EVALUATION OF PREBIOTIC AND PROBIOTIC FEED ADDITIVES AS A METHOD OF SALMONELLA MITIGATION IN BROILERS AND LAYING HENS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2021

Major Subject: Poultry Science

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ABSTRACT

For the last few decades, antibiotic usage in poultry production has been commonly implemented for increased production performance, as well as control of infectious diseases. However, changes in recent years both from legal entities and consumers have caused this usage to shrink in the last decade. These changes have resulted in food safety becoming a greater point of concern. As antibiotics continue to be phased out, alternative methods of pathogen control must be found in order to keep a growing population fed with nutritious and safe food sources. This study was conducted to evaluate the efficacy of prebiotics and probiotics as a pre-harvest method of mitigating *Salmonella* in broilers and laying hens. The objectives were to: 1) evaluate the effects of yeast prebiotics, *Bacillus* probiotics, and their combinations as a method of reducing *Salmonella* counts in the ceca of broilers; 2) determine the effects of yeast prebiotics, *Bacillus* probiotics, and their combinations as a method of reducing *Salmonella* counts in the ceca of early production laying hens; 3) determine the effects of yeast prebiotics and *Bacillus* probiotics as a method of reducing *Salmonella* translocation to internal organs including liver, spleen and ovaries; and 4) determine the effects of yeast prebiotics and *Bacillus* probiotics as a method of reducing *Salmonella* counts and ovary prevalence in late production laying hens.

Results from the laying hen experiments (experiments 1 and 2) showed positive benefits with the supplementation of yeast cell wall (YCW) and *Bacillus* products in laying hens, with reductions in cecal load being seen across all three different serovars of

Salmonella. In the first experiment, supplementation of YCW at 500ppm resulted in a reduction of over 1.8 logs of *Salmonella enterica* serovar Typhimurium (ST) and a nominal reduction of approx. 0.5 logs of *Salmonella enterica* serovar Braenderup (SB) in the ceca of early production laying hens. Similar reductions were seen in the second experiment. On an individual room basis, in room 3 YCW supplementation resulted in a 1.2 log reduction in *Salmonella enterica* serovar Enteritidis (SE), and the *Bacillus* product supplementation resulted in a 1-log reduction in SE in the same room. In a cumulative overview, the YCW treatment resulted in a cumulative reduction approaching 1 log. While these did not achieve statistical significance, they did achieve a biologically relevant reduction in *Salmonella* colonization of >1.0 Log_{10} when compared to the control treatment.

Results from the broiler experiments (experiments 3 and 4) were mixed and varied by the *Salmonella* serovar the birds were challenged with. In experiment 3, small *Salmonella* reductions were noted in ST challenged broilers, with a biologically relevant reduction of >1.3 logs at day 14 in the YCW treated birds. Other nominal reductions were seen with the supplementation of YCW in the diet, with a 0.8 log reduction in ST colonization at day 21 when compared to the control treatment. In experiment 4, birds challenged with *Salmonella enterica* serovar Infantis (SI) and supplemented with the YCW treatment experienced the greatest benefit, with a 4-point reduction in feed to weight ratio (0.04), as well as a >0.6 log reduction in SI load in the ceca at both sampling time points (days 14 and 21) when compared to the control treatment. While

this value was not statistically significant, YCW did achieve a slight reduction in *Salmonella* load in the ceca at both sampling time points.

While statistically significant reductions were not achieved in in many of the aspects observed in these experiments, biologically relevant reductions of *Salmonella* were observed with the inclusion of YCW or *Bacillus* products into the diet across a wide range of *Salmonella* serovars. Because of these reductions, both the Safmannan YCW and MicroSaf *Bacillus* show promise in improving food safety as a feed additive.

DEDICATION

I dedicate this dissertation to all of those I love;

To my wife, Shelby, for all the faith, love and advice I have ever needed, and then some. Without your constant support throughout my program, I would not be where I am today. I love you, and here's to the next phase of our lives, together;

To my parents, Billy and Shannon Padgett, for their continuous prayers and support. I cannot begin to describe how much I appreciate the drive that you instilled in me;

To my brother, Kade, and my niece Lillian, thank you for always being there when I need someone to talk to, or brighten my day.

ACKNOWLEDGEMENTS

I would first like to give my greatest gratitude to my supervisor, Dr. Christopher Bailey, for his support, encouragement, and guidance throughout my program. Thank you for always being willing to step outside the box and support my sometimes-off-the-wall ideas. Without your support and mentorship, I would not be where I am today. I am ever grateful for the opportunity you provided me.

I would also like to extend my gratitude to my committee members, Dr. John Carey, Dr. Allen Byrd, and Dr. Keri Norman. Dr. Carey, thank you for first introducing me to research, and for your continued support through both of my degrees here at Texas A&M. Dr. Byrd, thank you for constantly pushing me outside of my comfort zone in the pursuit of greater knowledge. Dr. Norman, thank you for your wealth of knowledge, readiness to help, and continued support.

Special acknowledgements go out to Denise Caldwell. Thank you for everything you have ever done, you have been one of my biggest supporters throughout this program. I cannot even begin to express my gratitude for your assistance, I am forever thankful for you.

Thanks to all my friends and colleagues, the Department of Poultry Science faculty, and staff, for taking a chance on me when I needed it most, and for helping me realize my true passion.

Finally, thank you to my beautiful wife Shelby, my parents Billy & Shannon, my brother Kade, and my niece Lillian. Your love and support over these few years has been the greatest blessing I could have ever asked for.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Christopher Bailey (advisor) and the committee members: Dr. John Carey, Dr. James Allen Byrd, and Dr. Keri Norman under the supervision of the Interim Department Head of Poultry Science at Texas A&M University, Dr. Audrey McElroy. All the work for this dissertation was completed independently by the student in collaboration with Dr. Bailey's laboratory: Dr. Akram ul-Haq, Dr. Hector Leyva-Jimenez, Dr. Akhil Alsadwi, Kimberly Gardner, Yansoon Al-Jumaa, Carl Crittendon, Alexis Thomas, Madalynn Hare, Rachel Schrank, and Micah Osburn.

Special thanks for the assistance on all this research as well as many other projects to Denise Caldwell. Without your assistance, absolutely none of this would have been possible. I am forever grateful for your help throughout my time at Texas A&M.

Funding Sources

The graduate study program was supported by an assistantship provided by the Texas A&M Department of Poultry Science, in collaboration with Phileo by Lesaffre (formerly Phileo Lesaffre Animal Care).

NOMENCLATURE

ABF	Antibiotic Free
ANOVA	Analysis of Variance
APIR	Acute Phase Immune Response
BW	Body Weight
CFU	Colony Forming Units
FCR	Feed Conversion Radio (Feed-to-Gain Ratio)
FWR	Feed to Weight Ratio
GIT	Gastrointestinal Tract
IACUC	Institutional Animal Care and Use Committee
MOS	Mannan-oligosaccharide
PFU	Plaque-forming Units
RCBD	Randomized Complete Block Design
RV	Rappaport Vassiliadis
SB	*Salmonella enterica* serovar Braenderup
SE	*Salmonella enterica* serovar Enteritidis
SI	*Salmonella enterica* serovar Infantis
ST	*Salmonella enterica* serovar Typhimurium
VFD	Veterinary Feed Directive
XLT-4	Xylose Lysine-Tergitol 4
YC	Yeast Culture
YCW	Yeast Cell Wall

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION v

ACKNOWLEDGEMENTS vi

CONTRIBUTORS AND FUNDING SOURCES....................vii

NOMENCLATURE.................... viii

TABLE OF CONTENTS ix

LIST OF FIGURES....................xii

LIST OF TABLES xiii

INTRODUCTION AND LITERATURE REVIEW 1

1.1. Introduction 1
1.2. Literature Review....................4
1.2.1. Salmonella in Broilers....................4
1.2.2. Salmonella in Laying Hens 7
1.2.3. Antibiotics in Poultry Production....................10
1.2.4. Yeast Cell Wall as an Antibiotic Alternative 12
1.2.5. Bacillus as an Antibiotic Alternative....................15

2. EVALUATION OF *SALMONELLA* COLONIZATION OF LAYING HENS FED A PARIETAL YEAST FRACTION WITH HIGH LEVELS OF MANNANS AND β-GLUCANS 18

2.1. Introduction 18
2.2. Materials and Methods....................20
2.2.1. Birds, Diets and Management 20
2.2.2. Prevalence Determination 23
2.2.3. Enumeration Determination 24
2.2.4. Statistical Analysis 25
2.3. Results and Discussion....................25
2.3.1. Ceca & Ovary Prevalence 25

2.3.2. Ceca Enumeration 27
2.3.3. Discussion 28

3. EVALUATION OF *SALMONELLA* eNTERITIDIS COLONIZATION OF LATE PRODUCTION LAYING HENS FED A PARIETAL YEAST FRACTION WITH HIGH LEVELS OF MANNANS AND β-GLUCANS OR BACILLUS PROBIOTICS 31

3.1. Introduction 31
3.2. Materials and Methods 34
3.2.1. Birds, Diets and Management 34
3.2.2. Prevalence Determination 38
3.2.3. Enumeration Determination 39
3.2.4. Statistical Analysis 39
3.3. Results and Discussion 40
3.3.1. Ceca & Ovary Prevalence 40
3.3.2. Ceca Enumeration 41
3.3.3. Discussion 43

4. Comparison of *salmonella* shedding and organ contamination of broiler chickens challenged with *salmonella enterica* serovar typhimurium in birds supplemented with yeast cell wall prebiotics, bacillus probiotics, or bacteriophages....................................45

4.1. Introduction 45
4.2. Materials and Methods 46
4.2.1. Birds, Diets and Management 46
4.2.2. Prevalence Determination 50
4.2.3. Enumeration Determination 51
4.2.4. Statistical Analysis 51
4.3. Results and Discussion 52
4.3.1. Performance Parameters 52
4.3.2. Ceca, Liver & Spleen Prevalence 59
4.3.3. Ceca Enumeration 60
4.3.4. Discussion 62

5. COMPARISON OF *SALMONELLA* SHEDDING AND ORGAN CONTAMINATION OF BROILER CHICKENS CHALLENGED WITH *SALMONELLA ENTERICA* SEROVAR INFANTIS IN BIRDS SUPPLEMENTED WITH YEAST CELL WALL PREBIOTICS, *BACILLUS* PROBIOTICS, OR BACTERIOPHAGES 65

5.1. Introduction 65
5.2. Materials and Methods 66
5.2.1. Birds, Diet and Management 66
5.2.2. Prevalence Determination 69
5.2.3. Enumeration Determination 70

5.2.4. Statistical Analysis 70
5.3. Results and Discussion 71
5.3.1. Performance Parameters 71
5.3.2. Ceca, Liver & Spleen Prevalence 73
5.3.3. Ceca Enumeration 75
5.3.4. Discussion 77

6. Conclusion 80

References 83

LIST OF FIGURES

Page

Figure 2-1 - Young Hen *Salmonella* Trial Schedule 23

Figure 2-2 - Young Hen Cecal *Salmonella* Prevalence (%) across servoars and treatments. 26

Figure 2-3 - Young Hen Ovary *Salmonella* Prevalence (%) across serovars and treatments. 26

Figure 3-1 - Late Production Hens Trial Schedule 37

Figure 3-2 - *Salmonella* Enteritidis Prevalence (%) in Cecal Content across rooms and treatments. 40

Figure 3-3 - *Salmonella* Enteritidis Prevalence (%) in Ovary Samples across rooms and treatments. 41

Figure 4-1 - *Salmonella* Typhimurim Challenged Broilers Trial Schedule 49

Figure 4-2 - Day 14 *Salmonella* Typhimurium Prevalence (%) across sample types and treatments 59

Figure 4-3 - Day 21 *Salmonella* Typhimurium Prevalence (%) across sample types and treatments 60

Figure 5-1- *Salmonella* Infantis Challenged Broilers Trial Schedule 68

Figure 5-2 - Day 14 *Salmonella* Infantis Prevalence (%) across sample types and treatments. 74

Figure 5-3 - Day 21 *Salmonella* Infantis Prevalence (%) across sample types and treatments 75

LIST OF TABLES

Page

Table 2-1 - Early Production Pullet and Hen Diet Composition....................................21

Table 2-2 - Early Production Hen Treatment Groups and Associated *Salmonella* Challenge 23

Table 2-3 - Young Hen *Salmonella* Log_{10} CFU/g in Cecal Content across serovars and treatments. 27

Table 3-1 - Late Production Lohmann Brown Laying Hen Diet 36

Table 3-2 - Late Production Hen Treatment Groups and *Salmonella* Challenge Dose ...37

Table 3-3 – ANOVA Comparing Late Production Hens *Salmonella* Log_{10} CFU/g of Ceca Content....................................42

Table 3-4 – ANOVA Comparing Late Production Laying Hens *Salmonella* Log_{10} CFU/g of Cecal Content in Room 3 43

Table 4-1 - *Salmonella* Typhimurium Challenged Broilers Diet Composition 48

Table 4-2 - *Salmonella* Typhimurium Challenged Broiler Treatment Groups and Product Application Methods....................................49

Table 4-3 – ANOVA comparing Phase Average Bird Body Weight (g) for treatment across days....................................53

Table 4-4 – ANOVA Comparing Average Phase Weight Gain per Bird (g) across days....................................55

Table 4-5 – ANOVA Comparing Average Phase and Cumulative Feed to Gain Ratio Across Days....................................56

Table 4-6 – ANOVA Comparing Average Cumulative Feed to Weight Ratio Across Days 58

Table 4-7 – ANOVA Comparing Day 14 *Salmonella* Typhimurium Enumeration Log_{10} CFU/g of Cecal Content 61

Table 4-8 – ANOVA Comparing Day 21 *Salmonella* Typhimurium Enumeration Log_{10} CFU/g of Cecal Content....................................62

Table 5-1 - *Salmonella* Infantis Challenged Broilers Diet Composition 67

Table 5-2 - *Salmonella* Infantis Challenged Broilers Treatment Groups and Product Application Methods 68

Table 5-3 – ANOVA Comparing Phase Average Body Weight (g) Across Days...........71

Table 5-4 – ANOVA Comparing Phase Average Weight Gain per Bird (g) Across Days 72

Table 5-5 – ANOVA Comparing Phase and Cumulative Average Feed to Gain Ratio Across Days 72

Table 5-6 – ANOVA Comparing Cumulative Average Feed to Weight Ratio Across Days 73

Table 5-7 – ANOVA Comparing Day 14 *Salmonella* Infantis Log_{10} CFU/g of Cecal Content Across Treatments...76

Table 5-8 – ANOVA Comparing Day 21 *Salmonella* Infantis Log_{10} CFU/g of Cecal Content Across Treatments...76

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

The vast majority of improvements in the poultry industry over the last 100 years have come from genetic selection for improved feed efficiency, coupled with a streamlining of the production model, and an improved understanding of poultry nutrition (Athrey, 2020). For more than sixty years, dietary antibiotics have been used in commercial poultry production for improved growth performance and control of infectious diseases (Gadde *et al.,* 2018). While antibiotic usage in conjunction with increasingly struct biosecurity measures may be sufficient for pathogen management in some situations, other situations may require the use of antibiotics in order to effectively treat diseases. In the North American poultry market, several antibiotics have been commonly used to keep up with the intensive production methods. A few of these include bacitracin, salinomycin, tetracycline, and virginiamycin (Diarra and Malouin, 2014). While these antibiotics may continue to be used in poultry diets for disease prevention in certain situations, some antibiotics including tetracycline and virginiamycin now require a prescription for use under the Veterinary Feed Directive program (VFD) and can only be used for the treatment of disease. This program was enacted due to fear that bacteria could develop antimicrobial resistance (AMR) to these drugs that are important for human health. Because of this, their use has decreased in recent years (Khalaifah, 2018). Due to the concern of AMR, and a growing movement by the end consumer towards poultry production without the inclusion of antibiotics,

prebiotics and probiotics have risen in popularity as antibiotic alternatives to fill the gap both in production and disease prevention previously filled by antibiotics.

