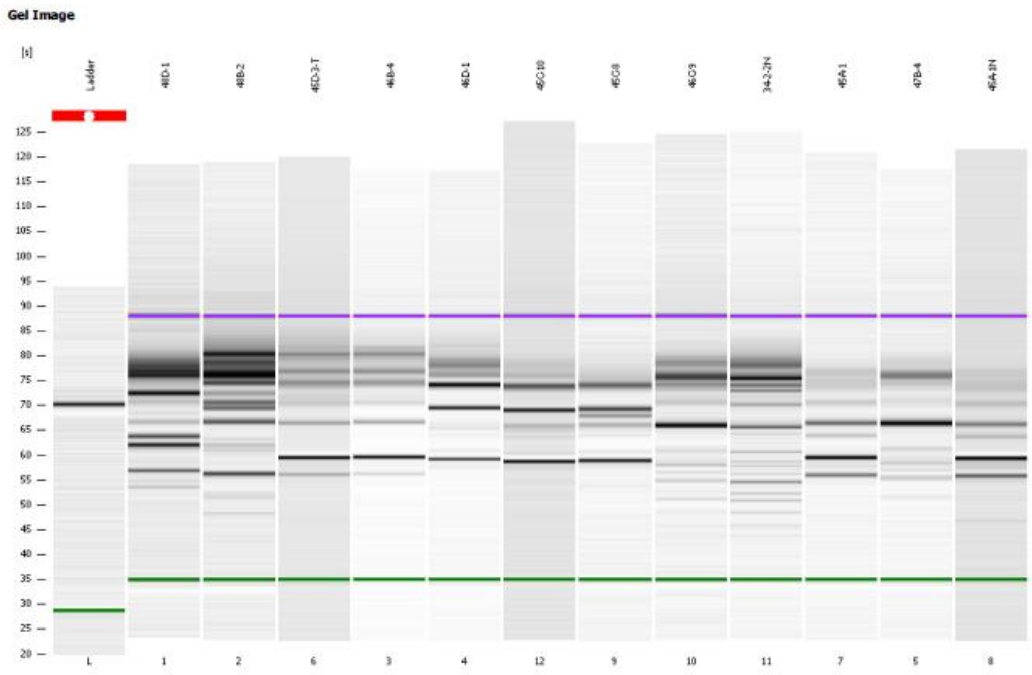

FIGURE 21-Digital gel image comparing *Salmonella* strains isolated using a Bioanalyzer (Agilent 7500). Strain 34-2-2N was the inoculated strain, and the other strains (48D-1) was naturally occurring.

TABLE 1- Distribution of *Salmonella* and DAPI-stained cells compiled via *in situ* hybridization within the top region of cow feces pats. "Dry" indicates samples without parafilm. "Wet" denotes samples with parafilm. "Treatment" signifies days 0 to 3, days 4 through 6, and days 7 to 10. "Average % Moisture" indicates the average moisture percentage of each treatment. Values under the column "N x 10^5" indicate averages taken from 20 trials each day, including standard deviations in parentheses. Results of One-way ANOVA's are listed under columns "F" and "P-value". Degrees of freedom are (2,8) for all one-way ANOVA's. Fisher's LSD results are listed as "Pairwise contrasts" for statistically significant One-Way ANOVA's, with letters indicating significant differences in treatment levels.

			Treatment	Average % Moisture	N x 10^5 (± SD)	F	P-value	Pairwise contrasts
Salmonella	Dry	Natural	0-3	81.2	8.0 (1.5)	12.3	<0.01	b
			4-6	79.5	8.7 (5.9)			a
			7-10	64.5	1.8 (0.8)			c
		Inoculated	0-3	81.2	163.0 (97.1)	2.2	0.18	
			4-6	79.5	7.5 (6.7)			
			7-10	64.5	6.3 (4.3)			
	Wet	Natural	0-3	80.5	8.8 (1.9)	0.6	0.58	
			4-6	81.6	7.5 (4.7)			
			7-10	82.0	6.3 (1.5)			
		Inoculated	0-3	80.5	484.1 (435.5)	13.6	<0.01	a
			4-6	81.6	118.3 (34.6)			b
			7-10	82.0	35.2 (30.2)			c
DAPI	Dry	Natural	0-3	81.2	13567.1 (3073.5)	12.4	<0.01	a
			4-6	79.5	9315.9 (2547.1)			b
			7-10	64.5	5217.5 (1649.0)			c
		Inoculated	0-3	81.2	16075.9 (6439.5)	12.6	<0.01	a
			4-6	79.5	4334.9 (1496.5)			c
			7-10	64.5	4351.7 (1812.4)			b
	Wet	Natural	0-3	80.5	17456.7 (5291.7)	9.1	<0.01	a
			4-6	81.6	10796.6 (733.8)			b
			7-10	82.0	10513.0 (74.2)			c
		Inoculated	0-3	80.5	15584.3 (2187.5)	30.9	<0.01	a
			4-6	81.6	10393.1 (1403.2)			b
			7-10	82.0	7133.2 (972.8)			c

