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Project Report

Evaluation of a Triple Buffered Peptone Broth for Detection of *Salmonella* in Broiler Feed

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Abstract: The pH of pre-enrichment media containing feed/ingredients can become acidic during incubation due to bacterial utilization of feed carbohydrates. This decrease in pH can result in cell injury or death, negatively impacting the detection of *Salmonella*. Our objective was to evaluate a new triple buffered peptone (**TBP**) against buffered peptone water (**BPW**) and lactose broth (**LB**) for the recovery of *Salmonella* from feed. Liquid cultures of nalidixic acid resistant strains of *Salmonella* (Enteritidis, Heidelberg, Kentucky or Typhimurium) were added to the pre-enrichment media alone, to pre-enrichment media containing feed or to artificially inoculated feed stored 1 or 7 d to evaluate the effect of the medium on the recovery of *Salmonella*. Three replicates per treatment were conducted. After incubation at 37 °C for 24 h, the pH of the medium was measured prior to plating onto brilliant green sulfa agar plates supplemented with 200 ppm nalidixic acid (**BGS$_{NA}$**). Plates were incubated and evaluated for presence of typical *Salmonella* colonies. The experiment was replicated. TBP was observed to exhibit significantly better buffering capacity than **BPW** or **LB**. Additionally, TBP was able to recover *Salmonella* 100% of the time compared to **BPW** (97.9%) and **LB** (61.5%). TBP shows promise to maintain neutral pH during pre-enrichment which may allow for a more accurate detection of *Salmonella* in feed.

Keywords: poultry; feed; *Salmonella*; pre-enrichment; detection

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1. Introduction

Salmonella enterica is a zoonotic pathogen readily passed from animal to man through the consumption of contaminated food. *Salmonella* species are commonly associated with the alimentary tract of animals and are considered a common commensal member of the gut microflora of poultry species [1]. Non-typhoidal *Salmonella* accounted for approximately 1.0 million cases of foodborne illnesses in the United States [2] with poultry meat products being associated with a higher percentage of outbreaks and infections than other food sources [1,2].

Salmonella contamination of broiler chickens can occur during grow-out, which can lead to contaminated birds arriving at the slaughter/processing plant. Despite elaborate post-harvest intervention strategies, contaminated poultry products on occasion reach the supermarket shelf and thus pose a health risk to the consumer. The poultry industry has long understood that pre-harvest intervention is necessary to control human enteropathogens, such as *Salmonella* and *Campylobacter* associated with poultry products. The grow-out farm is a horizontal transmission site, and these bacterial human pathogens can be recovered from multiple sources. Feed is one of the possible sources for the introduction of *Salmonella* into the farm. Numerous published studies have reported poultry feed as a potential source of *Salmonella* colonization of poultry [3–8]. However, only a low percentage of feed samples tested are typically reported as *Salmonella* positive [5,7].

Recovering *Salmonella* from feed poses many challenges. It is well known that *Salmonella* in feed is not uniformly distributed and the level of *Salmonella* in feed is

<20 cfu/100 g. Mitchell and McChesney in 1991 suggested that at least 30 individual test samples would be required to adequately determine that a particular lot of feed was *Salmonella* negative [9]. A second challenge is that *Salmonella* in feed may exist in a stressed or injured state and therefore require a pre-enrichment step for resuscitation. Recent research has indicated some pre-enrichment media become acidic during the incubation periods due to fermentation of carbohydrates by background microflora. Cox et al. (2013) reported that the pH of various pre-enrichment media could decrease from an initial pH of 6.1–7.2 to a final pH of 3.9–4.1 depending on the type of pre-enrichment media and feed/ingredient type [10]. The inability of the pre-enrichment media to maintain a near neutral pH impacts the recovery and detection of *Salmonella* [11–13].

Berrang et al. in 2015 developed and reported a triple buffered peptone (**TBP**) medium which they found was able to maintain a pH closer to neutral than lactose broth (**LB**) or buffered peptone water (**BPW**) when used to incubate poultry feed [14]. The hypothesis is that by maintaining a pH closer to neutral, the TBP will have a better recovery rate of poultry related *Salmonella* serovars. The objective of the current study is to compare LB, BPW and TBP pre-enrichment broths for their ability to maintain a near neutral pH and determine their impact on recovery of poultry related *Salmonella* strains (unstressed and stressed) from feed. The authors approached this objective by incubating each broth with feed inoculated with one of four poultry relevant *Salmonella* serovars or incubating each broth with the addition of feed and one of four poultry relevant serovars of *Salmonella*. Broth pH was monitored, and the recovery of *Salmonella* was compared for all test broths at 1- and 7-days post inoculation.