Probiotics have been explored for decades as a viable alternative to antibiotics and are defined as "a live microbial feed supplement which beneficially affects the host by improving its intestinal balance" (Fuller 1989). In more recent years, probiotics have been redefined as "Direct Fed Microbials (DFM)", which is defined as a source of live, naturally occurring microorganisms which may include bacteria, fungi and yeast (Miles and Bootwalla, 1991). Prebiotics on the other hand are defined as "non-digestible feed ingredients that can have a beneficial action due to selective stimulation of the growth or metabolic activity of a limited number of beneficial intestinal microbiota species" (Gibson and Roberfroid 1995). Probiotics and prebiotics have several proposed beneficial mechanisms in poultry including: (1) maintaining normal intestinal microflora by competitive exclusion; (2) altering metabolism by increasing digestive enzyme activity; (3) stimulating the immune system; and (4) improving feed intake and digestion (Nahashon *et al.,* 1994, Patterson and Burkholder 2003).

One specific sect of antibiotic alternatives that has gained traction in recent years are yeast probiotics and yeast fractions. Yeast probiotics and yeast fractions possess multiple modes-of-action that provide a health benefit to poultry, including the direct binding of toxins and stimulation of the host's immune system (Posadas *et al.* 2017). Yeast prebiotics exhibit similar effects on the host's immune system due to the mannan-oligosaccharides (MOS) obtained from the mannans found in the cell wall of the yeast strain *Saccharomyces cerevisiae* (Spring *et al.,* 2000). Yeast prebiotics exhibit a

competitive binding effect on pathogenic microbes by binding of the type-1 fimbriae to the mannose in the yeast cell wall (Akhtar *et al*., 2016).

In 2019 the U.S. poultry probiotics market topped $80 million and is projected to exceed $125 million USD by 2025 (Ahuja and Mamtani, 2019). While the popularity and promise of prebiotics and probiotics as antibiotic alternatives continues to grow, the major limitation of widespread adoption of these products is the apparent inconsistencies in their expected effects on performance and health benefits to poultry (Ajuwon, 2016). There is also a knowledge gap that exists on the precise mechanisms or mode-of-action of these products, which must be determined to ensure that as their use increases, producers are still able to ensure continued improvements in food safety from farm-to-fork. In order to implement these prebiotic and probiotic products into the marketplace as a predominant player, more in-depth research is needed on the subject matter to appropriately assess their effects in all types of poultry.

The goal of this project is to determine the efficacy of yeast cell wall prebiotics and *Bacillus* probiotics as an alternative to antibiotics as a method of *Salmonella* mitigation in broilers and laying hens. We will accomplish this through the following four specific objectives: 1) evaluate the effects of yeast prebiotics, *Bacillus* probiotics and their combinations as a method of reducing *Salmonella* counts in the ceca of broilers; 2) determine the effects of yeast prebiotics and *Bacillus* probiotics as a method of reducing *Salmonella* counts in the ceca of early production laying hens; 3) determine the effects of yeast prebiotics and *Bacillus* probiotics as a method of reducing *Salmonella* translocation to internal organs including the liver, spleen and ovaries; and 4)

determine the effect of yeast prebiotics and *Bacillus* probiotics as a method of reducing *Salmonella* counts in late production laying hens.

1.2. Literature Review

1.2.1. Salmonella in Broilers

The CDC estimates that annually 1.35 million illnesses and 420 deaths that occur are linked to *Salmonella* in the United States (CDC, 2020). While their numbers are staggering, these illnesses can be caused by a variety of serotypes of *Salmonella*. Monitoring data from the Foodborne Diseases Active Surveillance Network, also known as FoodNet, reported that even in 2019 *Salmonella* was still not under control in the United States. While incidence varies by serotype, *Salmonella* Enteritidis is still the most common cause of *Salmonella* infections in the United States and has been since 2007. *Salmonella* Infantis related infections also increased in 2019 due to a highly resistant strain that was linked to chicken products (CDC, 2019).

While more than 2,500 different serotypes of *Salmonella* have been identified, less than 100 of those account for illness or infections in humans (CDC, 2020). The largest issues with *Salmonella* stem from the product chain, in which control is needed at each of the three overarching points reached from production to consumption. The first and largest issue stems from the poultry production process, in which *Salmonella* resides both inside the bird as well as the housing system as a ubiquitous organism, making it difficult to easily control. The second issues stem from the cold-chain process, in which products must be kept at temperatures meant to hinder *Salmonella* growth in raw meat

products. The final issue and likely most common stems from consumer education on food handling and preparation practices (Landinez, 2019).

In order to effectively control *Salmonella* infections linked to poultry products, as in most cases, control should start at the source. If *Salmonella* can be controlled or reduced at the source, then the interventions already in place in the processing plants will be able to work more effectively.

The avian gastrointestinal tract is made of a diverse and dynamic population of microorganisms creating a symbiotic relationship with the host. This relationship is importantly mutualistic for host nutrition, metabolism and immunity (Al-Khalaifah, 2019). However, this dynamic population can change rapidly when exposed to the wrong type of pathogen, ultimately resulting in poor performance, intestinal stress, and in extreme situations, mortality.

For many years it has been believed and commonly noted that chickens are considered sterile at hatch, their gastrointestinal tract completely untouched. However, in recent years it has been shown that several species of bacteria can colonize the cecum in late embryonic development (Pedroso, 2008; Kizenwetter, 2008). In terms of overall population density and diversity, these only represent a fraction of the bacteria that the chick will contact in its' lifetime. Consequently, chickens are far more sensitive to *Salmonella* infection in the first few weeks of life due to delayed onset of flora development (Schneitz *et al.,* 2004).

As seen in the heat map from Ballou *et al.,* 2016, there is a significant increase in the microbial population density and diversity as the birds age, specifically increasing

later in life, after approximately the first week of life. Due to this delayed development, *Salmonella* exposure early in life allows for a swift and barrier-less entry into the intestinal tract. Without minimal bacteria present in the intestinal tract, *Salmonella* and other pathogens alike can colonize with little resistance.

Although *Salmonella* is considered to be a ubiquitous organism in the intestinal tract of poultry, excessive amounts of this bacteria as well as many others can trigger innate immune responses that can cause undesirable effects. This is especially important in young birds that lack a developed intestinal flora; with lower levels of intestinal flora the bird has less ability to resist pathogens such as *Salmonella*, resulting in an even greater stress on the bird. These undesirable effects stem from inflammation of the intestinal tract, which is a key portion of the acute phase immune response (APIR). This acute immune response, as well as normal maintenance, growth and development of birds are all linked at a singular point, which is the nutrients that bird's intake through feed consumption. When APIR is activated due to an intestinal pathogen, those nutrients needed for growth and maintenance are diverted away to the immune response. This not only causes birds to have reduced nutrients available for growth and maintenance, but also results in suppression of appetite, and in severe cases, catabolism of host tissues (Broom and Kogut, 2019). The specific "cost" of this APIR is hard to quantify, as it depends on multiple factors including pathogen exposure and virulence level, as well as other environmental factors and dietary components (Sandberg *et al*., 2006). In order to combat the pathogens that are the root of the problem, the intentional manipulation of the intestinal microbiota through several methods has surfaced in the interest of

preventing intestinal infection, improving overall gut health, and promoting broiler performance (Chambers *et al.,* 2011).

Intestinal manipulation is a term not often used in the study of pre- and probiotics, as it is a term that can easily be misunderstood. When considering prebiotics and probiotics however, it is something that nutritionists and others in the poultry industry are doing every day. Many pre- and probiotics stimulate the growth and production of specific bacteria in the intestinal tract. With delayed flora development in poultry, and most additives being supplemented from day-of-age, the commercial poultry industry is regularly manipulating the intestinal microbiota population.

1.2.2. Salmonella in Laying Hens

Contamination of eggs and eggshells have been identified as one of the major causes of foodborne *Salmonella* infections (Howard *et al*., 2012). In the United States alone between 1985 and 2002, 53% of all *Salmonella* cases reported to the Centers for Disease Control and Prevention (CDC) were attributed to contamination of eggs and egg products (FDA, 2009). The top two causative *Salmonella* serovars found to be contaminating eggs and eggshells are *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium (Galis *et al*., 2013). Both of these serotypes are able to colonize the ovary and oviduct of laying hens, making them a major concern as a causative agent of foodborne illness (Whiley and Ross, 2015).The largest issue with controlling *Salmonella* in laying hens, regardless of serovar, is the high number of variables affecting colonization and contamination. *Salmonella* sp. can colonize multiple organs in poultry with little to no clinical signs in commercial birds, which can result in

unknown contamination of eggs and chicken meat (Gast *et al*., 2013). While not all of these species will colonize in the same way, or at the same level, those serotypes of concern become an even greater concern in laying hens.

Chicken eggs are readily contaminated by *Salmonella* Enteritidis of enteric origin through two primary routes; either by penetration of the outer shell after oviposition or by contamination of egg products prior to shell formation in the reproductive tract (Krueger *et al.,* 2020; Gantois *et al.,* 2009).To add to this problem, there has been a shift over the past several years towards more natural methods of production, including cage free and free-range production. Coupled with this, there has also been a shift in consumer eating habits with increasing demand for unprocessed foods (Broglia and Kapel, 2011, Krester *et al.,* 2014). This increasing popularity often spills over to unprocessed home-made foods containing raw eggs such as mayonnaise, and egg-based desserts such as ice cream that can potentially increase the risk for salmonellosis (Krester *et al.,* 2014, Fearnley *et al.,* 2011, Mitchell *et al.,* 1989). While field evidence suggests that vaccination of layers and broiler breeders provides at least partial protection against *Salmonella* Enteritidis, and can reduce loads and/or prevalence under commercial conditions in meat and egg production, foodborne illness statistics still show that there is considerable room for improvement (Hofacre *et al.,* 2018).

Several factors have been identified as possible variables that can affect *Salmonella* prevalence in laying hen flocks, however, with conflicting evidence on each point. Those include egg production processes, environmental contamination, direct contamination, and storage/transport contamination. Each has the potential to be an area

of infection or an area of control; however, in order to effectively reduce *Salmonella* contamination a cohesive approach is needed. Studies comparing *Salmonella* contamination in varying egg production processes show conflicting evidence. This is due to the complexity of confounding factors and variables including flock size, flock age and stress caused by rehousing, weather, transportation, initiation of lay and molting (Holt *et al.,* 2011). These results become even more complicated when considering the factors that affect *Salmonella* contamination within the ovaries and indirect contamination from the environment (Gast *et al.,* 2014). These factors will also differ between direct contamination within the bird and indirect contamination from environmental contamination outside of the bird.

When considering contamination at its two most basic points, both direct and indirect contamination can be considered equally as points of concern. Direct contamination occurs primarily inside of the egg, during its formation in the reproductive tract, prior to shell formation. Two serovars are of the greatest concern for direct contamination; *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Typhimurium (ST). Both *Salmonella* Enteritidis and *Salmonella* Typhimurium are known to colonize the reproductive tract of laying hens (Gantois *et al.,* 2008), however *Salmonella* Enteritidis is more commonly isolated from the internal contents of eggs due to its ability to better adhere to the reproductive mucosa than *Salmonella* Typhimurium (Wales and Davies, 2011). Publications regarding housing effects on direct contamination are varied, with results not showing a concise answer as

to if and how housing affects this internal contamination (Gast *et al.,* 2014, De Vylder *et al.,* 2011).

Indirect contamination on the other hand occurs after oviposition. In this case, indirect contamination can also be interchanged synonymously with environmental contamination. Environmental contamination has the potential to occur throughout the rest of the egg's journey from the point it is laid until it is consumed. However, the most common place that environmental contamination occurs would be on farm, as farm prevalence of *Salmonella* is likely to be significantly higher than a standard U.S. household kitchen. Interestingly though, several studies suggest that environmental sources of contamination of *Salmonella* in free-range housing are lower than cage housing (Namata *et al.,* 2008, Wales *et al.,* 2007). AS noted in Wales *et al.,* (2007), samples from cage housing were found to have higher levels of *Salmonella* (19%) when compared to free-range housing (10%). While this is not always the case, these results could likely be due to increased floor space per bird, as well as ventilation practices and other unknown environmental confounders.

1.2.3. Antibiotics in Poultry Production

For more than sixty years, dietary antibiotics have been commonly used in commercial poultry production for improved growth performance and disease protection (Gadde *et al.,* 2018). In the North American Poultry market specifically, several antibiotics have been used in order to keep up with the intensive production methods. These antibiotics include bacitracin, salinomycin, tetracycline and virginiamycin (Diarra

and Malouin, 2014). However, with a growing consumer push towards production without the inclusion of antibiotics, alternatives have been taking their place slowly over the last few decades. The largest switch towards antibiotic free (ABF) poultry production has occurred in the last decade, with the movement being pushed primarily due to the implementation of the Veterinary Feed Directive (VFD) in 2017 (21 C.F.R. § 514). The VFD final rule created a category of feed known as veterinary feed directive drugs. These VFD drugs are those which in order to be used in the process of animal production are limited to use under the professional supervision of a licensed veterinarian (Veterinary Feed Directive Final Rule, 2015). This rule applies to those drugs which are classified as "essential for human health", and the goal of this ruling was to hopefully attempt to limit the proliferation of the number of antibiotic resistant strains of bacteria and pathogens that are of concern to human health.

While some of the common antibiotics are still legal without the use of a prescription, some antibiotics including tetracycline and virginiamycin now require a prescription for use under the VFD program and can only be used specifically for the treatment of disease. Thus, alternatives such as pre- and probiotics have stepped up to fill the gap left in the wake of the VFD.

The concept of probiotics initially came to fruition as a method for competitively excluding pathogens from the chicken's intestinal tract (Nurmi *et al.,* 1973). Although the ability of pre- and probiotics to improve performance as well as reduce foodborne pathogens is widely reported, their overall effectiveness in-vivo with poultry is mixed.

Often, the positive benefits of a few of these additives are improperly attributed to all prebiotics across the spectrum (Froebel et al., 2019).

1.2.4. Yeast Cell Wall as an Antibiotic Alternative

Collectively, compounds classified as prebiotics have been defined as non-digestible food ingredients that promote one or more number of beneficial bacteria in the gastrointestinal tract, enhance gastrointestinal health, and potentially improve host health (Gibson and Roberfroid, 1995). In order to be classified as a prebiotic, there are three main qualifying factors, including; (1) the prebiotic cannot be hydrolyzed or absorbed in the upper GIT; (2) the prebiotic must serve as a selective nutrient source for the growth and/or metabolic activity of beneficial microbes in the GIT; and (3) the prebiotic must induce luminal or other systemic physiological responses benefitting the host (Ricke *et al.,* 2020).

Yeast products have been used in poultry production for several decades, however, their exact mode of action is not fully understood. The primary driver behind this lack of knowledge stems from a lack of understanding, and more specifically, a lack of perceived differentiation between varying yeast products. Not all yeast products are the same, and their differences stem from their composition. Because of this, not all yeast products can be considered as mannan oligosaccharides (MOS), and MOS is not an accurate description of YCW products. Prebiotic MOS is only a portion of the cell wall of *Saccharomyces cerevisiae*, which contains a mixture of oligosaccharides, β-glucans, and mannoproteins (Fowler *et al.,* 2015).