TABLE 2- Distribution of *Salmonella* and DAPI-stained cells compiled via *in situ* hybridization within the middle region of cow pats. "Dry" indicates samples without parafilm. "Wet" denotes samples with parafilm. "Treatment" signifies days 0 to 3, days 4 through 6, and days 7 to 10. "Average % Moisture" indicates the average moisture percentage of each treatment. Values under the column "N x 10^5" indicate averages taken from 20 trials each day, including standard deviations in parentheses. Results of One-way ANOVA's are listed under columns "F" and "P-value". Degrees of freedom are (2,8) for all one-way ANOVA's. Fisher's LSD results are listed as "Pairwise contrasts" for statistically significant One-Way ANOVA's, with letters indicating significant differences in treatment levels.

			Treatment	Average % Moisture	N x 10^5 (± SD)	F	P-value	Pairwise contrasts
Salmonella	Dry	Natural	0-3	81.2	16.0 (3.1)	0.2	0.78	
			4-6	79.5	21.8 (7.4)			
			7-10	64	20.7 (16.9)			
		Inoculated	0-3	81.2	171.6 (121.8)	6.2	0.02	a
			4-6	79.5	54.7 (12.8)			b
			7-10	64	32.7 (21.1)			c
	Wet	Natural	0-3	80.6	12.7 (4.9)	1.7	0.23	
			4-6	81.7	8.2 (3.6)			
			7-10	82.2	14.2 (3.7)			
		Inoculated	0-3	80.6	247.6 (169.8)	26.1	<0.01	a
			4-6	81.7	45.2 (8.5)			b
			7-10	82.2	22.5 (8.7)			c
DAPI	Dry	Natural	0-3	81.2	13285.3 (4072.4)	7.9	0.01	a
			4-6	79.5	10027.2 (2924.3)			b
			7-10	64	5667.8 (2379.2)			c
		Inoculated	0-3	81.2	14968.6 (4709.5)	11.3	<0.01	a
			4-6	79.5	9105.1 (778.6)			b
			7-10	64	5370.2 (2033.5)			c
	Wet	Natural	0-3	80.6	14845.5 (4579.0)	7.7	0.01	a
			4-6	81.7	9832.6 (319.1)			b
			7-10	82.2	8111.5 (1620.8)			c
		Inoculated	0-3	80.6	12978.0 (3968.4)	1.7	0.23	
			4-6	81.7	8840.8 (986.8)			
			7-10	82.2	10180.0 (2299.2)			

TABLE 3- Distribution of *Salmonella* and DAPI-stained cells compiled via *in situ* hybridization within the bottom region of cow pats. "Dry" indicates samples without parafilm. "Wet" denotes samples with parafilm. "Treatment" signifies days 0 to 3, days 4 through 6, and days 7 to 10. "Average % Moisture" indicates the average moisture percentage of each treatment. Values under the column "N x 105" indicate averages taken from 20 trials each day, including standard deviations in parentheses. Results of One-way ANOVA's are listed under columns "F" and "P-value". Degrees of freedom are (2,8) for all one-way ANOVA's. Fisher's LSD results are listed as "Pairwise contrasts" for statistically significant One-Way ANOVA's, with letters indicating significant differences in treatment levels.

			Treatment	Average % Moisture	N x 10^5 (± SD)	F	P-value	Pairwise contrasts
Salmonella	Dry	Natural	0-3	81.2	11.1 (4.1)	1.5	0.27	
			4-6	81.1	14.6 (2.4)			
			7-10	80.1	14.2 (1.4)			
		Inoculated	0-3	81.2	180.3 (28.1)	8.1	0.01	a
			4-6	81.1	131.8 (16.8)			b
			7-10	80.1	101.1 (27.8)			c
	Wet	Natural	0-3	80.3	17.1 (5.2)	0.3	0.77	
			4-6	81.1	14.9 (1.1)			
			7-10	80	17.0 (3.7)			
		Inoculated	0-3	80.3	314.2 (139.3)	14.9	<0.01	a
			4-6	81.1	119.4 (38.7)			b
			7-10	80	66.0 (18.7)			c
DAPI	Dry	Natural	0-3	81.2	14270.2 (4479.4)	3.2	0.09	
			4-6	81.1	10013.9 (1422.1)			
			7-10	80.1	9142.4 (2219.5)			
		Inoculated	0-3	81.2	10684.7 (1641.5)	4.1	0.06	
			4-6	81.1	8117.4 (500.8)			
			7-10	80.1	8385.0 (1403.7)			
	Wet	Natural	0-3	80.3	11356.0 (2138.6)	1	0.4	
			4-6	81.1	9356.3 (3685.8)			
			7-10	80	8796.2 (2099.0)			
		Inoculated	0-3	80.3	11168.4 (2805.9)	0.8	0.47	
			4-6	81.1	9954.6 (743.5)			
			7-10	80	9084.9 (2354.2)			