2. Materials and Methods

2.1. Preparation of Broths

Two commonly used pre-enrichment buffers, buffered peptone water (**BPW**; Neogen Culture Media, Lansing, MI, USA) and lactose broth (**LB**; Becton-Dickinson, Sparks, MD, USA) were prepared according to the manufacturer's directions and autoclaved for 15 min at 121 °C. Triple buffered peptone (**TBP**) was prepared according to the method of Berrang et al. [14] and filter sterilized using 0.22 µm polyethersuflone, low protein binding membrane filters (PES Membrane Filters, Corning Costar, Corning, NY, USA). The pH of each broth was measured using a pH meter (SevenCompact, Mettler Toledo, Columbus, OH, USA) and found to be within the appropriate specifications.

2.2. Salmonella Cultures and Liquid Inoculum

Four nalidixic acid resistant serovars of *Salmonella* (Enteritidis, Heidelberg, Kentucky and Typhimurium) were grown on brilliant green sulfa agar (**BGS_{NA}**; Becton-Dickinson, Sparks, MD, USA) plates supplemented with 200 ppm nalidixic acid (**NA**; Sigma-Aldrich Chemicals, St. Louis, MO, USA) at 35 °C for 24 h. Cells on the plate were harvested and a liquid inoculum of each *Salmonella* serotype was prepared by suspending the cells in phosphate buffered saline. *Salmonella* was enumerated by serial dilution and plating on BGS_{NA} agar plates. Plates were incubated for 24 ± 2 h at 35 °C prior to enumerations. Cell suspensions were stored at −80 °C in tryptic soy broth (**TSB**; Becton-Dickinson, Sparks, MD, USA) with 15% glycerol (Sigma-Aldrich Chemicals, St. Louis, MO, USA) until use.

2.3. Inoculation of Feed

Non-medicated grower feed obtained from a local research farm was used in the experiments (Table 1). One hundred g of feed (n = 4 per replication) was placed into sterile plastic freezer bags (Ziploc, S.C. Johnson and Johnson, Sturtevant, WI, USA) and inoculated with 10 mL of the 10^3 cfu/mL cell suspension while mixing. Inoculum was prepared for each serotype and contained ~10^3 cfu *Salmonella*/g of feed. Inoculated feed was held at 22 ± 2 °C for 1 and 7 days.

Table 1. Composition of Broiler Starter Grower Feed.

Ingredient	**Percentage (%)**
Corn, yellow	59.46
Soybean meal	34.18
Dicalcium phosphate	2.06
Fat, vegetable	1.95
Calcium carbonate	1.19
Sodium chloride	0.44
Methionine	0.33
L-lysine	0.18
Nutrient	Percentage (%)
Dry matter	88.05
Crude protein	21.19
Crude fat	4.36
Crude fiber	2.16
Calcium	0.98
Total phosphate	0.75
Available phosphate	0.45
Total Methionine	0.63
Total Lysine	1.32

2.4. Evaluation of Broths

Pre-enrichment broths were evaluated for their ability to maintain a near neutral pH during incubation and the subsequent impact on the recovery of each strain of *Salmonella*. Broths (45 mL) were dispensed into individual sterile specimen cups containing the following treatments: (1) pre-enrichment media + 5 mL of the 10^1 cfu ml cell suspension, (2) pre-enrichment media + 5 mL of the 10^1 cfu ml cell suspension + 5 g uninoculated feed, (3) pre-enrichment media + 5 g of inoculated feed (10^2 cfu/g) stored for 1 day or (4) pre-enrichment media + 5 g of inoculated feed (10^2 cfu/g) stored for 7 days. *Salmonella* inoculated feed samples were prepared with a higher cfu/g to ensure desiccation did not eliminate all the viable *Salmonella* in the feed samples. Broths were incubated for 24 $\pm$ 2 h at 37 °C. Three replicates per treatment in two replicate studies were conducted (N = 288).