The cell wall of yeast and other fungi determines the shape and integrity of the organism during growth and division. When considering yeast cell wall, there are three main groups of polysaccharides that form the cell wall; polymers of mannose (mannoproteins), polymers of glucose (β-glucans), and polymers of *N*-acetylglucosamine (chitin) (Figure 1.2). When considering *Saccharomyces cerevisiae* specifically, the mannoproteins comprise approximately 35-40% of the cell wall, β-glucans comprise 55-65%, and chitin accounts for only 1-2% of the cell wall by weight. (Aguilar-Uscanga and Francois, 2003).

In order to effectively understand how these YCW prebiotics work, we must first understand the two main components that affect animal health. Yeast β-glucans are a polysaccharide extracted from the cell wall of *Saccharomyces cerevisiae* and have been widely studied and shown to possess immunomodulatory activities involving receptor recognition, and are most effective at enhancing host protective immunity against infectious agents (Volmon *et al.,* 2008, Saleh *et al.,* 2015, Shao *et al.,* 2016). The other portion of the cell wall that is important to animal health would be the mannoproteins, or "mannans".

There is evidence that live yeasts such as *Saccharomyces cerevisiae* and their derivatives (extracts and YCW) can be effective in pathogen exclusion and promotion of intestinal health, as well as improvements in zootechnical performance parameters (Montzouris *et al.,* 2017).

One of the most common and important pathogens that these yeast products are used to control is *Salmonella*. The effectiveness of these products to control *Salmonella*

stems from the mannan portion of the yeast cell wall. This mannan protein assists in the control of *Salmonella* through direct agglutination, in which *Salmonella* possessing a type-1 fimbriae directly bind to the mannoproteins in the YCW, thus preventing adhesion to the GIT (Lourenco *et al.,* 2016). This, combined with a stimulation of the immune system attributed to the β-glucans in the YCW leads to an overall healthier bird than those left untreated in a challenged environment (Posadas *et al.,* 2017).

The use of YCW with high concentrations of mannans and β-glucans also shows other benefits that assist YCW in playing a key role as an antibiotic alternative. Several publications have shown that, on top of pathogen binding, YCW supplementation results in increases in villus height, width and area compared to birds without supplementation when challenged with *Salmonella* (Abudabos, 2020). This is especially important when considering nutrient digestion in a challenged situation. When birds are challenged with *Salmonella* or other intestinal pathogens normally found in chicken houses, they are less likely to fully utilize energy from the feed they consume, thus decreasing zootechnical performance.

While the benefits attributed to yeast and YCW are known to be true due to the great volume of research on the subject, it is not well understood how variation among products and their compositions affects their efficacy in commercial production. An ideal product composition has yet to be defined, and it has also yet to be fully understood how pathogen strain affects their interaction. Variability on this has been shown in in-vitro models as described by Posadas (2017). However, the results of this study and

others that have been conducted similarly are highly debatable, due to the inconsistent results in-vitro, as well as a great deal of variation between in-vitro and in-vivo results.

1.2.5. Bacillus as an Antibiotic Alternative

Bacillus feed additives unlike YCW fall into the category of probiotics, which are defined by The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) as "live organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). This definition encompasses a wide range of products, including *Bacillus* spp. as well as *Saccharomyces cerevisiae*, the live yeast product that is used in the production of YCW. Several probiotic strains have shown promise in altering activity in the intestinal tract of poultry through competitive exclusion (Kabir, 2009). These probiotics also have beneficial effects through facilitation of cross-talk between the gut microbes and the immune system, resulting in a decrease in gut inflammation (Al-Khalaifah, 2018).

Probiotics in the form of bacteria have been explored as alternatives to antibiotics in ABF production systems, with mixed results. When considering probiotics, two general classifications can be considered: Spore-forming or non-spore-forming. The inclusion of non-spore-forming bacteria in poultry diets causes challenges in terms of shelf life as well as stability during the pelleting process (Shanmugasundaram *et al.,* 2020) so their use in the commercial poultry industry is somewhat limited. Spore-forming bacilli produce secretory proteins and antimicrobial compounds, and when

coupled with their tolerance of the conditions of the gastrointestinal tract makes them ideal candidates as a food industry probiotic (Elshaghabee *et al.,* 2017).

Two of the commonly used *Bacillus* strains in the poultry industry are *Bacillus subtilis* and *Bacillus lichenformis*. Both species are known to survive high temperatures, much higher than those sustained in the pelleting process, and both are referenced as being used to control specific pathogens that commonly plague poultry. *Bacillus subtilis* is documented showing stimulation of lactic acid bacteria as well as reductions in *Salmonella* colonization (Knap *et al.,* 2011). *Bacillus lichenformis* has been studied as a growth promoter and for the control of necrotic enteritis (Liu *et al.,* 2012). Another strain of *Bacillus* used in certain blends of products for commercial poultry is *Bacillus amyloliquefaciens*. This strain of *Bacillus*, when in spore form, is also heat resistant to well above pelleting temperatures (>170°F) and can survive the harsh conditions of the gastrointestinal tract. It has also been shown to provide other benefits such as the production of bacteriocins including subtilin and barnase, which act as antibacterials against several pathogenic microorganisms (Lee *et al.,* 2008). Similar to the other two strains, it also has been documented as a growth promoter (Ahmed *et al.,* 2014, Latorre *et al.,* 2015) and has shown positive effects with regard to production parameters in laying hens (Tang *et al.,* 2017).

Singularly, these strains can provide adequate benefits to commercial poultry to justify their inclusion, and several commercially available products use only singular strains of *Bacillus*. However, in some cases based on the pathogen that the birds are experiencing, a singular strain may not be enough to provide ample benefit. Because of this, many

products utilize a combination of two or more of these strains in order to achieve a multi-faceted approach towards preventing multiple diseases at the same time. Hence, the use of a blend of *Bacillus* products, or a combination *Bacillus* + yeast culture/yeast cell wall is implemented in many commercial poultry operations in the United States.

Due to *Salmonella*'s prevalence throughout poultry and the commercial poultry production process, alternative methods of controlling this pathogen are of the utmost importance. The studies encompassed in this dissertation seek to evaluate the efficacy of two commercially available antibiotic alternatives; a YCW prebiotic (Safmannan™) and a *Bacillus* blend probiotic (Microsaf™). This research was conducted in four separate experiments, two of which focused on their efficacy in broilers (chapters III and IV), and two focused on laying hens, both in early and late production (chapters I and II).

2. EVALUATION OF *SALMONELLA* COLONIZATION OF LAYING HENS FED A PARIETAL YEAST FRACTION WITH HIGH LEVELS OF MANNANS AND Β-GLUCANS

2.1. Introduction

Salmonella infections and illness around the world continue to be a concern, and the World Health Organization ranks it as one of the four key causes of diarrheal illness globally (WHO, 2018). *Salmonella*, as well as several other organisms are commonly considered ubiquitous within poultry due to being commonly found in the gastrointestinal tract (GIT), however they exhibit far greater pathogenicity when found in humans. In the last few years, outbreaks have continued to increase, with multiple outbreaks found stemming from live poultry and poultry products, including a recent outbreak of *Salmonella enterica* serovar Infantis (SI) linked to raw poultry across 32 states. In a three year period from 2017-2019, two of the largest egg recalls in the history of the commercial egg industry also took place involving *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Braenderup (SB). While *Salmonella* Enteritidis continues to remain atop the list of disease causing serovars in the United Staes, *Salmonella enterica* serovar Typhimurium remains number two. These two serovars are considered to be responsible for over half of all human foodborne *Salmonella* infections (CDC, 2018). For the most part, the focus has been on infection from poultry and poultry products as the source, due to the commonality of proliferation of *Salmonella* in poultry flocks. Prevalence surveys in some cases have reported as high as 49% prevalence of *Salmonella* on some laying hen farms (Hulaj *et al.,* 2016).

While colonization of *Salmonella* in the intestinal tract of poultry is common, and typically does not cause infection at a clinical level in many flocks, *Salmonella*'s prevalence in poultry meat and eggs becomes a specific concern in the processing plants. In the United States, the United States Department of Agriculture (USDA) sets performance standards for *Salmonella* prevalence within processing plants, which are divided into three categories. Plants that fail in regards to *Salmonella* prevalence more than 50% of the time fall into Category 3. In current standards, the allowable prevalence of *Salmonella* in poultry meat and egg products is 7 out of 52 samples (13.5%). As of November 28th, 2020, 12.3% of all United States chicken plants producing young chicken carcasses are ranked in Category 3 (FSIS, 2020). In the European Union (EU), the incidence of salmonellosis attributed to egg and egg products in 2011 reached 65%, despite poultry meat having a higher prevalence than eggs tested, indicating the high possibility of consumption of raw egg products (EFSA, 2011).

Eggs are widely considered to be one of the most nutritious and cost-effective sources of protein, and this has led to their expanded production in developing nations around the world. Per capita annual consumption in the United States reached an estimated 290 eggs in 2019. This consumption number has grown rapidly in recent years, with an indicated increase of over 45 eggs per capita over the last decade (Statista, 2020), and the existing U.S. layer flock reached over 340 million hens as of December 2019 (United Egg Producers, 2019).

In order to effectively combat these challenges, with a growing consumer movement towards production of meat and other animal products without the use of

antibiotics, alternative methods of pathogen control must be explored. *Salmonella* is especially of concern due to its growing prevalence linked to meat and egg products. Thus, this study was conducted to evaluate the efficacy of a parietal yeast fraction (Safmannan™) and its ability to control *Salmonella* in early production hens.

2.2. Materials and Methods

2.2.1. Birds, Diets and Management

This study was conducted in two phases; The first phase of the study was conducted at the Texas A&M Poultry Research Center in College Station, TX. A total of 90 Hy-Line W-36 replacement pullets were obtained from a commercial facility and transferred to the Texas A&M Poultry Research Center. Birds were divided into 2 groups of 45 birds each and housed in large floor pens equipped with hanging feeders and nipple drinkers. Birds were fed a basal pullet grower diet formulated to HyLine W-36 nutritional recommendations, which was divided into two batches. The first batch remained as a basal diet, which serves as the control group, while the second batch was supplemented with a proprietary parietal yeast fraction (Safmannan™) derived from the cell wall of *Sachharomyces cerevisiae* at 500ppm. At 17 weeks of age, both treatment groups were provided a layer diet formulated to HyLine W-36 recommendations (Table 2-1) (Hy-Line, 2020).

Table 2-1 - Early Production Pullet and Hen Diet Composition

Phase 1 – Pullet Diet		Phase 2 – Laying Hen Diet	
Ingredients	Percentage	Ingredients	Percentage
Corn	75.66	Corn	50.22
Dehulled Soybean Meal	18.97	Dehulled Soybean meal	29.88
DL-Methionine	0.11	DL-Methionine	1.31
L-Threonine	0.05	L-Threonine	0.05
Lysine HCL	0.02	Soybean Oil	4.88
Limestone	0.83	Limestone	11.68
Mono Di-Cal Phosphorus	3.58	Mono Di-Cal Phosphorus	2.23
Salt	0.21	Salt	2.16
Sodium Bicarbonate	0.26	Trace Minerals[1]	0.05
Trace Minerals[1]	0.05	Trace Vitamins[2]	0.25
Trace Vitamins[2]	0.25		

Vitamin Premix provided by DSM Animal Nutrition and Health. [1]Per pound of premix; Cu: Copper minimum 1.40%, I: Iodine minimum 800.0ppm, FE: Iron minimum 12.00%, Mm: Manganese minimum 12.00%, Zn: Zinc minimum 12.00%, [2]Per pound of premix; Vitamin A: 4,000,000 IU, Vitamin D3: 1,400,000 IU, Vitamin E: 16,666 IU, Vitamin B12: 6mg, Riboflavin (B2): 2166mg, Niacin (B3): 16,666mg, d-pantothenic acid (B5): 7334mg, Choline: 47383mg, Menadione: 534mg, Folic acid (B9):634mg, Pyridoxine (B6): 2,600mg, Thiamine (B1): 1,066mg, d-Biotin (B7): 200mg

The second phase of the study was conducted at the USDA-Agricultural Research Service (USDA-ARS) facilities in College Station, TX, and received approval from the USDA-ARS Institutional Animal Care and Use Committee (IACUC No. 2018-

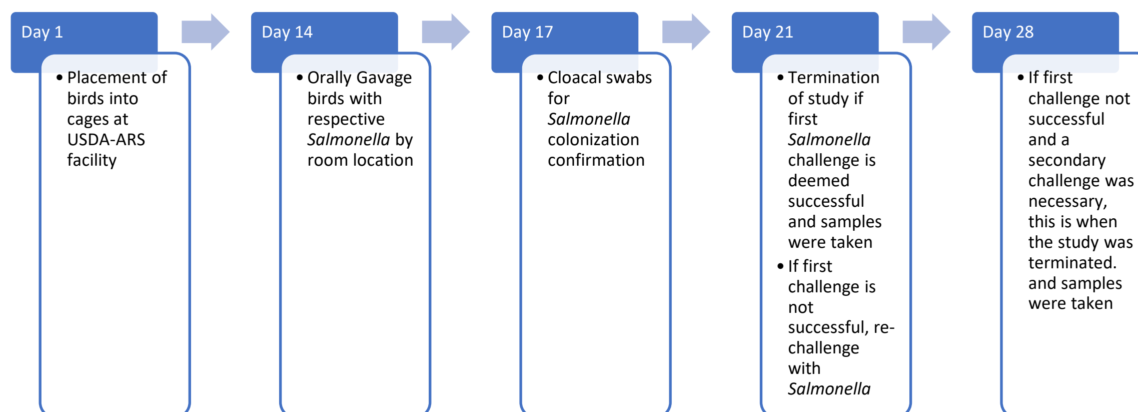
005). The second phase of the study began when the birds were 20 weeks of age, and birds were transferred from the Texas A&M Poultry Research Center to the USDA-Agricultural Research Service facility in College Station, TX. Birds were allocated into three environmentally controlled rearing rooms, each equipped with two stainless steel A-frame layer cages. Each room housed a total of 24 birds, with one bird per cage (12 per treatment) Birds were observed daily for general flock condition, feed, water, and egg production. Egg production factors were recorded daily.

After a two-week acclimation period (22 weeks-of-age), birds were challenged with their respective *Salmonella* strains via a 3mL oral gavage at the levels listed in Table 2-2. Three days after challenging with their respective *Salmonella*, a cloacal swab was taken from each bird and direct plated onto XLT-4 agar to ensure that the challenge was successful. If the prevalence of *Salmonella* was found to be less than 25% within a given room then the challenge would be considered unsuccessful and the birds would be re-challenged with their respective *Salmonella* strains four days later (7 days post initial challenge). If the first *Salmonella* challenge was found to be successful (>25% positive prevalence), one week after initially being challenged with *Salmonella*, birds were humanely euthanized, and samples including ceca and ovaries taken for further analysis.