TABLE 4- Distribution of bacteria and DAPI-stained cells compiled via *in situ* hybridization within the top region of cow pats. "Dry" indicates samples without parafilm. "Wet" denotes samples with parafilm. "Treatment" signifies days 0 to 3, days 4 through 6, and days 7 to 10. "Average % Moisture" indicates the average moisture percentage of each treatment. Values under the column "N x 10^7" indicate averages taken from 20 trials each day, including standard deviations in parentheses. Results of One-way ANOVA's are listed under columns "F" and "P-value". Degrees of freedom are (2,8) for all one-way ANOVA's. Fisher's LSD results are listed as "Pairwise contrasts" for statistically significant One-Way ANOVA's, with letters indicating significant differences in treatment levels.

			Treatment	Average % Moisture	N x 10^7 (± SD)	F	P-value	Pairwise contrasts
Bacteria	Dry	Natural	0-3	81.2	93.6 (52.8)	3.6	0.08	
			4-6	79.5	85.4 (49.6)			
			7-10	64.5	32.2 (18.7)			
		Inoculated	0-3	81.2	82.5 (27.7)	3.5	0.08	
			4-6	79.5	59.7 (18.9)			
			7-10	64.5	31.1 (25.5)			
	Wet	Natural	0-3	80.5	106.0 (58.8)	8.6	0.01	a
			4-6	81.6	104.4 (14.8)			b
			7-10	82.0	39.0 (12.1)			c
		Inoculated	0-3	80.5	84.1 (7.2)	2.9	0.11	
			4-6	81.6	62.2 (29.8)			
			7-10	82.0	46.3 (21.3)			
DAPI	Dry	Natural	0-3	81.2	143.9 (39.3)	10.6	<0.01	a
			4-6	79.5	125.4 (42.9)			b
			7-10	64.5	44.4 (22.9)			c
		Inoculated	0-3	81.2	143.9 (40.0)	4.7	0.04	a
			4-6	79.5	94.3 (18.3)			b
			7-10	64.5	49.9 (43.7)			c
	Wet	Natural	0-3	80.5	149.2 (61.1)	2.1	0.18	
			4-6	81.6	167.8 (18.6)			
			7-10	82.0	108.0 (28.9)			
		Inoculated	0-3	80.5	167.2 (20.2)	2.9	0.11	
			4-6	81.6	109.5 (48.0)			
			7-10	82.0	102.7 (35.9)			

TABLE 5- Distribution of bacteria and DAPI-stained cells compiled via *in situ* hybridization within the middle region of cow pats. "Dry" indicates samples without parafilm. "Wet" denotes samples with parafilm. "Treatment" signifies days 0 to 3, days 4 through 6, and days 7 to10. "Average % Moisture" indicates the average moisture percentage of each treatment. Values under the column "N x 10^7" indicate averages taken from 20 trials each day, including standard deviations in parentheses. Results of One-way ANOVA's are listed under columns "F" and "P-value". Degrees of freedom are (2,8) for all one-way ANOVA's. Fisher's LSD results are listed as "Pairwise contrasts" for statistically significant One-Way ANOVA's, with letters indicating significant differences in treatment levels.

			Treatment	Average % Moisture	N x 10^7 (± SD)	F	P-value	Pairwise contrasts
Bacteria	Dry	Natural	0-3	81.2	108.3 (18.6)	41.6	<0.001	a
			4-6	79.5	71.7 (28.5)			b
			7-10	64	20.9 (9.2)			c
		Inoculated	0-3	81.2	117.7 (38.9)	15.3	<0.01	a
			4-6	79.5	46.5 (17.4)			b
			7-10	64	24.6 (12.0)			c
	Wet	Natural	0-3	80.6	89.1 (7.2)	8.1	0.01	a
			4-6	81.7	46.0 (5.0)			b
			7-10	82.2	37.4 (25.8)			c
		Inoculated	0-3	80.6	89.5 (30.0)	7.5	0.01	a
			4-6	81.7	41.9 (10.4)			b
			7-10	82.2	33.7 (19.0)			c
DAPI	Dry	Natural	0-3	81.2	151.3 (20.5)	18.7	<0.001	a
			4-6	79.5	149.1 (23.4)			b
			7-10	64	58.1 (22.3)			c
		Inoculated	0-3	81.2	168.0 (52.6)	14.4	<0.01	a
			4-6	79.5	103.0 (27.3)			b
			7-10	64	65.2 (11.3)			c
	Wet	Natural	0-3	80.6	163.2 (11.6)	17.0	<0.01	a
			4-6	81.7	129.0 (12.1)			b
			7-10	82.2	99.1 (18.1)			c
		Inoculated	0-3	80.6	171.6 (25.6)	9.4	<0.01	a
			4-6	81.7	121.5 (25.4)			b
			7-10	82.2	105.8 (15.3)			c