2.5. Analysis

Due to laboratory standard operating procedures which prohibit the use of pH meter probes in samples which have been inoculated with known pathogens, the pH of the broths after incubation was measured using disposable pH test strips (colorpHast, EM Science, Gibbstown, NJ, USA). Two replicate test strips were analyzed per sample. *Salmonella* recovery was determined by streaking a 10 µL aliquot of the broth onto individual BGS^{NA} agar plates and incubation at 37 °C for 22 $\pm$ 2 h. Presumptive positive colonies on the BGS_{NA} agar plates were transferred to triple sugar iron (**TSI**, Becton-Dickinson, Sparks, MD, USA) and lysine iron agar (**LIA**, Becton-Dickinson, Sparks, MD, USA) slants for biochemical characterization. Slants with typical reactions were verified by O-antigen serogrouping (Becton-Dickinson, Sparks, MD, USA) to confirm the isolate as belonging to the same serogroup as the original nalidixic acid resistant *Salmonella* serovar (data not included in tables).

2.6. Statistics

Data (pH and *Salmonella* recovery) from the two experiments was combined for statistical analysis (n = 6). Data from the pH measurements of the broths was analyzed by least significant difference test *t*-test to determine differences among broths and treatments. *Salmonella* recovery (% recovery) data was compared using Fisher's Exact test. Significance was assigned at a *p*-value of <0.05.

3. Results

The initial pH of pre-enrichment broths (uninoculated and non-incubated) and the pH of the pre-enrichment broths containing the 10^1 cfu/mL of cell suspension of *Salmonella* that was incubated for 24 h were identical. However, differences were observed between broth types when feed was included in the treatment group (Table 2). No difference in pH was observed among *Salmonella* strains incubated in the same broth type and an average pH value for all strains was used for comparison purposes. It was observed that the pH of LB and BPW incubated with feed had become acidic at a pH of 4.3 and 5.1, respectively, during incubation, while the pH of TBP incubated with feed was near neutral at a pH of 6.8. The overall mean pH for each pre-enrichment broth was significantly ($p < 0.05$) different. No differences in broth pH were observed between broth containing feed and a cell suspension of *Salmonella* and broths containing *Salmonella* inoculated feed. Based on these data it appears that the decrease in pH of the broth is related to the production of acidic byproducts from the growth of other background microorganisms in the feed and not necessarily the resuscitation or growth of the *Salmonella* serovars [10].

Table 2. pH of pre-enrichment broths after 22 ± 2 h incubation at 37 °C [1].

Broth Type	*Salmonella* Isolate [2]	Broth + Cell Suspension	Broth + Cell Suspension+ Feed	Broth + Inoculated Feed (1 dpi)	Broth + Inoculated Feed (7 dpi)
		pH	pH	pH	pH
BPW	SE	7.3	5.1	5.1	5.0
	SH	7.3	5.2	5.0	5.1
	SK	7.3	5.1	5.1	5.0
	ST	7.3	5.1	5.0	5.0
	All Serovars	7.3 [a]	5.1 [a]	5.1 [a]	5.0 [a]
LB	SE	5.9	4.5	4.2	4.0
	SH	6.2	4.5	4.5	4.4
	SK	5.9	4.5	4.3	4.4
	ST	6.2	4.0	4.3	4.1
	All serovars	6.0 [b]	4.4 [b]	4.3 [b]	4.2 [b]
TBP	SE	8.3	6.50	6.9	6.9
	SH	8.3	6.60	6.9	6.8
	SK	8.3	6.8	7.0	6.8
	ST	8.5	6.8	6.8	6.8
	All serovars	8.4 [c]	6.7 [c]	6.9 [c]	6.8 [c]

[1] Values are the combined average of the 3 replicate samples/treatment from experiment 1 and 2. [2] SE—*Salmonella enteritidis*, SH—*Salmonella* Heidelberg, SK—*Salmonella* Kentucky, ST—*Salmonella* Typhimurium. [a–c] Values in columns with different superscripts differ significantly ($p < 0.05$).

Salmonella recovery data is presented in Table 3. In broths which were inoculated with only a cell suspension of *Salmonella*, broth type did not impact *Salmonella* recovery with *Salmonella* recovery from 100% of the inoculated buffer samples as expected. However, when uninoculated feed plus the cell suspension or inoculated feed were added to the broths, differences in recovery were observed among the three broths and among the four *Salmonella* strains. Of the strains evaluated, the recovery of *S. enteritidis* and Kentucky were most adversely affected.