Table 2-2 - Early Production Hen Treatment Groups and Associated *Salmonella* Challenge

	Room 1	Room 2	Room 3
Salmonella Challenge	*Salmonella* Enteritidis	*Salmonella* Typhimurium	*Salmonella* Braenderup
Control	12 birds	12 birds	12 birds
Safmannan	12 birds	12 birds	12 birds
Dosage Given	1.05×10^9 CFU/mL	2.9×10^9 CFU/mL	1.25×10^9 CFU/mL

Figure 2-1 - Young Hen *Salmonella* Trial Schedule



2.2.2. Prevalence Determination

Ceca and ovary samples taken from birds at termination were divided and split between two different culturing methodologies. For ceca samples, one cecum was placed into a conical tube containing Rappaport Vassiliadis (RV) (Difco, Franklin Lakes, NJ) broth as a method of enrichment for determination of *Salmonella* prevalence. For ovary

samples, the ovaries were weighed and divided in half according to weight. Half of the ovary was placed into a conical tube containing RV broth. Samples were homogenized by shaking and then incubated at 42°C for 24 hours. After 24 hours of incubation, samples were again homogenized by shaking, and a sterile loop was used to plate a sample onto Xylose Lysine-Tergitol 4 (XLT-4) agar (Hardy Diagnostics, Santa Maria, CA) containing novobiocin (20mg/mL) and naladixic acid (25mg/mL) for use as a selective growth media. Plates were then incubated at 37°C for 24 hours, and samples were deemed positive by visual identification of colonies on the plate. Colonies were positively identified as *Salmonella* if the colony presents as black or black-centered circular colony after 24 hours of incubation at 37°C.

2.2.3. Enumeration Determination

For ceca and ovary counts, the other ceca and remaining ovary samples were diluted weight to volume using a 10x dilution series resulting in dilutions of 1:10 to 1:10,000, of which 0.1mL was plated onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) for use as a selective growth media using a spread plating method. Enumeration counts were determined by visual identification of colonies after 48 hours of incubation. Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 48 hours of incubation at 37°C.

2.2.4. Statistical Analysis

All data obtained during this experiment were analyzed using a one-way Analysis of Variance (ANOVA) and means separated using students t-test were considered statistically significantly different at $p \leq 0.05$. Bonferroni multiple comparisons were used to make all pairwise comparisons for statistically significant ANOVA ($p \leq 0.05$) results. Mean and standard deviation values were used in the determination of outliers as well as for comparisons between treatment groups. Enumeration values were log transformed prior to analysis. These analyses were conducted for each of the sampling time points noted in Figure 2.1 All data were analyzed using STATA v.16.1 (StataCorp, College Station, TX).

2.3. Results and Discussion

2.3.1. Ceca & Ovary Prevalence

Samples obtained in this experiment were taken at termination of the study and *Salmonella* prevalence was determined by the methods specified previously. In reviewing the ceca prevalence data (Figure 2-2), although not significant, small decreases in ceca prevalence were noted in the *Salmonella* Typhimurium (16%) and the *Salmonella* Braenderup (9%) challenged groups with the inclusion of Safmannan YCW supplement. No differences were observed in the *Salmonella* Enteritidis challenge.

In reviewing the ovary prevalence data, although not significant, the Safmannan YCW treatment resulted in a nominal reduction in the *Salmonella* Enteritidis and

Salmonella Typhimurium challenge groups. The *Salmonella* Braenderup challenge group exhibited no ovary colonization in either treatment group (Figure 2-3).

Figure 2-2 - Young Hen Cecal *Salmonella* Prevalence (%) across servoars and treatments.

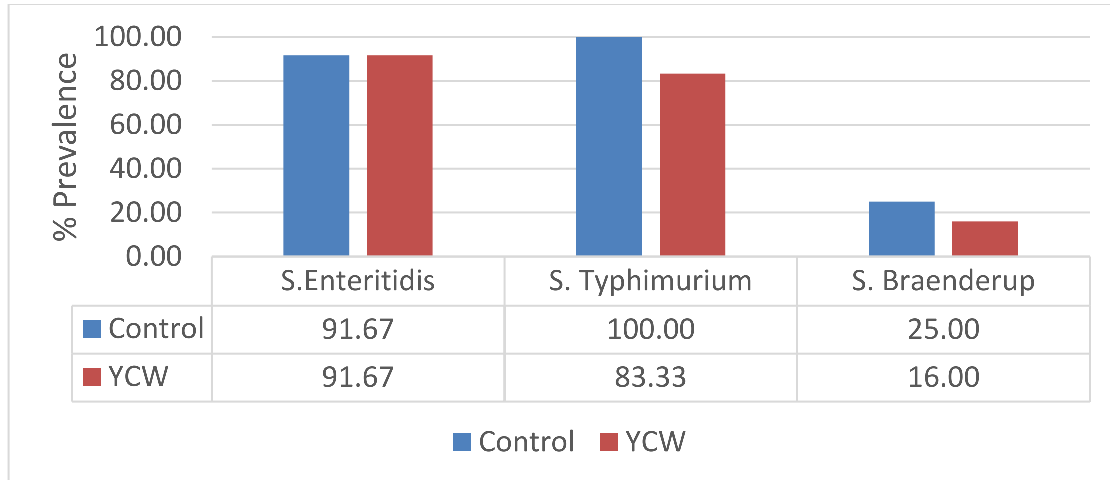
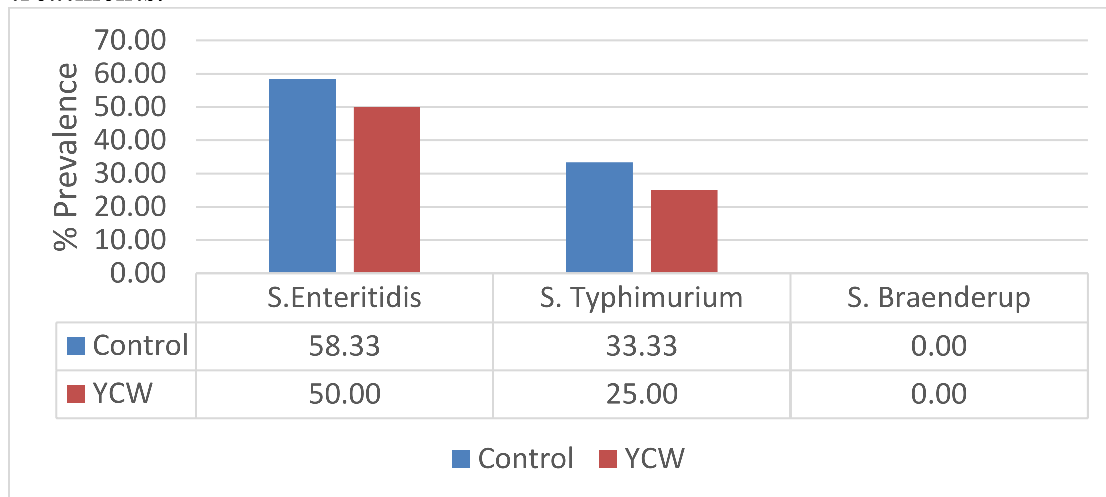


Figure 2-3 - Young Hen Ovary *Salmonella* Prevalence (%) across serovars and treatments.



2.3.2. Ceca Enumeration

In this study, the *S.*Enteritidis challenge group showed no reduction in ceca colonization when treated with the Safmannan YCW treatment. While not statistically significant, YCW supplementation did reduce the colonization of *S.*Braenderup by approximately 0.5 logs compared to the control treatment. In the *S.*Typhimurium challenged group, YCW supplementation did significantly reduce *Salmonella* colonization compared to the control group (p=0.033), with a >1.8 Log_{10} reduction. This reduction is both statistically significant, and biologically relevant from a food safety standpoint (Table 2-3).

Table 2-3 - Young Hen *Salmonella* Log_{10} CFU/g in Cecal Content across serovars and treatments.

Treatment	Challenge	Log_{10} CFU/g	Std. Err.	95% Conf. Interval
Control	*S.*Enteritidis	4.27	0.614	[3.001, 5.547]
Safmannan	*S.*Enteritidis	4.69	0.614	[3.415, 5.960]
Control	*S.*Typhimurium	4.49	0.568	[3.312, 5.667]
Safmannan	*S.*Typhimurium	2.66	0.568	[1.483, 3.838]
Control	*S.*Braenderup	1.02	0.473	[0.045, 2.005]
Safmannan	*S.*Braenderup	0.53	0.473	[-0.455, 1.505]
Treatment Comparison		Challenge		Bonferroni P>|t|
Safmannan vs Control		*S.*Enteritidis		0.638
Safmannan vs Control		*S.*Typhimurium		0.033
Safmannan vs Control		*S.*Braenderup		0.463

2.3.3. Discussion

Interestingly, minimal colonization was seen in the group subjected to the *Salmonella* Braenderup challenge when considering prevalence and enumeration compared to the other two challenge groups. *S.*Braenderup is considered more of an environmental pathogen rather than a GIT strain when considering poultry due to low colonization in the GIT/ovary. This is further validated by the statement from the CDC outbreak of *S.*Braenderup traced to shell eggs in 2018, which stated that the *S.*Braenderup identification was made from environmental samples at the farm (CDC, 2018). The article failed to reference if the *Salmonella* was found to contaminate the exterior shell only or if both shell and interior of eggs was contaminated. However, in the case of *S.*Braenderup, the YCW treatment was able to slightly reduce prevalence in the ceca, as well as a 0.5 Log_{10} reduction when compared to the control treatment.

Contradictory to other publications on the subject, the YCW treatment in this study failed to reduce *S.*Enteritidis counts or prevalence in the ceca, or prevalence in the ovary (Price *et al.,* 2020, Hofacre *et al.,* 2018). Due to the cage environment that the birds were housed in, it would be highly unlikely that coprophagy occurred, which would potentially skew the counts through reingestion of high doses of *S.*Enteritidis being shed through the feces. Considering the number of publications that experienced alternative results with supplementation of the specific YCW used in this experiment and other YCW products, as well as the controlled environment that the birds were in, it is reasonable to consider that exterior factors likely affected the validity of these results.

This could have been due to dosing issues either with the oral gavage, improper plating for determining the amount of *Salmonella* given, or other undetermined factors.

In this experiment, the *S.*Typhimurium challenged group is where the greatest changes were seen with the supplementation of YCW in the diet. Reductions in both ceca and ovary *Salmonella* prevalence favored the YCW treatment, which also translated over to cecal load. In the case of cecal *Salmonella* load, a statistically significant ($p=0.033$) reduction in *Salmonella* was seen when compared to the control treatment. YCW supplementation in the diet resulted in a >1.8 $\log_{10}$ reduction in *Salmonella* load, which was both statistically significant, and biologically relevant. While *S.*Typhimurium is rarely a problem in the internal contamination of eggs, it can become a problem when considering external contamination of shell eggs. Therefore, products that can effectively control *S.*Typhimurium in the intestinal tract and reduce the prevalence/load before encountering the exterior of eggs are extremely vital.

While this study showed some benefits with the supplementation of YCW in the diet, statistically significant reductions were only seen in the *S.*Typhimurium challenged group. This variation between challenge groups likely stems from several factors including the small size of the study, which limits the ability to view significance overall. The results of this study vary based on the strain of *Salmonella* used. This is to be expected based on previous results showing variability in binding affinity in-vitro of Saffmannan YCW (Posadas *et al.,* 2017). However, this study did provide positive results for controlling pathogens that are not as commonly studied in laying hens such as *S.*Typhimurium and *S.*Braenderup. This study also provided a direction for future

research for the control of *S.*Enteritidis, proving that a larger and more focused study is needed to determine whether this specific YCW possesses benefits in the control of *S.*Enteritidis in laying hens.

3. EVALUATION OF *SALMONELLA* ENTERITIDIS COLONIZATION OF LATE PRODUCTION LAYING HENS FED A PARIETAL YEAST FRACTION WITH HIGH LEVELS OF MANNANS AND B-GLUCANS OR BACILLUS PROBIOTICS

3.1. Introduction

Salmonellosis is currently one of the leading causes of foodborne illness in the United States and is responsible for over 26,500 hospitalizations and 400 deaths annually (CDC, 2020). Salmonellosis is caused by *Salmonella* enterica serovars and is typically accompanied by fever, stomach cramps and diarrhea. Although most people recover within a weeks' time, this is not always the case, especially for those who are at severe risk for illness including those with compromised immune systems, elderly patients over 65 years of age, and infants under 1 year of age. Infection typically occurs from consumption of food or water that is contaminated by animals and/or their feces (EFSA, 2014). Foodnet rankings in 2019 show *Salmonella* as the second leading cause of foodborne illness, causing approximately 17% of all cases, second only to *Campylobacter* (Foodnet, 2019).

Salmonella is a gram-negative facultative anaerobe with 2500+ known serovars, however only approximately 100 of these are known to cause disease in humans (Underwood *et al.,*2015). *Salmonella*'s ability to pass through the hostile environment of the GIT allows them the ability to invade mucosal and epithelial cells. Their uptake to macrophages and invasion of epithelial cells can result in the triggering of inflammation through the release of proinflammatory cytokines (Gianella, 1979). Because most

Salmonella enter the body orally, there are several defenses in place in order to prevent their colonization including the acidic environment of the stomach, peristaltic motility of the intestines, and the competitive microflora already present in the intestine. However, in cases where the normal microflora of the intestine become disrupted, *Salmonella* can more easily colonize and proliferate, resulting in an increased population that is hard to control (Gianella *et al.,*1973, Finlay *et al.,* 1992).

Salmonella Enteritidis (SE) has been the most commonly isolated serovar linked to reported illness since 2007 (CDC, 2020). Salmonellosis cases linked to this serovar have often been associated with the consumption of poultry and poultry products, and more specifically, eggs and egg containing foods (Gast *et al.,*2004). Due to the virulent genes that it possesses, SE can easily survive through the GIT, allowing it to multiply in the intestine through macrophages, ultimately migrating to and colonizing the ovaries and preovulatory follicles in laying hens (Velge *et al.,* 2005, Foley *et al.,* 2011). To assist in combatting the prevalence of SE in the food chain, the FDA issued the egg safety rule in 2009 mandating disinfection, rodent controls, environmental monitoring and pullet sourcing protocols. The FDA also advices producers to implement biosecurity procedures on farm, as well as vaccination protocols, in an attempt to control SE. It has been suggested that the utilization of feed and water additives that are known to impact the population of SE in the hen and environment are part of a successful plan (FDA, 2010).

Multiple feed additives including yeast, probiotics and butyrate have shown efficacy in the GIT of poultry as a method of controlling SE (Bailey *et al.,* 1991, Collins

and Gibson 1999, Van Immerseel *et al.,* 2002, Fernandez-Rubio 2009). Yeast products including yeast fractions and yeast cultures often contain high levels of β1-3, and 1-6 glucans, which as an individual component have shown to be effective against SE colonization across broilers and layers (Attia *et al.,* 2012, Lourenco *et al.,* 2015, Hofacre *et al.,* 2018, Price *et al.,* 2019, Girgis *et al.,* 2020). Both *Bacillus* and yeast products are already often utilized in commercial poultry production for both production benefits and animal health, and several different *Bacillus* strains have shown an effect on reducing SE (Murate *et al.,* 2015, Zhen *et al.,* 2018, Adhikari *et al.,* 2019). *Bacillus* are known to sporulate into a protective state and are sought for this ability due to their resistance to the extreme environments of poultry feed delivery. *Bacillus* are known to secrete enzymes that can aid in the digestion of non-starch polysaccharides (NSP), effecting the gut microflora, and hydrolyzing bacterial toxins (Khan *et al.,* 2020, Shanmugasundaram *et al.,* 2020). In addition to their survivability in the harsh conditions of the GIT, as well as against challenges of coccidiosis induced necrotic enteritis, *Bacillus* probiotics have the capability to secrete antimicrobial compounds that result in the suppression of SE (Knarreborg *et al.,* 2008, Knap *et al.,* 2011). Due to their previously published efficacy in pullets, an experiment was conducted to test the efficacy of a commercially available parietal yeast fraction (Safmannan) and *Bacillus* probiotic (MicroSaf) in inhibiting SE colonization in late production laying hens (Price *et al.,* 2020, Price *et al.,* 2020).