TABLE 6- Distribution of bacteria and DAPI-stained cells compiled via *in situ* hybridization within the bottom region of cow pats. "Dry" indicates samples without parafilm. "Wet" denotes samples with parafilm. "Treatment" signifies days 0 to 3, days 4 through 6, and days 7 to10. "Average % Moisture" indicates the average moisture percentage of each treatment. Values under the column "N x 10^7" indicate averages taken from 20 trials each day, including standard deviations in parentheses. Results of One-way ANOVA's are listed under columns "F" and "P-value". Degrees of freedom are (2,8) for all one-way ANOVA's. Fisher's LSD results are listed as "Pairwise contrasts" for statistically significant One-Way ANOVA's, with letters indicating significant differences in treatment levels.

			Treatment	Average % Moisture	N x 10^7 (± SD)	F	P-value	Pairwise contrasts
Bacteria	Dry	Natural	0-3	81.2	121.2 (33.3)	10.3	<0.01	a
			4-6	81.1	82.6 (20.2)			b
			7-10	80.1	57.6 (6.2)			c
		Inoculated	0-3	81.2	114.7 (31.6)	6.6	0.02	a
			4-6	81.1	82.6 (32.5)			b
			7-10	80.1	56.8 (8.3)			c
	Wet	Natural	0-3	80.3	108.4 (14.0)	3.9	0.07	
			4-6	81.1	96.0 (34.5)			
			7-10	80	68.4 (12.4)			
		Inoculated	0-3	80.3	106.6 (29.8)	2.2	0.17	
			4-6	81.1	94.1 (17.9)			
			7-10	80	73.7 (12.8)			
DAPI	Dry	Natural	0-3	81.2	208.2 (30.8)	4.4	0.05	a
			4-6	81.1	182.4 (14.2)			b
			7-10	80.1	162.2 (15.8)			c
		Inoculated	0-3	81.2	196.1 (16.6)	18.9	<0.001	a
			4-6	81.1	175.1 (19.5)			b
			7-10	80.1	137.8 (7.3)			c
	Wet	Natural	0-3	80.3	171.9 (22.2)	2.9	0.11	
			4-6	81.1	157.1 (28.3)			
			7-10	80	136.2 (9.7)			
		Inoculated	0-3	80.3	168.6 (34.8)	1.1	0.37	
			4-6	81.1	171.8 (177.2)			
			7-10	80	145.4 (19.2)			

TABLE 7: Enumerations of *Salmonella*, and bacteria cells in the top layer of artificial cow pats. Samples were exposed to natural or inoculated treatments, with or without parafilm. Values under the columns "%" indicate percent averages taken from 20 trials. Values under the "1 x10" column indicate average cells counted taken from 20 trials with standard deviations in parentheses. Values under the column "DAPI" represent the bacterial percentage within each segment with total numbers in parentheses and their respective standard deviations in brackets.

Top Layer

	Natural Feces								Inoculated Feces							
	Dry				Wet				Dry				Wet			
	Salmonella		Bacteria		Salmonella		Bacteria		Salmonella		Bacteria		Salmonella		Bacteria	
Day	%	n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)
0	0.05	8.7 (17.8)	30.0	37.9 (17.4)	0.04	10.4 (22.8)	68.9	84.2 (32.8)	1.16	286.4 (107.5)	24.8	45.9 (20.3)	1.37	215.8 (88.9)	49.5	83.5 (39.2)
1	0.06	9.5 (16.9)	81.5	163.1 (46.7)	0.06	8.2 (18.1)	65.3	56.7 (42.3)	0.99	168.8 (65.7)	81.4	80.0 (52.9)	6.22	1134.0 (412.2)	59.9	83.5 (46.8)
2	0.07	7.9 (20.8)	66.7	73.6 (36.2)	0.07	10.2 (18.2)	58.0	91.7 (50.0)	1.40	146.8 (59.1)	74.8	92.6 (36.1)	2.49	321.3 (106.4)	53.9	93.5 (38.6)
3	0.06	6.0 (14.6)	72.0	100.1 (50.9)	0.04	6.3 (15.3)	83.2	191.3 (42.8)	0.42	50 (38.7)	66.4	112 (52.5)	1.71	265.5 (118.5)	40.5	75.9 (31.3)
4	0.06	3.8 (11.8)	44.5	39.4 (18.9)	0.09	10.2 (22.4)	64.2	121.5 (54.8)	0.25	15.3 (33.7)	53.9	39.5 (19.8)	1.08	128.4 (117.0)	58.8	96.6 (28.1)
5	0.13	15.3 (22.8)	79.9	137.9 (61.9)	0.09	10.2 (18.2)	62.6	97.0 (54.0)	0.12	3.82 (11.8)	61.0	62.5 (26.8)	0.88	79.8 (64.3)	50.5	45.0 (24.2)
6	0.08	6.9 (14.2)	68.6	79.1 (31.1)	0.02	2.1 (9.37)	59.5	95.0 (32.4)	0.09	3.5 (10.7)	72.0	77.2 (25.2)	1.42	146.6 (81.0)	60.0	45.0 (17.8)
7	0.04	2.6 (8.0)	81.8	51.2 (20.3)	0.08	7.9 (16.2)	34.3	31.9 (13.0)	0.15	7.8 (12.2)	58.9	64.4 (23.0)	1.10	76.9 (71.8)	36.5	19.0 (8.8)
8	0.02	1.3 (5.6)	69.1	45.3 (19.0)	0.06	6.8 (16.5)	35.3	52.3 (15.3)	0.12	7.54 (11.8)	66.1	36.6 (13.5)	0.50	38.3 (36.6)	58.4	70.3 (37.1)
9	0.02	0.9 (4.0)	54.2	15.4 (14.2)	0.04	4.4 (13.4)	42.3	45.9 (25.8)	0.25	9.9 (12.3)	73.1	17.6 (7.3)	0.22	13.1 (32.0)	39.2	52.5 (22.1)
10	0.07	2.6 (6.4)	79.6	16.8 (14.7)	0.06	6.4 (15.6)	31.8	26.1 (16.1)	0.00	0	54.6	6.017 (4.4)	0.16	12.7 (20.0)	41.8	43.5 (30.4)