Table 3. *Salmonella* recovery from pre-enrichment broths after 22 ± 2 h incubation at 37 °C [1].

Broth Type	*Salmonella* Isolate [2]	Broth + Cell Suspension		Broth + Cell Suspension + Feed		Broth + Inoculated Feed (1 dpi)		Broth + Inoculated Feed (7 dpi)	
		+/−	% Positive	+/−	% Positive	+/−	% Positive	+/−	% Positive
BPW	SE	6/6	100	6/6	100	6/6	100	4/6	66.7
	SH	6/6	100	6/6	100	6/6	100	6/6	100
	SK	6/6	100	6/6	100	6/6	100	6/6	100
	ST	6/6	100	6/6	100	6/6	100	6/6	100
	All serovars	24/24	100	24/24	100 [a]	24/24	100	22/24	91.7 [a]
LB	SE	6/6	100	0/6	0	6/6	33.3	0/6	0
	SH	6/6	100	1/6	16.7	6/6	100	6/6	100
	SK	6/6	100	0/6	0	6/6	66.7	3/6	50
	ST	6/6	100	1/6	16.7	6/6	100	6/6	100
	All serovars	24/24	100	2/24	8.3 [b]	24/24	75	15/24	62.5 [b]
TBP	SE	6/6	100	6/6	100	6/6	100	6/6	100
	SH	6/6	100	6/6	100	6/6	100	6/6	100
	SK	6/6	100	6/6	100	6/6	100	6/6	100
	ST	6/6	100	6/6	100	6/6	100	6/6	100
	All serovars	24/24	100	24/24	100 [a]	24/24	100	24/24	100 [a]

[1] Values are the combined average of the 3 replicate samples/treatment from experiment 1 and 2. [2] SE—*Salmonella enteritidis*, SH—*Salmonella* Heidelberg, SK—*Salmonella* Kentucky, ST—*Salmonella* Typhimurium. [a,b] Values in columns with different superscripts differ significantly ($p < 0.05$).

No *S. enteritidis* was recovered from lactose broth in either the broth with feed and *S. enteritidis* or the broth with feed stored for 7 days after inoculation with *S. enteritidis*. Additionally, no *S.* Kentucky was recovered from lactose broth or the broth with feed and *S.* Kentucky inoculation and there was only 50% recovery from broth with feed stored for 7 days after inoculation with *S.* Kentucky. Overall, the recovery rates for lactose broth were 100, 8.3, 75 and 62.5 % from the broth plus cell suspension, broth plus *Salmonella* culture plus feed, broth plus feed stored for 1 day after inoculation with the *Salmonella* culture, and broth plus feed stored 7 days after inoculation with the *Salmonella* culture. A significant difference was noted between the recovery rates for lactose broth plus feed and *Salmonella* suspension and lactose broth plus inoculated feed stored for 7 days after inoculation when compared to the same two treatments for both buffered peptone water and triple buffered phosphate broth.

4. Discussion

Research on *Salmonella* and acid exposure is often focused on adaptation due to continuous exposure. In some analytical methods, a short incubation period can preclude any acid adaptation and sensitivity to pH must be considered. Our data agrees with the data of Cox et al., who observed that the decrease in pH of the broth is related to the production of acidic byproducts from the growth of other background microorganisms in the feed and not the resuscitation or growth of the *Salmonella* serovars present in the feed or feed ingredients [10]. Blankenship reported that at a pH of 3–3.5 cell injury and cell death occurred in *Salmonella* Bareilly [15]. The percentages of cell injury and/or death were dependent on temperature and time. Cox et al. examined the sensitivity of cell suspensions of various *Salmonella* serotypes to acidic conditions (pH 4.0–5.5) at commonly used pre-enrichment times (18–48 h) [11]. It was observed that even at a pH of 5.5, the recovery of *Salmonella* could be negatively impacted. Recent research by Richardson et al. has shown that the recovery of *Salmonella* is dependent on pH, strain of *Salmonella* and stress status (i.e., cell suspension vs. naturally contaminated) [12]. Data indicated that if

the pH of a pre-enrichment medium was not maintained at a near neutral pH, then >50% of the population of some strains of *Salmonella* would die or be injured at a pH of <5.8.