3.2. Materials and Methods

3.2.1. Birds, Diets and Management

This study was conducted at the USDA-ARS facility in College Station, TX and was approved by the local Institutional Animal Care and Use Committee (IACUC No. 2021-002).

A total of 144 Lohmann brown laying hens were obtained from a commercial facility at approximately 75 weeks of age and transported to the Texas A&M Poultry Research Center in College Station, TX. Birds were divided into 3 pens of 48 birds per pen and housed in large floor pens equipped with hanging feeders and nipple drinkers. Birds were fed a basal layer diet formulated to Lohmann Brown hen nutritional recommendations, which was divided into three batches (Table 3-1). The first batch remained as a basal diet, which served as the control group, while the second batch was supplemented with a proprietary parietal yeast fraction derived from the cell wall of *Saccharomyces cerevisiae* at 500ppm. The third batch was supplemented with a proprietary *Bacillus* probiotic blend supplemented at 500ppm. After a 3-week period of receiving treatment feed, birds were transferred to the USDA-ARS facility in College Station, TX. Prior to transferring, birds were tested for *Salmonella* prevalence via a cloacal swab, which is described in the prevalence determination section below. Upon arrival at the USDA facility, birds were allocated into three environmentally controlled rearing rooms, each equipped with two stainless steel A-frame layer cages. Each room housed a total of 48 birds, with two birds per cage (16 per treatment). Birds were banded

with a leg band to identify individual birds within a cage unit. Birds were observed daily for general flock condition, feed, water and egg production. Egg production data were recorded at the end of each day.

After a one-week acclimation period, birds were challenged with *Salmonella enterica* serovar Enteritidis via oral gavage in 3mL at the levels listed in Table 3-2. Three days after challenging with *Salmonella* Enteritidis, a cloacal swab was taken from each bird and direct plated onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) to ensure that the challenge was successful. If the prevalence of *Salmonella* was found to be less than 25%, then the challenge would be considered unsuccessful and birds would be re-challenged with *Salmonella* Enteritidis 4 days later (7 days post initial challenge). If the first *Salmonella* challenge was found to be successful, one week after initially being challenged with *Salmonella* the birds were humanely euthanized, and samples including the ceca and ovaries were taken for further analysis.

Table 3-1 - Late Production Lohmann Brown Laying Hen Diet

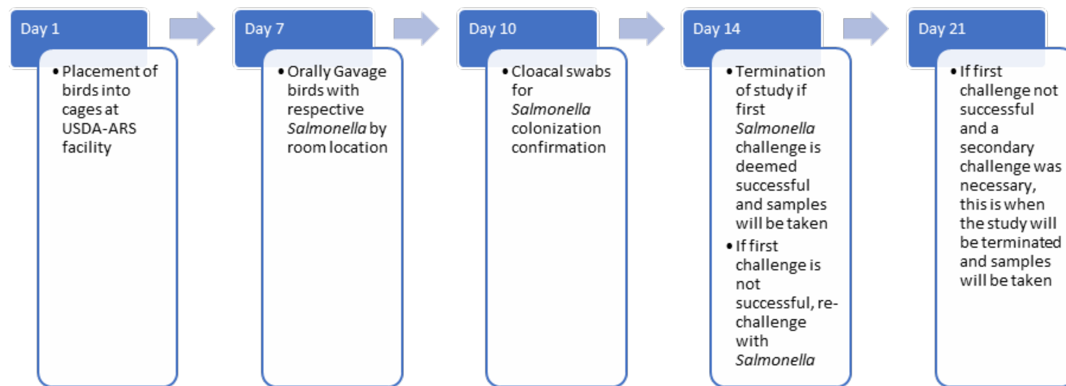
Ingredients	Percentage
Corn	63.62
Dehulled Soybean Meal	20.59
DL-Methionine	0.17
Soybean Oil	1.86
Limestone	11.66
Mono-Dical Phosphorus	1.33
Salt	0.24
Sodium Bicarbonate	0.23
Trace Minerals[1]	0.05
Trace Vitamins[2]	0.250

[1]Per pound of premix; Cu: Copper minimum 1.40%, I: Iodine minimum 800.0ppm, FE: Iron minimum 12.00%, Mm: Manganese minimum 12.00%, Zn: Zinc minimum 12.00%, [2]Per pound of premix; Vitamin A: 4,000,000 IU, Vitamin D3: 1,400,000 IU, Vitamin E: 16,666 IU, Vitamin B12: 6mg, Riboflavin (B2): 2166mg, Niacin (B3): 16,666mg, d-pantothenic acid (B5): 7334mg, Choline: 47383mg, Menadione: 534mg, Folic acid (B9):634mg, Pyridoxine (B6): 2,600mg, Thiamine (B1): 1,066mg, d-Biotin (B7): 200mg

Table 3-2 - Late Production Hen Treatment Groups and *Salmonella* Challenge Dose

	Room 1	Room 2	Room 3
Salmonella Challenge	*Salmonella* Enteritidis		
Control	16 Birds	16 Birds	16 Birds
Safmannan (500g/T)	16 Birds	16 Birds	16 Birds
MicroSaf (500g/T)	16 Birds	16 Birds	16 Birds
Salmonella Challenge Dose	2.2 x 10^9 CFU/mL		

Figure 3-1 - Late Production Hens Trial Schedule



3.2.2. Prevalence Determination

Cloacal samples were obtained prior to transferring birds to the USDA facility in order to ensure that birds are *Salmonella* free prior to the start of the experiment. Cloacal swabs were placed into conical tubes containing RV broth, homogenized by shaking, and incubated at 42°C for 24 hours. After 24 hours of incubation, samples were again homogenized by shaking, and a 10 µL sterile loop was used to streak plate a sample onto XLT-4 agar. Plates were then incubated at 37°C for 24 hours, and samples were deemed positive by visual identification of colonies on the plate.

Ceca and ovary samples taken from the birds at termination were divided and split between the different culturing methodologies. For ceca samples, one cecum was placed into a conical tube containing RV (Difco, Franklin Lakes, NJ) broth as a method of enrichment for determination of *Salmonella* prevalence. For ovary samples, a sample of the ovary was placed into a conical tube containing RV broth. Both ceca and ovary samples were homogenized by shaking and incubated for 24 hours at 42°C. After 24 hours of incubation, samples were again homogenized by shaking, and a 10 µL sterile loop was used to streak plate a sample onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) for use as a selective growth media. Plates were then incubated at 37°C for 24 hours, and samples deemed positive by visual identification of colonies on the plate. All prevalence colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after incubation on XLT-4 agar.

3.2.3. Enumeration Determination

In this study, only ceca samples were enumerated. Ceca samples were diluted weight to volume using a 10x dilution series resulting in dilutions of 1:10 to 1:10,000, of which 0.1mL was plated onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) for use as a selective growth media using a spread plating method. Enumeration counts were determined by visual identification of colonies after 48 hours of incubation at 37°C. Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 48 hours of incubation.

3.2.4. Statistical Analysis

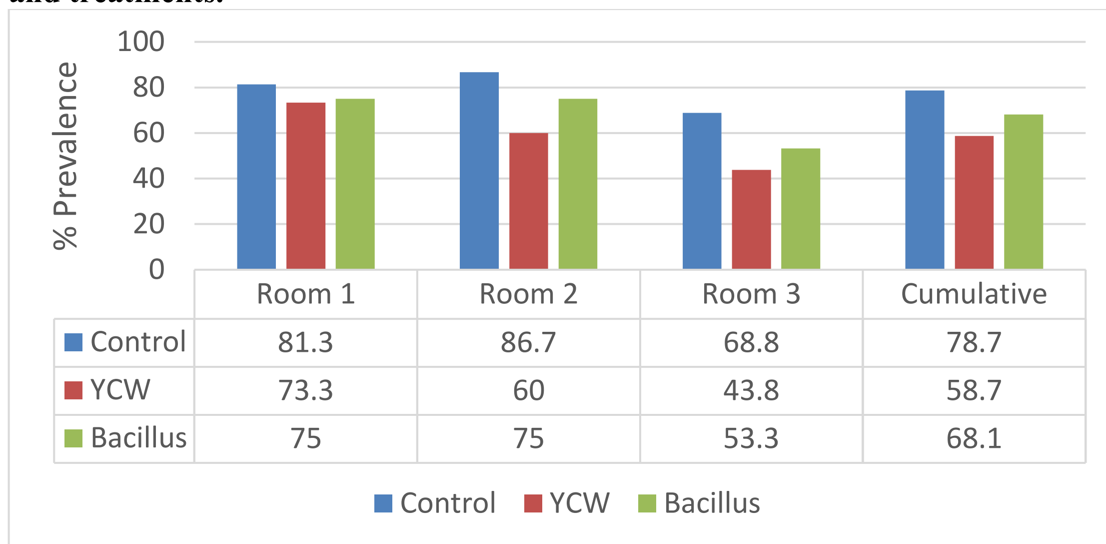
All data obtained during this experiment were analyzed using a one-way Analysis of Variance (ANOVA) and means separated using students t-test were considered statistically significantly different at $p \leq 0.05$. Bonferroni multiple comparisons were used to make all pairwise comparisons for statistically significant ANOVA ($p \leq 0.05$) results. Mean and standard deviation values were used in the determination of outliers as well as for comparisons between treatment groups. Enumeration values were log transformed prior to analysis. These analyses were conducted for each of the sampling time points noted in Figure 2.1 All data were analyzed using Stata v.16.1 (StataCorp, College Station, TX).

3.3. Results and Discussion

3.3.1. Ceca & Ovary Prevalence

Although not statistically significant, both the Safmannan YCW and Microsaf *Bacillus* products resulted in a decrease in cecal prevalence across all three study rooms, as well as cumulatively. Safmannan resulted in a 20% reduction in *Salmonella* prevalence in the ceca, or over a 25% reduction when compared to the control group. The Microsaf *Bacillus* treatment resulted in a 10% reduction in *Salmonella* prevalence in the ceca, or over a 13% reduction when compared to the control group. When analyzing each room individually, both Safmannan and Microsaf showed reductions in ceca prevalence across all three rooms (Figure 3-2).

Figure 3-2 - *Salmonella* Enteritidis Prevalence (%) in Cecal Content across rooms and treatments.

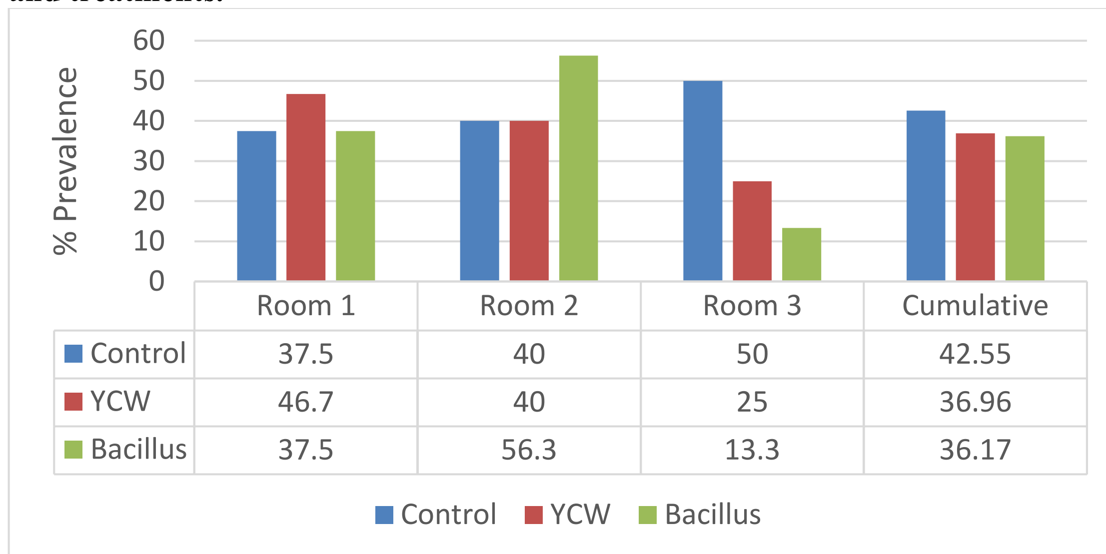


	Room 1	Room 2	Room 3	Cumulative
Control	81.3	86.7	68.8	78.7
YCW	73.3	60	43.8	58.7
Bacillus	75	75	53.3	68.1

When reviewing the cumulative ovary prevalence, although not statistically significant, both the Safmannan YCW and Microsaf *Bacillus* groups resulted in nominal

reductions of *Salmonella* prevalence in the ovary. Safmannan exhibited a 6% reduction in ovary colonization, or a 15% reduction compared to the control treatment, while Microsaf exhibited a 5.5% reduction in ovary colonization, or a 13% reduction when compared to the control group. When analyzing each room individually, Safmannan YCW showed a nominal reduction in ovary prevalence only in room 3, with a 25% reduction in total prevalence, or a 50% reduction when compared to the control group. Microsaf *Bacillus* showed reductions in the same room, with a 36.7% reduction in total ovary prevalence, or a 73% reduction when compared to the control group (Figure 3-3).

Figure 3-3 - *Salmonella* Enteritidis Prevalence (%) in Ovary Samples across rooms and treatments.



	Room 1	Room 2	Room 3	Cumulative
Control	37.5	40	50	42.55
YCW	46.7	40	25	36.96
Bacillus	37.5	56.3	13.3	36.17

3.3.2. Ceca Enumeration

Cumulative ceca enumeration values showed a significant reduction in *S.*Enteritidis colonization when comparing the Safmannan YCW treatment group to the

control treatment (p=0.009), with a reduction of approximately 0.9 logs. Although not statistically significant (p=0.084), the Microsaf *Bacillus* treatment reduced *Salmonella* load in the ceca by approximately 0.67 logs (Table 3-3).

Table 3-3 – ANOVA Comparing Late Production Hens *Salmonella* Log_{10} CFU/g of Ceca Content

Treatment	Log_{10} CFU/g	Std. Err.	95% Conf. Interval
Control	2.53	0.214	[2.112, 2.958]
Safmannan	1.61	0.216	[1.187, 2.042]
Microsaf	1.86	0.219	[1.424, 2.288]
	P=0.0086		
Treatment Comparison			Bonferroni P>|t|
Safmannan vs Control			0.009
Microsaf vs Control			0.084

In reviewing each of the rooms individually, the Safmannan YCW treatment resulted in *Salmonella* load reductions in all three of the experimental rooms, and the Microsaf *Bacillus* treatment resulted in reductions in two of the three experiment rooms. However, Room 3 resulted in the greatest decrease in *Salmonella* colonization in the ceca for both of the treatment groups. The Safmannan YCW treatment resutled in a biologically relevant reduction (1.26 log_{10}) in *S.*Enteritidis load when compared to the control group (p=0.096). Although not significant, the Microsaf *Bacillus* treatment group resulted in a biologically relevant reduction of approximately 1.0 log_{10} when compared to the control treatment (Table 3-4). Although these were not always significantly

different, a 1-log reduction in *Salmonella* load in poultry is considered a biologically relevant valuae, which was exhibited by both Safmannan Microsaf in room 3.