TABLE 8: Enumerations of *Salmonella*, and bacterial cells in the middle layer of artificial cow pats. Samples were exposed to natural or inoculated treatments, with or without parafilm. Values under the columns "%" indicate percent averages taken from 20 trials. Values under the "1 x10" column indicate average cells counted taken from 20 trials with standard deviations in parentheses. Values under the column "DAPI" represent the bacterial percentage within each segment with total numbers in parentheses and their respective standard deviations in brackets.

Middle Layer

	Natural Feces								Inoculated Feces							
	Dry				Wet				Dry				Wet			
	Salmonella		Bacteria		Salmonella		Bacteria		Salmonella		Bacteria		Salmonella		Bacteria	
Day	%	n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)
0	0.1	15.2 (25.5)	74.0	115.4 (41.8)	0.1	16.6 (24.8)	60.2	94.9 (50.3)	0.8	154.0 (88.5)	69.6	170.0 (55.0)	2.9	489.6 (298.5)	62.5	127.8 (63.5)
1	0.1	19.0 (31.4)	80.5	105.9 (44.2)	0.1	11.5 (19.3)	49.0	78.7 (39.2)	2.1	339.5 (167.6)	65.7	80.1 (36.4)	1.9	238.6 (111.4)	45.5	81.2 (38.2)
2	0.1	11.9 (22.7)	60.0	83.9 (31.5)	0.2	16.4 (20.6)	58.8	90.7 (33.8)	1.0	144.8 (105.6)	65.3	97.7 (39.8)	1.0	149.4 (92.3)	63.3	93.3 (29.3)
3	0.2	18.0 (27.4)	71.8	127.9 (57.6)	0.0	6.3 (15.3)	51.2	92.2 (35.4)	0.5	48.0 (51.2)	78.8	123.5 (42.2)	1.4	112.9 (91.1)	35.6	55.5 (19.2)
4	0.2	28.7 (30.0)	55.6	83.3 (27.2)	0.0	4.1 (12.5)	38.2	49.0 (19.7)	0.8	63.0 (63.5)	45.8	61.5 (24.9)	0.7	55.0 (63.8)	35.5	37.1 (16.2)
5	0.3	22.9 (38.0)	53.8	92.6 (32.9)	0.1	10.2 (18.2)	34.3	40.2 (12.2)	0.6	61.1 (54.6)	59.7	50.5 (21.0)	0.5	40.9 (39.8)	31.8	34.8 (10.3)
6	0.2	13.9 (23.6)	31.3	39.3 (15.3)	0.1	10.5 (18.6)	34.5	48.7 (19.1)	0.4	39.9 (46.8)	30.5	27.4 (12.5)	0.4	39.8 (31.8)	35.7	53.8 (13.9)
7	0.1	5.19 (10.7)	31.9	21.2 (4.8)	0.2	17.7 (29.9)	21.7	27.2 (11.1)	0.3	18.2 (20.8)	24.0	17.5 (5.8)	0.4	35.5 (31.1)	21.1	25.7 (10.8)
8	0.1	8.8 (12.3)	22.8	19.5 (22.6)	0.1	9.01 (18.5)	26.9	22.6 (9.5)	0.2	15.1 (20.6)	17.7	13.5 (3.6)	0.2	30.3 (27.3)	24.1	27.1 (8.3)
9	1.0	41.2 (34.5)	51.8	21.1 (11.2)	0.2	15.3 (21.4)	24.9	23.8 (11.4)	1.6	60.9 (43.7)	51.6	26.9 (10.3)	0.2	17.5 (29.7)	19.7	20.2 (7.6)
10	0.6	27.9 (32.7)	54.9	21.1 (7.4)	0.2	14.9 (31.7)	83.1	75.9 (29.0)	1.0	36.6 (35.7)	68.4	40.7 (10.6)	0.1	17.0 (28.9)	72.0	61.9 (21.3)