Our data supports the findings of previous researchers that lactose broth, a non-buffered broth, is not satisfactory for recovering *Salmonella* from feed and other dry, environmental samples. In the experiments of Richardson et al., the ability to recover *Salmonella* from the pre-enrichment broth was directly related to the buffering capacity of the media [12]. Lactose broth is an unbuffered medium, and the observation was that it was not particularly effective in recovering *Salmonella* from feed.

The difference in the recovery of *Salmonella* from lactose broth amended with a cell suspension and uninoculated feed versus inoculated feed in the current study may be due to the stress status (presumption is of dry-stress or desiccation) of the organism. In the current work, the lack of recovery of *S. enteritidis* from lactose broth and inoculated feed stored either 1 or 7 days after inoculation and the lack of recovery of *S.* Kentucky from inoculated feed stored 1 day after inoculation and 50% recovery from lactose broth and feed stored 7 days after inoculation were expected due to the unbuffered capacity of lactose broth. Richardson et al. demonstrated that the acid sensitivity of the same isolate of *Salmonella* grown in cell suspension (unstressed) and in contaminated feed (presumably dry-stressed) was different and was dependent on the pH of the enrichment broth and serovar of the *Salmonella* isolate [16]. Longer storage time of inoculated feed has been reported to result in more desiccation of the cell suspensions that were added to feed resulting in a reduction in viable cell numbers present in the feed for recovery [17]. This may explain the observation of lower *S. enteritidis* recovery in BPW from inoculated feed (7dpi). Reduced recovery may be a combination of dry stress in feed stored for 7 days after inoculation and the acid stress associated with low pH of the pre-enrichment broth during incubation.

The use of a pre-enrichment medium that maintains a near neutral pH, such as TBP, is essential for evaluating the incidence and types of *Salmonella* in feed. Acidic conditions can bias both the recovery of *Salmonella* and the serotype recovered. In the present study, TBP was the only broth with 100% recovery of all four *Salmonella* serovars from all broth, *Salmonella*, and feed. BPW had a near identical recovery, with a 97.9% recovery rate. This supports the finding of Cox et al. [10] and Richardson et al. [12].

In addition, detection of recovered *Salmonella* can be impacted by the pH buffering capability of the broth used to inoculate plates for incubation and identification of colonies. Blankenship [15] showed that *S.* Bareilly lost the ability to decarboxylate lysine and produce H_2S when acid-injured at a pH of 4.0. Richardson et al. reported that the ability to produce H_2S on selective agar occurred at a less acidic pH and was dependent on the isolate of *Salmonella* and stress status (dry vs. liquid culture) [18]. Laboratory technicians and personnel are trained to select colonies based on phenotypic characteristics and the inability of injured *Salmonella* to produce typical reactions on selective media could limit detection. This low pH has been shown to be detrimental to the recovery of *Salmonella* [11]. Therefore, a properly pH buffered pre-enrichment broth may preserve the typical colonial morphology and characteristics of *Salmonella* species making the recognition and identification of *Salmonella* more likely in feed samples and other dry environmental samples abundant in background microflora. Selection of pre-enrichment media and methods may be critical to prevent false negative test results for *Salmonella* testing and the coinciding misunderstanding or lower expectations for the potential of *Salmonella* species to be transmitted to growing poultry from feed and feed ingredients.

5. Conclusions

(1) Significant decreases in the pH of broiler feed pre-enriched in lactose broth or buffered peptone water during incubation at 37 °C may injure or kill *Salmonella*, thereby preventing recovery of the pathogen and possibly producing false negative results.

(2) Triple buffered peptone used as a pre-enrichment was able to maintain a neutral pH and allowed the most recovery of *Salmonella* after incubation. Use of this newly

developed medium may allow for recovery of more *Salmonella* from feed samples. Improved detection of *Salmonella* from feed could allow the poultry industry to find new and/or better intervention strategies for feed and may eventually help to lead to a reduction of *Salmonella* on the finished carcass.

(3) Pre-enrichment of feed in LB produced a more drastic change in pH than pre-enrichment in BPW or TBP. The low pH level of feed in LB has been shown to kill and injure *Salmonella*. The continued use of lactose broth for the evaluation of feed may need to be re-evaluated by regulatory agencies to ensure that *Salmonella* is recovered from feed samples contaminated by this pathogen.

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