Table 3-4 – ANOVA Comparing Late Production Laying Hens *Salmonella* Log_{10} CFU/g of Cecal Content in Room 3

Treatment	Log_{10} CFU/g	Std. Err.	95% Conf. Interval
Control	2.49	0.403	[1.684, 3.307]
Safmannan	1.23	0.403	[0.424, 2.047]
Microsaf	1.49	0.416	[0.656, 2.332]
	P=0.0770		

3.3.3. Discussion

Yeast components including yeast fractions and yeast cell wall have a proven record of binding affinity across a wide range of pathogens including multiple *Salmonella* serotypes. This binding affinity promotes adhesion by direct agglutination of pathogenic microbes to the mannose in yeast via the type-1 fimbriae, resulting in the removal of the pathogens from the GIT (Akhtar *et al.,* 2016, Posadas *et al.,* 2017). *Bacillus* probiotics have shown promising activity in affecting *Salmonella* colonization through other methods including competitive exclusion as well as facilitation of cross-talk between GIT microbes and the immune system, resulting in decreased gut inflammation (Kabir 2009, Al-Khalaifah *et al.,* 2016, Al-Khalaifah 2018).

It has also been observed that most SE contaminated eggs contain extremely low CFU counts, much lower than the level of detection utilized in most controlled studies (Humphrey *et al.,* 1989, Gast *et al.,* 1990). With low levels of infection present, a

reduction in the GIT can potentially indicate a biological significance for food safety. Since yeast and *Bacillus* products have been shown to reduce colonization sites in the microvilli of the intestine, thus decreasing "leaky gut" conditions that allow *Salmonella* to enter the macrophages and consequently the blood stream, there is a lower chance of these pathogens reaching the reproductive tract. In this case, consequentially a reduction in cecal load of SE should evidentially lead to a reduction in positive samples in the ovary (Wang *et al.,* 2016, Nopovichai *et al.,* 2019, Gharib-Naseri *et al.,* 2020).

The results of this study are in agreement with previous studies that show supplementation of Safmannan yeast fraction result in SE reductions in the ceca, as well as ST reductions by over 1 log (Price *et al.,* 2020, Price *et al.,* 2020). MicroSaf *Bacillus* also has showed similar reductions approaching 1 log in the ceca (Price *et al.,* 2020). While the reductions in this case were only statistically significant with the Safmannan treatment both cumulatively and in Room 3, a 1-log reduction in the ceca in Room 3 with both Safmannan and MicroSaf, as well as the cumulative approach to 1 log with Safmannan are biologically significant. From a food safety perspective, any reduction, especially those of approximately 1 log or greater are biologically significant and are important in the continued improvement of food safety in the United States. Combined, these results show that both Safmannan and MicroSaf possess excellent potential as food safety interventions in the commercial egg industry supply chain.

4. COMPARISON OF *SALMONELLA* SHEDDING AND ORGAN CONTAMINATION OF BROILER CHICKENS CHALLENGED WITH *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM IN BIRDS SUPPLEMENTED WITH YEAST CELL WALL PREBIOTICS, BACILLUS PROBIOTICS, OR BACTERIOPHAGES

4.1. Introduction

Salmonella enterica serovar Typhimurium is among the top 5 *Salmonella* serovars linked to poultry associated human salmonellosis, and is joined by *Salmonella enterica* serovars Enteritidis, Newport, Heidelberg and I 4,[5],12:i:- (CDC, 2008). However, this has not always been the case, and has only recently come to fruition due to the control of *Salmonella enterica* serovars Gallinarum and Pullorum which were prolific in the early 20th century. With the 20th century shift from backyard flocks to commercial production came increasing use of vaccination in chicks as well as voluntary programs, which led to the ultimate eradication of fowl typhoid and pullorum disease. Because these serovars were eradicated, an ecological niche was opened and allowed for the proliferation of other serovars, most importantly *Salmonella* Enteritidis and *Salmonella* Typhimurium (Foley *et al.,*2011).

While *Salmonella* Typhimurium (ST) is not solely associated with poultry, there are multiple instances in the last decade where outbreaks of ST are associated with live poultry. Recently, an outbreak of ST occurred in 2017 in which ST along with several other serovars led to a 48-state outbreak of *Salmonella* infections that hospitalized nearly

250 people (CDC, 2017). In this instance, the outbreak was linked to backyard poultry, but this does not mean that ST is not a commercial production issue. However, unlike backyard flocks, many commercial poultry producers use a multi-hurdle approach to mitigating *Salmonella* in both live production as well as processing.

In order to effectively reduce *Salmonella* infections linked to poultry products, a multi-hurdle approach is necessary. This requires both reductions of *Salmonella* in the processing plant, as well as in the live farm production. However, not all strains of *Salmonella* are the same, and even within a given serovar mutations can occur that can change a products efficacy at mitigating the pathogen. Therefore, this study was conducted in order to effectively evaluate the efficacy of yeast prebiotics, *Bacillus* probiotics, their combinations, and bacteriophages at reducing *Salmonella* Typhimurium colonization and translocation in broilers.

4.2. Materials and Methods

4.2.1. Birds, Diets and Management

This study was conducted at the USDA-ARS facility in College Station, TX and was approved by the local Institutional Animal Care and Use Committee (IACUC No. 2019-009).

A total of 240 day-old straight run Cobb-500 broiler chicks were obtained from a commercial hatchery and transported to the USDA-ARS facility in College Station, TX. Chicks were weighed on day-of-age and divided into pen replicates according to an average pen weight. Treatments were distributed amongst the pens using a randomized

complete block design (RCBD) to reduce variability. Birds were fed diets containing commercially available pre- and probiotic supplements, their combinations, or had access to water supplemented with bacteriophages (Table 4-2). Treatments include a parietal yeast fraction (YCW) at 500ppm, a *Bacillus* blend at 500ppm, their combinations, and bacteriophages at $1x10^9$ PFU. Birds were observed daily for general flock condition, feed, water and mortality. This study utilized a two-phase feeding program, in which a starter diet was used for Days 1 to 9 and a grower diet for Days 10 to 21. Diets were formulated according to the Cobb-500 nutritional recommendations (Table 4-1), and additives were supplemented either via feed or water at the rates specified in Table 4-2.

On Day 3, birds were challenged with *Salmonella enterica* serovar Typhimurium at $2.0x10^7$ CFU/mL in 0.5mL by oral gavage. Bird weights and feed consumption were recorded weekly and at feed changes. Fecal samples and cloacal swabs were taken on the same days as bird weights and feed consumption. On Day 14, one bird per pen was removed, euthanized, and necropsied for desired samples including liver, spleen and ceca. On Day 21, all remaining birds were euthanized, and samples obtained utilizing the same methods as on Day 14 (Figure 4-1).

Table 4-1 - *Salmonella* Typhimurium Challenged Broilers Diet Composition

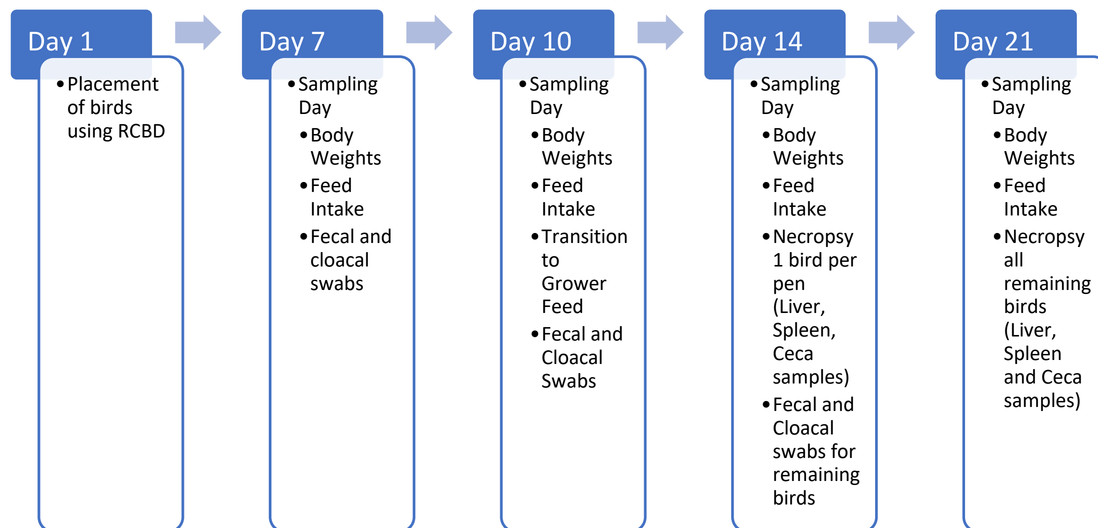
Phase 1 – Broiler Starter		Phase 2 – Broiler Grower	
Ingredients	Percentage	Ingredients	Percentage
Corn	60.83	Corn	64.82
Dehulled Soybean Meal	33.91	Dehulled Soybean Meal	28.92
Limestone	1.47	Limestone	1.44
Mono-Dical Phosphorus	1.54	Mono-Dical Phosphorus	1.43
Salt	0.44	Salt	0.44
Lysine HCL	0.15	Lysine HCL	0.20
DL-Methionine	0.21	DL-Methionine	0.25
L-Threonine	0.06	L-Threonine	0.04
Fat, Animal-Vegetable Blend	1.17	Fat, Animal-Vegetable Blend	2.24
Trace Minerals[1]	0.05	Trace Minerals[1]	0.05
Trace Vitamins[2]	0.175	Trace Vitamins[2]	0.175

Vitamin premix provided by DSM Animal Nutrition and Health. [1]Per pound of premix; Cu: Copper minimum 1.40%, I: Iodine minimum 800.0ppm, FE: Iron minimum 12.00%, Mm: Manganese minimum 12.00%, Zn: Zinc minimum 12.00%, [2]Per pound of premix; Vitamin A: 4,000,000 IU, Vitamin D3: 1,400,000 IU, Vitamin E: 16,666 IU, Vitamin B12: 6mg, Riboflavin (B2): 2166mg, Niacin (B3): 16,666mg, d-pantothenic acid (B5): 7334mg, Choline: 47383mg, Menadione: 534mg, Folic acid (B9):634mg, Pyridoxine (B6): 2,600mg, Thiamine (B1): 1,066mg, d-Biotin (B7): 200mg

Table 4-2 - *Salmonella* Typhimurium Challenged Broiler Treatment Groups and Product Application Methods

Treatment	Bird # per Treatment group	Initial # Birds per pen	# Pens	Application Method
Positive Control	40	5	8	
MicroSaf (500g/T)	40	5	8	Feed
Safmannan (500g/T)	40	5	8	Feed
MicroSaf (125g/T) + Safmannan (125g/T)	40	5	8	Feed
MicroSaf (125g/T) + Safmannan (250g/T)	40	5	8	Feed
Bacteriophages (40mL/10L of drinking water daily, 10^9 PFU)	40	5	8	Water
Total Birds in Study	240		48	

Figure 4-1 - *Salmonella* Typhimurim Challenged Broilers Trial Schedule



4.2.2. Prevalence Determination

Fecal and cloacal samples obtained on Days 7, 10 and 14 were used for determination of *Salmonella* prevalence post-inoculation. Fecal and cloacal samples were taken using a sterile cotton swab and placed into a conical tube containing Rappaport Vassiliadis (RV) broth, homogenized by shaking, and incubated at 42°C for 24 hours. After 24 hours of incubation, samples were again homogenized by shaking, and a 10 µL sterile loop was used to streak plate a sample onto XLT-4 agar. Plates were then incubated at 37°C for 24 hours, and samples were deemed positive by visual identification of colonies on the plate. Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 24 hours of incubation.

Samples obtained during necropsy on Days 14 and 21 for *Salmonella* prevalence included ceca, liver and spleen. Ceca pair samples were divided in half upon necropsy, and one cecum was used for prevalence determination. Spleen samples utilized the entire spleen, and liver samples utilized one lobe of the liver. Samples were placed into a conical tube containing RV broth, homogenized by shaking, and incubated at 42°C for 24 hours. After 24 hours of incubation, samples were again homogenized by shaking, and a 10 µL sterile loop was used to plate a sample onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) for use as a selective growth media using a streak plating method. Plates were then incubated at 37°C for 24 hours, and samples were deemed positive by visual identification of colonies on the plate.

Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 24 hours of incubation.

4.2.3. Enumeration Determination

In this study, only ceca samples were enumerated. Ceca samples were diluted weight to volume using a 10x dilution series resulting in dilutions of 1:10 to 1:10,000, of which 0.1mL was plated onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) for use as a selective growth media using a spread plating method. Enumeration was determined by visual identification of colonies after 48 hours of incubation at 37°C. Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 48 hours of incubation.

4.2.4. Statistical Analysis

All data obtained during this experiment were analyzed using a one-way Analysis of Variance (ANOVA) and means separated using students t-test were considered statistically significantly different at $p \leq 0.05$. The ANOVA was used to compare observations between the treatment groups for each of the factors (ceca, liver & spleen prevalence, and performance parameters) observed during this study noted in the materials and methods section. Bonferroni multiple comparisons were used to make all pairwise comparisons in results that showed a statistically significant ANOVA ($p \leq 0.05$) result. Mean and standard deviation values were used in the determination of outliers as well as for comparisons between treatment groups. Enumeration values were log

transformed prior to analysis. These analyses were conducted for each of the sampling time points noted in Figure 4.1 All data were analyzed using Stata v.16.1 (StataCorp, College Station, TX).

4.3. Results and Discussion

4.3.1. Performance Parameters

All data obtained in this study was done so by the methods specified in the materials and methods section of this chapter and analyzed according to the statistical methods detailed previously. When reviewing average body weight per bird, at Day 10 we see that the inclusion of Safmannan YCW in the diet resulted in an increased average body weight per bird when compared to the control treatment. The inclusion of bacteriophages in the water in the same phase resulted in nominal increases in body weights (Tables 4-3). While neither of these increases attributed to the Safmannan YCW or the Bacteriophages were statistically significant, other significant differences noted in the treatment comparison section of table 4-3 were primarily noticed between treatment groups; rather than between treatments and control.

Table 4-3 – ANOVA comparing Phase Average Bird Body Weight (g) for treatment across days

Treatment	D0 (g) ±SD	D7 (g) ±SD	D10 (g) ±SD	D14(g) ±SD	D21(g) ±SD
Control	40.1 ± 1.5	121.6 ±6.9	210.1 ±10.2	399.4 ±17.7	922.8 ±38.8
Microsaf	40.4 ± 1.2	119.3 ±7.5	202.7 ±13.1	391.7 ±19.3	881.1 ±46.8
Safmannan	40.6 ± 1.1	127.9 ±10.1	226.2 ±15.1	416.5 ±29.4	927.8 ±54.7
Microsaf 125 + Safmannan 125	39.5 ± 1.7	123.2 ±9.0	213.1 ±14.7	403.7 ±19.6	899.7 ±39.9
Microsaf 125 + Safmannan 250	40.3 ± 1.5	125.4 ±8.1	220.1 ±13.3	413.8 ±20.4	938.6 ±50.4
Bacteriophages	40.5 ± 1.3	125.7 ±6.3	222.1 ±9.2	418.6 ±13.4	931.5 ±42.2
P-Value	0.6436	0.3270	0.0072	0.0746	0.1258
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

Treatment Comparison	Day 10 Bonferroni P>|t|
Microsaf vs Control	1.000
Safmannan vs Control	0.229
Microsaf 125 + Safmanann 125 vs Control	1.000
Microsaf 125 + Safmannan 250 vs Control	1.000
Bacteriophages vs Control	0.996
Safmannan vs Microsaf	0.010
Microsaf 125 + Safmannan 125 vs Microsaf	1.000
Microsaf 125 + Safmannan 250 vs Microsaf	0.141
Bacteriophages vs Microsaf	0.062
Microsaf 125 + Safmannan 125 vs Safmannan	0.693
Microsaf 125 + Safmannan 250 vs Safmannan	1.000
Bacteriophages vs Safmannan	1.000
Microsaf 125+Safmannan 125 vs Microsaf 125+Safmannan 250	1.000
Microsaf 125+Safmannan 125 vs Bacteriophages	1.000
Microsaf 125 + Safmannan 250 vs Bacteriophages	1.000

This trend of increased body weight with supplementation continued through the Day 14 sampling period, in which we see, although not significant, an increase in average body weight per bird, with the greatest differences observed in the Safmannan YCW and Bacteriophages treatment groups. When reviewing the bacteriophage treatment, birds weighed an average of 19 grams higher at this phase when compared to the control, and the Safmannan YCW treated birds weighed an average of 17 grams higher when compared to the control (Table 4-3).