TABLE 9: Enumerations of *Salmonella*, and bacterial cells in the bottom layer of artificial cow pats. Samples were exposed to natural or inoculated treatments, with or without parafilm. Values under the columns "%" indicate percent averages taken from 20 trials. Values under the "1 x10" column indicate average cells counted taken from 20 trials with standard deviations in parentheses. Values under the column "DAPI" represent the bacterial percentage within each segment with total numbers in parentheses and their respective standard deviations in brackets.

Bottom Layer

	Natural Feces								Inoculated Feces							
	Dry				Wet				Dry				Wet			
	Salmonella		Bacteria		Salmonella		Bacteria		Salmonella		Bacteria		Salmonella		Bacteria	
Day	%	n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)		n x 10^5(±)	%	n x 10^7(±)	%	n x 10^5(±)	%	n x 10^7(±)
0	0.07	14.3 (24.1)	59.9	136.0 (42.9)	0.13	16.5 (24.6)	64.6	116.7 (84.2)	1.71	215.2 (108.8)	61.3	131.1 (60.3)	1.9	281.8 (122.5)	69.4	138.8 (57.2)
1	0.04	5.66 (13.8)	66.0	158.0 (64.7)	0.11	14.8 (22.6)	63.1	87.7 (39.0)	1.47	162.2 (74.5)	73.5	151.1 (74.6)	5.7	476.3 (184.4)	67.4	103.2 (49.7)
2	0.12	14.3 (20.0)	56.5	110.4 (31.3)	0.15	12.5 (27.3)	62.4	117.5 (47.0)	1.79	153.4 (90.8)	47.7	89.4 (32.4)	3.1	355.3 (146.1)	59.9	116.4 (33.5)
3	0.11	10.1 (22.3)	47.1	80.6 (36.4)	0.21	24.6 (36.1)	62.0	111.5 (37.5)	1.8	190.3 (116.1)	49.1	87.2 (30.2)	1.4	143.3 (92.5)	53.4	67.7 (32.7)
4	0.18	16.1 (24.1)	40.8	68.5 (25.3)	0.16	14.3 (24.1)	72.6	127.2 (66.0)	1.48	112.7 (91.0)	38.8	62.3 (14.8)	0.9	90.2 (58.9)	59.6	110.6 (36.5)
5	0.14	15.8 (23.7)	53.8	106.0 (40.5)	0.12	16.1 (24.1)	59.5	102.7 (55.2)	1.71	138.4 (77.5)	60.9	120.1 (44.9)	1.5	163.4 (78.0)	54.3	96.6 (44.9)
6	0.13	11.9 (18.6)	40.3	73.5 (32.0)	0.24	14.1 (23.6)	47.3	58.9 (23.4)	1.67	144.3 (54.8)	39.1	65.4 (18.0)	1.1	104.7 (64.6)	49.5	75.1 (26.8)
7	0.17	12.9 (24.8)	38.8	56.0 (26.2)	0.16	17.2 (23.2)	42.5	63.0 (20.2)	1.77	135 (68.2)	41.3	58.3 (19.6)	1.0	88.0 (65.8)	44.2	58.1 (18.8)
8	0.13	16.1 (24.0)	36.3	59.9 (22.3)	0.17	12.0 (18.2)	62.0	86.6 (31.4)	0.99	102.5 (70.8)	50.9	67.1 (23.3)	0.8	46.5 (30.9)	60.8	80.0 (3.84)
9	0.15	13.4 (22.5)	35.5	64.6 (17.9)	0.25	18.1 (24.9)	51.6	65.3 (30.5)	1.2	99.8 (48.9)	41.5	54.7 (15.6)	0.7	74.3 (38.1)	50.7	87.4 (28.4)
10	0.19	14.5 (21.7)	31.6	49.9 (21.6)	0.21	20.9 (31.4)	45.0	58.8 (20.5)	0.93	67.0 (33.8)	32.1	47.1 (10.7)	0.5	55.1 (43.5)	47.3	69.1 (27.4)

TABLE 10: Detection of Salmonella in feces incubated for 11 days

		Natural Feces		Inoculated Feces	
		Dry	Wet	Dry	Wet
I	*In situ* hybridization				
	a) Top	2.6 (6.4) *	6.4 (15.6)	0 (0)	12.7 (20.0)
	b) Middle	27.9 (32.7)	14.9 (31.6)	36.6 (35.7)	17.0 (28.9)
	c) Bottom	14.5 (21.7)	20.9 (31.4)	67.0 (33.8)	55.1 (43.5)
II	Enrichment	0	100% (n=5) **	100% (n=5)	80% (n=4)
III	Colonies	42% (n=21)***	92% (n=46)	90% (n=45)	68% (n=34)
	a) Strain 1^{+}	20	41	31	36
	b) Strain 2	-	5	10	11

* $1x10^{5}$ cells [g feces dry wt]$^{-1}$, ** Numbers in brackets for enrichment are the amount of positives out of 5 total samples, ***Numbers in brackets for Colonies are the amount of positives out of 50 total samples, $^{+}$ indicates the inoculated strain.

IV. DISCUSSION

January detection of salmonellae- Initially, determining the overall micro-organism community within cow feces was of vital importance. Fluorescence *in situ* hybridization allowed counting and identifying organisms within complex environmental samples [46, 2] such as feces. Targeting specific oligonucleotide sequences using 16S, 18S and 23S rRNA-targeted probes for Archaea (Arch915), Eukarya (Euk516), *Escherichia.coli* (EC) initially did not yield any results in detections during the January feces sampling. Bacteria (EUB338), and *Salmonella* (Sal 3) probes consistently detected bacterial cells within January samples. DAPI-stained cells provided enumeration on the overall microorganism content within bovine feces. Based on DAPI counts, overall organisms increased from pre-inoculation numbers within cow feces, to after inoculation. Gradually, each day the total number of organisms decreased based on exposure to room temperature conditions and loss of moisture.

Probe EUB338 is complementary to a portion of 16S rRNA gene conserved in the domain Bacteria [44] and targets many bacterial species including *Desulfovibrio gigas, Escherichia.coli, Desulfobacter hydrogenophilus* and others [1]. Using the initial cow feces samples from January, most organisms detected were from the Domain Bacteria, composing 73% to 90% of the total community. Upon inoculation and using DAPI staining techniques, overall bacterial counts were noted to increase in numbers largely within samples. Over three days, numbers of organisms decreased while the cow

pats desiccated at room temperature. *Salmonella* composed of approximately 3% of the total community on day 0 after inoculation, and decreased to <1 % after 3 days of moisture loss.

July detection of salmonellae -Top, middle, and bottom layers of cow pats were analyzed to assess microbial fate within each layer. *Salmonella* counts had contrasting results within the top feces layer. Naturally occurring *Salmonella* in drying feces had a significant decrease in numbers over the 10-day experimental period resulting in a 22% survival rate in salmonellae. Other studies have shown during the drying out phase on feces pat tops, a 90% reduction in populations occurred over 25 days [15]. Inoculated feces in the top layer under similar moisture conditions also had a large decrease in salmonellae, although these results were statistically insignificant. Protected inoculated feces showed a significant reduction over time in the number of salmonellae counted throughout the experiment. Researchers found within limited moisture loss environments, a 90% reduction in *Salmonella* populations have occurred within 9 days, despite the continued moisture. Desiccation has also been noted to greatly lower populations of specific *Salmonella* serotypes such as *S. Newpor*t or *S. Hadar*, but not greatly affect *S. enteriditis* [21]. *S. enterica* population decreases occurred when pat water content decreased to 70-75% [42].

Within the middle layer, samples were noted to have significant decreases in both moist and dry inoculated feces. This significant decrease was unrelated to feces moisture content. The bottom layers of both moist and dry inoculated feces also had a significant decrease in *Salmonella* counts, despite maintenance of moisture within this layer. Within both middle and bottom layers, dry and wet natural populations maintained Salmonella

populations, or even increased populations slightly (although not significantly). Despite similar moisture conditions to the top layer, natural *Salmonella* counts in middle pats exhibited greater survivorship with limited decreases in counts. Researchers noted that despite continual moisture contents of >80% in cow pat content, *Salmonella enterica* growth sometimes occurred [42]. Cow pat water contents of >80% generally had increases in *E. coli, enterococci* and *fecal streptococci* counts. Thus, conflicting results do not support the hypothesis that *Salmonella* persist in moist cow feces over time.