For average phase weight gain per bird, nominal increases in average body weight during the Day 7-10 phase were seen with supplementation of the Safmannan YCW treatment, as well as a nominal increase in the bacteriophage treatment. However, unlike average body weight per bird, these significant differences did not transfer over to the Day 10-14 phase, leading to the conclusion that the differences noted in the average body weight per bird likely stemmed from the Day 7-10 phase and carried over to the Day 14 body weights.(Table 4-4).

Table 4-4 – ANOVA Comparing Average Phase Weight Gain per Bird (g) across days

Treatment	D0-7 (g) ±SD	D7-10 (g) ±SD	D10-14 (g) ±SD	D14-21 (g) ±SD
Control	81.5 ±5.9	88.5 ±5.9	189.3 ±8.9	593.2 ±26.7
Microsaf	78.9 ±7.6	83.4 ±6.1	189.0 ±14.4	565.1 ±35.4
Safmannan	87.3 ±9.9	98.3 ±16.3	190.3 ±31.5	585.0 ±36.7
Microsaf 125 + Safmannan 125	83.8 ±8.0	89.9 ±6.6	190.6 ±7.8	563.7 ±49.0
Microsaf 125 + Safmannan 250	85.2 ±8.1	94.7 ±7.2	193.7 ±9.7	602.7 ±35.8
Bacteriophages	85.3 ±6.5	96.4 ±3.2	196.5 ±5.7	592.0 ±33.7
P-Value	0.3244	0.0117	0.9212	0.2107
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

Treatment Comparison	Day 7-10 Bonferroni $P>\vert t\vert$
Microsaf vs Control	1.000
Safmannan vs Control	0.409
Microsaf 125 + Safmanann 125 vs Control	1.000
Microsaf 125 + Safmannan 250 vs Control	1.000
Bacteriophages vs Control	1.000
Safmannan vs Microsaf	0.019*
Microsaf 125 + Safmannan 125 vs Microsaf	1.000
Microsaf 125 + Safmannan 250 vs Microsaf	0.183
Bacteriophages vs Microsaf	0.065
Microsaf 125 + Safmannan 125 vs Safmannan	0.864
Microsaf 125 + Safmannan 250 vs Safmannan	1.000
Bacteriophages vs Safmannan	1.000
Microsaf 125+Safmannan 125 vs Microsaf 125+Safmannan 250	1.000
Microsaf 125+Safmannan 125 vs Bacteriophages	1.000
Microsaf 125 + Safmannan 250 vs Bacteriophages	1.000

*-Indicates significant values at $P<0.05$ from Bonferroni multiple comparisons

Table 4-5 – ANOVA Comparing Average Phase and Cumulative Feed to Gain Ratio Across Days

Treatment	D0-7 ±SD	D7-10 ±SD	D10-14 ±SD	D14-21 ±SD	Cumulative ±SD
Control	1.32 ±0.04	1.16 ±0.03	1.19 ±0.03	1.34 ±0.05	1.28 ±0.03
Microsaf	1.34 ±0.12	1.21 ±0.08	1.17 ±0.07	1.34 ±0.04	1.28 ±0.01
Safmannan	1.29 ±0.08	1.18 ±0.04	1.20 ±0.03	1.34 ±0.04	1.28 ±0.02
Microsaf 125 + Safmannan 125	1.31 ±0.15	1.18 ±0.03	1.22 ±0.06	1.34 ±0.04	1.28 ±0.04
Microsaf 125 + Safmannan 250	1.36 ±0.10	1.17 ±0.04	1.23 ±0.07	1.33 ±0.05	1.29 ±0.04
Bacteriophages	1.34 ±0.05	1.13 ±0.03	1.19 ±0.04	1.34 ±0.04	1.27 ±0.02
P-Value	0.7215	0.0001	0.2609	0.9952	0.9230
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

Treatment Comparison	Day 7-10 Bonferroni P>|t|
Microsaf vs Control	0.004*
Safmannan vs Control	1.000
Microsaf 125 + Safmanann 125 vs Control	1.000
Microsaf 125 + Safmannan 250 vs Control	1.000
Bacteriophages vs Control	1.000
Safmannan vs Microsaf	0.090
Microsaf 125 + Safmannan 125 vs Microsaf	0.077
Microsaf 125 + Safmannan 250 vs Microsaf	0.030*
Bacteriophages vs Microsaf	0.000*
Microsaf 125 + Safmannan 125 vs Safmannan	1.000
Microsaf 125 + Safmannan 250 vs Safmannan	1.000
Bacteriophages vs Safmannan	0.219
Microsaf 125+Safmannan 125 vs Microsaf 125+Safmannan 250	1.000
Microsaf 125+Safmannan 125 vs Bacteriophages	0.253
Microsaf 125 + Safmannan 250 vs Bacteriophages	0.570

*-Indicates significant values from Bonferroni multiple comparisons at P<0.05

Similar to previous trials, the only significant differences noticed in feed to gain ratio were during the Day 7-10 observation period. In this case, the significant difference was between a treatment (Microsaf *Bacillus*) and control, however the significance was in favor of the control treatment, and the Microsaf treatment had significantly worse (higher) feed to gain ratio (p=0.004) than the control treatment. Otherwise, the treatments observed in this trial resulted in minimal benefits compared to the control group, with cumulative feed to gain ratio being cumulatively within 1-point (0.01) of the control (Table 4-5).

Similar to the other factors noted previously, significant differences were only noted in the Day 7-10 observations for feed to gain ratio (Table 4-5). However in this case, all treatments except the bacteriophages resulted in higher feed to gain ratios than the control treatment. In this case, it is likely that the benefits seen previously were the result of increased feed consumption in the Safmannan YCW treatment group. Similar results were seen in the cumulative feed to weight ratio data, where all treatments had an observed higher feed to weight ratio than the control treatment in the Day 0-10 data, confirming the notion that the increased body weight mentioned previously was due to increased feed consumption, rather than an improvement in digestibility (Table 4-6).

Table 4-6 – ANOVA Comparing Average Cumulative Feed to Weight Ratio Across Days

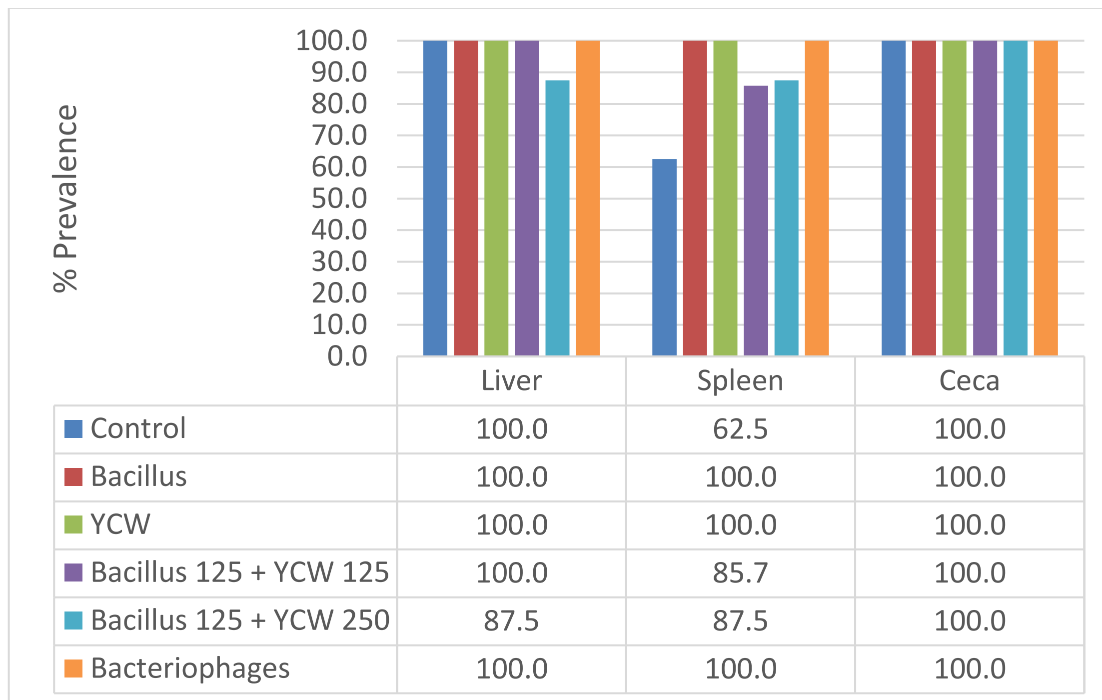
Treatment	D0-7 ±SD	D0-10 ±SD	D0-14 ±SD	D0-21 ±SD
Control	0.879 ±0.022	0.994 ±0.019	1.086 ±0.011	1.328 ±0.025
Microsaf	0.871 ±0.069	1.023 ±0.018	1.089 ±0.037	1.343 ±0.017
Safmannan	0.878 ±0.027	1.003 ±0.019	1.096 ±0.011	1.338 ±0.012
Microsaf 125 + Safmannan 125	0.869 ±0.034	0.996 ±0.017	1.101 ±0.031	1.329 ±0.041
Microsaf 125 + Safmannan 250	0.919 ±0.072	1.023 ±0.036	1.118 ±0.043	1.354 ±0.043
Bacteriophages	0.898 ±0.031	0.997 ±0.022	1.088 ±0.024	1.339 ±0.027
P-Value	0.2406	0.0388	0.2878	0.5383
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

Treatment Comparison	Day 0-10 Bonferroni P>|t|
Microsaf vs Control	0.258
Safmannan vs Control	1.000
Microsaf 125 + Safmanann 125 vs Control	1.000
Microsaf 125 + Safmannan 250 vs Control	0.258
Bacteriophages vs Control	1.000
Safmannan vs Microsaf	1.000
Microsaf 125 + Safmannan 125 vs Microsaf	0.432
Microsaf 125 + Safmannan 250 vs Microsaf	1.000
Bacteriophages vs Microsaf	0.552
Microsaf 125 + Safmannan 125 vs Safmannan	1.000
Microsaf 125 + Safmannan 250 vs Safmannan	1.000
Bacteriophages vs Safmannan	1.000
Microsaf 125+Safmannan 125 vs Microsaf 125+Safmannan 250	0.432
Microsaf 125+Safmannan 125 vs Bacteriophages	1.000
Microsaf 125 + Safmannan 250 vs Bacteriophages	0.552

4.3.2. Ceca, Liver & Spleen Prevalence

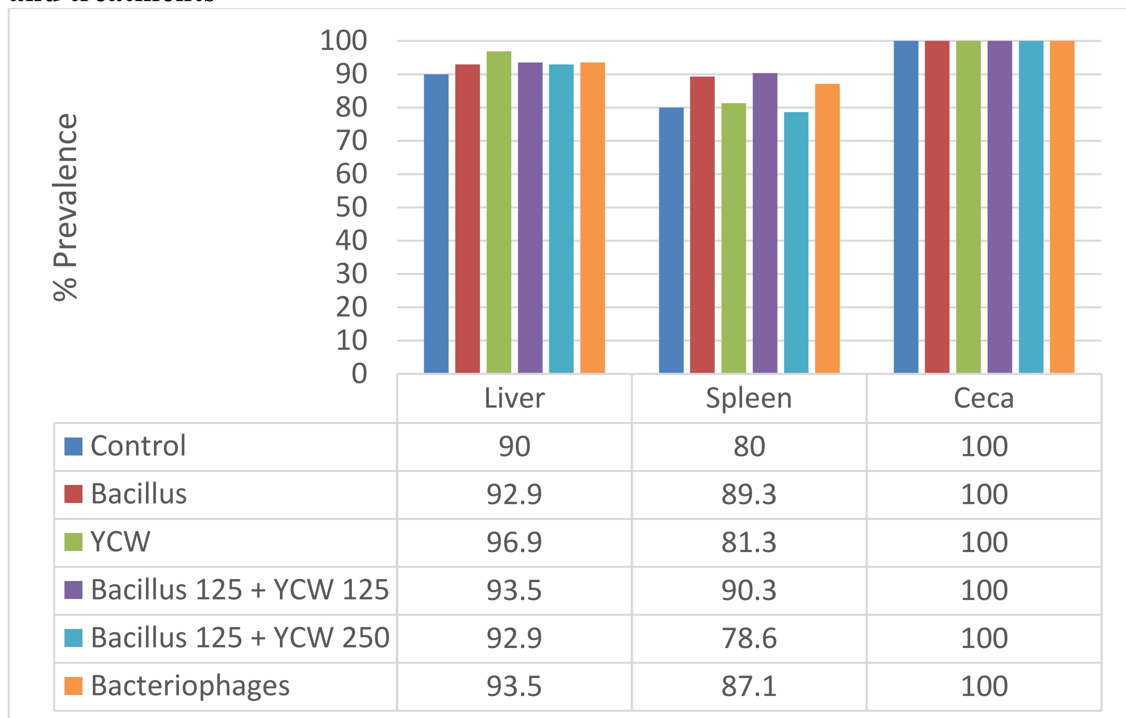
No significant differences were noticed in either the Day 14 or Day 21 observations related to prevalence. Small reductions were noted on the Day 14 observations with the Microsaf 125ppm + Safmannan 250ppm treatment on liver prevalence only (Figure 4-2). The same treatment also showed reductions in spleen prevalence on Day 21 (Figure 4-3). However, in the case of this experiment, no reductions in cecal prevalence were seen with any treatment inclusion at either of the time points that were considered for sampling.

Figure 4-2 - Day 14 *Salmonella* Typhimurium Prevalence (%) across sample types and treatments



	Liver	Spleen	Ceca
Control	100.0	62.5	100.0
Bacillus	100.0	100.0	100.0
YCW	100.0	100.0	100.0
Bacillus 125 + YCW 125	100.0	85.7	100.0
Bacillus 125 + YCW 250	87.5	87.5	100.0
Bacteriophages	100.0	100.0	100.0

Figure 4-3 - Day 21 *Salmonella* Typhimurium Prevalence (%) across sample types and treatments



	Liver	Spleen	Ceca
Control	90	80	100
Bacillus	92.9	89.3	100
YCW	96.9	81.3	100
Bacillus 125 + YCW 125	93.5	90.3	100
Bacillus 125 + YCW 250	92.9	78.6	100
Bacteriophages	93.5	87.1	100

4.3.3. Ceca Enumeration

Although not statistically significant, the Safmannan YCW, and both combination treatment showed nominal reductions in *Salmonella* Typhimurium colonization when compared to the control treatment at Day 14. Nominal reductions of 0.7 and 0.5 logs were seen with the Microsaf 125+Safmannan 125 treatment and the Microsaf 125+Safmannan 250 treatments, respectively compared to the control. The Safmannan individual treatmenct resulted in a biologically relevant reduction of >1.3 logs compared to the control group at the same phase (Table 4-7).