Using *in situ* hybridization, overall bacterial community counts were also assessed using the EUB338 probe. Bacterial numbers decreased insignificantly, within the top cow pat layer over 11 days. Within unprotected samples, counts decreased to approximately 30-40% of their original averages. Wet natural feces did have a significant decrease especially after day 6. Within the middle section of pats, all treatments decreased significantly over the experimental time period. The largest differences were noted within the dry natural and inoculated feces, with final samples having approximately 20% bacterial survival. Within moist conditions, there was 37-40% bacterial survival after 11 day. Within the bottom layer, significant decreases in overall bacterial counts were noted in dried natural and inoculated samples only with overall bacterial survivorship at approximately 50%. In comparison, overall bacterial communities declined in time, but *Salmonella* counts declined much more rapidly. Bacterial communities have shown differences within individual cattle [8]. Main bacterial types within beef cattle are *Bacteroidetes Prevotella* and *Bacteroides*; the *Firmicutes Faecalibacterium*, *Ruminococcus*, *Roseburia*, and *Clostridium*; and the proteobacterium *Succinovibrio* [8]. Dairy cattle show similarities in major bacterial groups, but have

prevalence in *Clostridium lituseburense, Ruminococcus bromii, Acinetobacter johnsonii, Bacillus silvestris,* and *Eubacterium tenue* [31]. Further microbial community analysis was conducted using probes targeting the Domains Archaea and Eukarya to further investigate organisms comprising the total DAPI-counts. Eukarya made up approximately 2-3% of the total DAPI population, and Archaea were approximately 1-2% of total DAPI populations with minimal decreases over time. Thus, in time, overall bacterial communities decline, but *Salmonella* communities decrease at a quicker rate.

The random detection by end-point PCR in cattle feces collected at Freeman Ranch suggests that *Salmonella* is a common occurrence on ranches. Within central Texas, up to 70% of cattle on farms can be infected with *Salmonella* [3]. This supports the hypothesis that *Salmonella* can be detected regularly in cow feces despite no outward signs of illness from the affected animal. Feces samples with 2 natural *Salmonella* strains were collected randomly from Freeman Ranch. Despite being inoculated, a natural strain isolated from another ranch within San Marcos, Tx was found in day 10 natural and inoculated samples. A total of 61 isolates were noted with the natural strain predominant. In comparison, by day 10 the inoculated feces had 67 isolates of the inoculated strain 34-2-2. The natural detection of salmonellae in cow feces at Freeman Ranch by end-point PCR and *in situ* hybridization suggest that cattle can easily be considered reservoirs for salmonellae, and be transmitted through the environment. Environmental transmission can also lead to waterbody contamination, causing illness in humans. Despite dry inoculated samples having negligible *Salmonella* counts by day 10, upon placing samples into enrichment broth, *Salmonella* colonies regrew. We were therefore unable to reject

the hypothesis that salmonellae can stay viable in feces over time, although undetectable using conventional molecular tools.

V. FUTURE PERSPECTIVES

Initial data indicating that the large decreases of salmonellae were related to a diminution of cow pat moisture content, proved incorrect. A more detailed analysis of moisture content, and *Salmonella* populations throughout the cow pats revealed conflicting results between both natural and inoculated samples (Tables 1, 2, and 3). Results showed that despite similar moisture contents in top and middle layers, *Salmonella* populations did not decrease in a comparable fashion. Middle *Salmonella* populations responded corresponding to bottom salmonellae counts. Also, despite wet and dry treatments, the majority of significant results occurred in inoculated feces, both wet and dry (Tables 2 and 3). This indicates other factors influencing *Salmonella* populations. Other researchers tested influencing factors such as pH, ammonia content, and water activity and cow pat moisture content. Within cow pats or manure slurries, temperature and oxygen content have large impacts on *Salmonella* populations. Conducting further studies examining these factors would greatly clarify their impacts on the persistence of salmonellae within the environment.

Within natural cow feces collected at Freeman Ranch, multiple *Salmonella* strains were noted. Once determining unique strains using rep-PCR techniques, focusing on antibiotic sensitivity and resistance around Texas would be beneficial. Of the antibiotics used on farms or ranches, approximately 10% are used for active infections while 90%

are used for preventative purposes and growth promotion [48]. Antibiotics once excreted, can enter aquatic systems [25], or at low continual exposures cause bacteria to become resistant by killing wild-type bacteria and promoting bacterial mutations [19]. Recent studies suggest that *Salmonella* have gained antibiotic resistance to many of the common treatments, or change resistance within a region depending on the antibiotics commonly used at the time [6]. Within the United States, studies show salmonellae resistance to drugs such as Ampicillin and Streptomycin [6], Tetracycline [5, 6] and strong sensitivity to Amikacin, Apramycin, Gentamicin and Ciprofloxacin is an important tool for physicians treating salmonellosis.

Using *in situ* hybridization, methodology is extremely important when counting cells or determining whether specific cell types are within a sample. Defining whether cleavage furrows determine one or multiple chain forming cells can be difficult due to probe strength, and the inability to differentiate between individual cells. Counting in the same method on a day to day basis can also be difficult, and may alter counts on specific days. Once cells become dormant in less than ideal conditions, rRNA within the cell cannot become hybridized, making visualization difficult. A researcher is then unable to differentiate between dormant cells and the overall environment that the cells live. Dormancy is temporary, and once cells are in an ideal environment, they become active again.

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