Table 4-7 – ANOVA Comparing Day 14 *Salmonella* Typhimurium Enumeration Log_{10} CFU/g of Cecal Content

Treatment	Log_{10} CFU/g	Std. Err.	95% Conf. Interval
Control	6.01	0.590	[4.818, 7.203]
Safmannan	4.65	0.590	[3.458, 5.843]
Microsaf	6.18	0.590	[4.987, 7.371]
Microsaf 125 + Safmannan 125	5.32	0.631	[4.045, 6.595]
Microsaf 125 + Safmannan 250	5.51	0.590	[4.320, 6.704]
Bacteriophages	6.43	0.590	[5.239, 7.624]

On Day 21, only the Safmannan YCW treatment was able to achieve a nominal reduction in *S.*Typhimurium colonization in the ceca, with a resulting reduction of approximately 0.8 logs when compared to the control treatment. All other treatments showed nominally higher log_{10} CFU/g than the control treatment in this case (Table 4-8).

Table 4-8 – ANOVA Comparing Day 21 *Salmonella* Typhimurium Enumeration Log_{10} CFU/g of Cecal Content

Treatment	Log_{10} CFU/g	Std. Err.	95% Conf. Interval
Control	4.40	0.302	[3.806, 4.997]
Safmannan	3.59	0.312	[2.975, 4.208]
Microsaf	4.61	0.292	[4.028, 5.182]
Microsaf 125 + Safmannan 125	5.04	0.297	[4.457, 5.629]
Microsaf 125 + Safmannan 250	4.48	0.312	[3.862, 5.095]
Bacteriophages	4.93	0.297	[4.344, 5.516]
	P=0.0178		

Treatment Comparison	Day 21 Bonferroni P>|t|
Safmannan vs Control	0.956
Microsaf vs Control	1.000
Microsaf 125 + Safmanann 125 vs Control	1.000
Microsaf 125 + Safmannan 250 vs Control	1.000
Bacteriophages vs Control	1.000
Microsaf vs Safmannan	0.283
Microsaf 125 + Safmannan 125 vs Safmannan	0.014
Microsaf 125 + Safmannan 250 vs Safmannan	0.692
Bacteriophages vs Safmannan	0.033
Microsaf 125 + Safmannan 125 vs Microsaf	1.000
Microsaf 125 + Safmannan 250 vs Microsaf	1.000
Bacteriophages vs Microsaf	1.000
Microsaf 125+Safmannan 125 vs Microsaf 125+Safmannan 250	1.000
Microsaf 125+Safmannan 125 vs Bacteriophages	1.000
Microsaf 125 + Safmannan 250 vs Bacteriophages	1.000

4.3.4. Discussion

While some benefits were observed with the supplementation of either the Safmannan YCW, the Microsaf *Bacillus* blend, and their combinations, the results of this

study do not lend to definitive results with selecting a single treatment as having performed better than the others. Safmannan showed small improvements in performance parameters when compared to the control group, which were only found at the Day 7 and 10 observations. This was mirrored in the *Salmonella* observations as well, with biologically relevant reductions in cecal load at day 14; however, with nominal benefits at the Day 21 sampling. In both the performance parameters and *Salmonella* colonization, benefits of product supplementation were lost in the later phases as birds continued to age. Combination treatment groups in some cases showed sporadic marginal benefits when compared to the control group.

The bacteriophage group showed only minimal benefits in body weight per bird, however, this benefit was negated by the higher cecal load of *Salmonella* Typhimurium compared to the control group at both Day 14 and 21. The lack of benefit of this product in this instance was likely due to a reduced efficacy of the product. The bacteriophages utilized in this study are sensitive to high temperatures and require a cold storage environment to maintain efficacy. In the early growth phases of these broilers, especially around the time when the birds were challenged with *Salmonella* Typhimurium it was hot, with average temperatures hovering close to 90°F, which would cause the product to degrade, thus resulting in a loss of efficacy. This combined with the low inclusion rate of the bacteriophages in the water may be the reason for the lack of benefit in this case.

Overall, this study only provided minimal benefits regarding the control of *Salmonella* Typhimurium. Minimal reductions were seen with the inclusion of the Microsaf *Bacillus* product and the Safmannan yeast fraction with regard to reducing

Salmonella Typhimurium load in the ceca, with an approximately 0.5 log reduction each. However, what this study was able to prove is that the combination of Safmannan and Microsaf together did not provide additional benefits at the concentrations listed. In order for these products to work effectively, it is likely that the inclusion rates would need to be increased compared to those trialed in the combination groups to provide ample benefit. Cumulatively, these results showed minimal benefit and that some alterations to products may be necessary in order to effectively control *Salmonella* Typhimurium from a food safety perspective.

5. COMPARISON OF *SALMONELLA* SHEDDING AND ORGAN CONTAMINATION OF BROILER CHICKENS CHALLENGED WITH *SALMONELLA ENTERICA* SEROVAR INFANTIS IN BIRDS SUPPLEMENTED WITH YEAST CELL WALL PREBIOTICS, *BACILLUS* PROBIOTICS, OR BACTERIOPHAGES

5.1. Introduction

Salmonella is a gram-negative pathogen known to colonize the intestinal tract of poultry and is commonly associated with poultry or poultry products. When considering the serogroups of *Salmonella* that most commonly cause infections, *Salmonella enterica* serovar Infantis historically was not considered a pathogen of concern. However, in recent years, *Salmonella* Infantis has become a pathogen of greater concern, with multidrug-resistant strains being isolated from outbreaks in 32 states across the United States (CDC, 2019). Additionally, in 2016, a separate *Salmonella* Infantis outbreak occurred involving backyard poultry that caused people to become ill across 13 states (CDC, 2016).

Salmonella Infantis prevalence has been increasing in the past few years and shows no signs of slowing down. In an article published in 2019, *Salmonella* Infantis prevalence was the 4th highest prevalence behind only *Salmonella* Gallinarum (1st), *Salmonella* Enteritidis (2nd) and *Salmonella* Typhimurium (3rd)(Kumar *et al.,*.2019). While 4th in line typically would not be considered highly significant in most cases, for *Salmonella* Infantis to be 4th out of over 2500 known serovars of *Salmonella* is a great point of concern. Therefore, a study was conducted to determine the efficacy of several

commercially available YCW/YC products, *Bacillus* blend products, and bacteriophages at reducing *Salmonella* Infantis colonization in broiler chickens.

5.2. Materials and Methods

5.2.1. Birds, Diet and Management

This study was conducted at the USDA-ARS facility in College Station, TX and was approved by the local Institutional Animal Care and Use Committee (IACUC No. 2019-009).

A total of 240 day-old Cobb-500 straight-run broiler chicks were obtained from a commercial hatchery and transported to the USDA-ARS facility in College Station, TX. Chicks were weighed on day-of-age and divided into pen replicates according to an average pen weight. Treatments were distributed amongst the pens using a randomized complete block design (RCBD). Birds were fed diets containing commercially available pre- and probiotic supplements or had access to water supplemented with bacteriophages (Table 5-2). Birds were observed daily for general flock condition, feed, water and mortality. This study utilized a 2-phase feeding program, in which a starter diet was used for Days 1 to 9 and a grower diet for Days 10 to 21 (Table 5-1). Diets were formulated according to the Cobb-500 nutritional recommendations, and additives were supplemented via feed or water at the rates specified in Table 5-2.

On Day 3, birds were challenged with *Salmonella enterica* serovar Infantis at 2.0×10^6 CFU/mL in 0.5mL by oral gavage. Bird weights and feed consumption were recorded weekly and at feed changes (Figure 5-1). Fecal samples and cloacal swabs were

taken on the same days as bird weights and feed consumption. On Day 14, one bird per pen was removed, euthanized, and necropsied for desired samples including ceca, liver and spleen. On Day 21, all remaining birds were euthanized, and samples obtained utilizing the same methods as on Day 14.

Table 5-1 - *Salmonella* Infantis Challenged Broilers Diet Composition

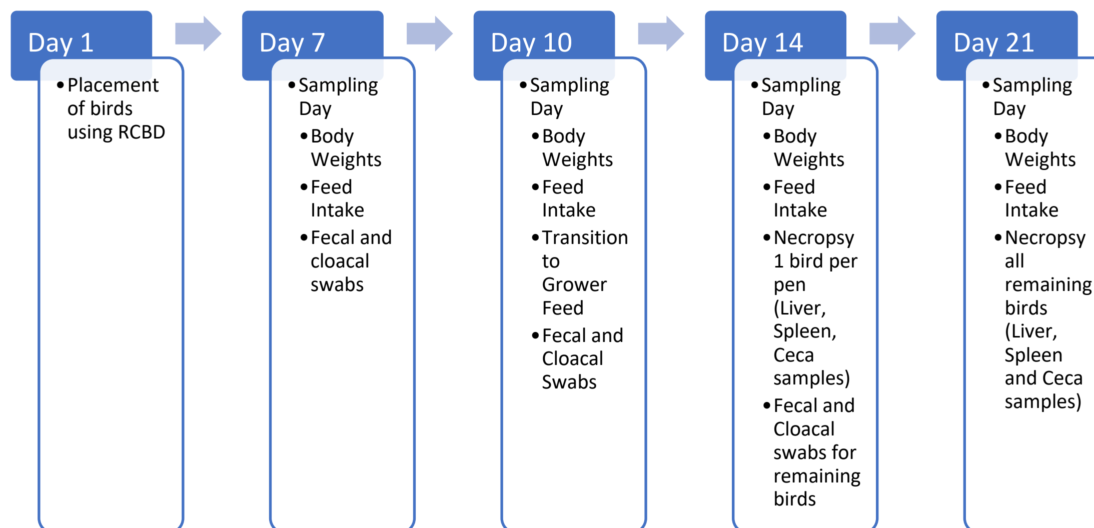
Phase 1 – Broiler Starter		Phase 2 – Broiler Grower	
Ingredients	Percentage	Ingredients	Percentage
Corn	60.83	Corn	64.82
Dehulled Soybean Meal	33.91	Dehulled Soybean Meal	28.92
Limestone	1.47	Limestone	1.44
Mono-Dical Phosphorus	1.54	Mono-Dical Phosphorus	1.43
Salt	0.44	Salt	0.44
Lysine HCL	0.15	Lysine HCL	0.20
DL-Methionine	0.21	DL-Methionine	0.25
L-Threonine	0.06	L-Threonine	0.04
Fat, Animal-Vegetable Blend	1.17	Fat, Animal-Vegetable Blend	2.24
Trace Minerals[1]	0.05	Trace Minerals[1]	0.05
Trace Vitamins[2]	0.175	Trace Vitamins[2]	0.175

Vitamin Premix provided by DSM Animal Nutrition and Health. [1]Per pound of premix; Cu: Copper minimum 1.40%, I: Iodine minimum 800.0ppm, FE: Iron minimum 12.00%, Mm: Manganese minimum 12.00%, Zn: Zinc minimum 12.00%, [2]Per pound of premix; Vitamin A: 4,000,000 IU, Vitamin D3: 1,400,000 IU, Vitamin E: 16,666 IU, Vitamin B12: 6mg, Riboflavin (B2): 2166mg, Niacin (B3): 16,666mg, d-pantothenic acid (B5): 7334mg, Choline: 47383mg, Menadione: 534mg, Folic acid (B9):634mg, Pyridoxine (B6): 2,600mg, Thiamine (B1): 1,066mg, d-Biotin (B7): 200mg

Table 5-2 - *Salmonella* Infantis Challenged Broilers Treatment Groups and Product Application Methods

Treatment	*Salmonella* Infantis Challenge	Bird # per Treatment group	Initial # Birds per pen	# Pens	Application Method
Positive Control	Yes	40	5	8	
Positive Control + MicroSaf Log 5 (500g/T)	Yes	40	5	8	Feed
Positive Control + Safmannan (500g/T)	Yes	40	5	8	Feed
Positive Control + Diamond V XPC (1250g/T)	Yes	40	5	8	Feed
Positive Control + Bacteriophages (40mL/10L of drinking water daily)	Yes	40	5	8	Water
Positive Control + LY26-50a Log 7	Yes	40	5	8	Feed
Total Birds in Study		240		48	

Figure 5-1- *Salmonella* Infantis Challenged Broilers Trial Schedule



5.2.2. Prevalence Determination

Fecal and cloacal samples obtained on all feed days (7, 10 and 14) were used for determination of *Salmonella* prevalence post-inoculation. Fecal and cloacal samples were placed into conical tubes containing RV broth, homogenized by shaking, and then incubated at 42°C for 24 hours. After 24 hours of incubation, samples were again homogenized by shaking, and a 10 µL sterile loop was used to streak plate a sample onto XLT-4 agar. Plates were then incubated for 24 hours at 37°C, and samples were deemed positive by visual identification of colonies on the plate after incubation. Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 24 hours of incubation.

Samples obtained on Days 14 and 21 for *Salmonella* prevalence included ceca, liver and spleen. Ceca pair samples were divided in half upon necropsy, and one ceca was used for prevalence determination. Samples were placed into conical tubes containing RV broth, homogenized by shaking, and incubated at 42°C for 24 hours. After 24 hours of incubation, samples were again homogenized by shaking, and a 10 µL sterile loop was used to plate a sample onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) for use as a selective growth media using a spread plating method. Plates were then incubated at 37°C for 24 hours, and samples were deemed positive by visual identification of colonies on the plate after incubation. Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 24 hours of incubation.

5.2.3. Enumeration Determination

In this study, only ceca samples were enumerated. Ceca samples were diluted weight to volume using a 10x dilution series resulting in dilutions of 1:10 to 1:10,000, of which 0.1mL was plated onto XLT-4 agar treated with novobiocin (20mg/mL) and nalidixic acid (25mg/mL) for use as a selective growth media using a spread plating method. Enumeration counts were determined by visual identification of colonies after 48 hours of incubation at 37°C. Colonies were positively identified as *Salmonella* if the colony presented as a black or black-centered circular colony after 48 hours of incubation.

5.2.4. Statistical Analysis

All data obtained during this experiment were analyzed using a one-way Analysis of Variance (ANOVA) and means separated using students t-test were considered statistically significantly different at $p \leq 0.05$. The ANOVA was used to compare observations between the treatment groups for each of the factors (ceca, liver & spleen prevalence, performance parameters) observed during this study noted in the materials and methods section. Bonferroni multiple comparisons were used to make all pairwise comparisons in results that showed a statistically significant ANOVA ($p \leq 0.05$). Mean and standard deviation values were used in the determination of outliers as well as for comparisons between treatment groups. Enumeration values were log transformed prior to analysis. These analyses were conducted for each of the sampling time points noted in Figure 5.1 All data were analyzed using Stata v.16.1 (StataCorp, College Station, TX).

5.3. Results and Discussion

5.3.1. Performance Parameters

All data obtained in this study was done so by the methods specified in the materials and methods section of this chapter and analyzed according to the statistical methods detailed previously. When reviewing phase average body weight per bird, no significant differences were seen across any of the observation timepoints. However, notably the Safmannan YCW and XPC YC treatments showed an interesting trend of compensatory growth in the later phases of production, starting out similarly to the control treatment through Day 10, and ending with a 20g+ benefit when compared to the control treatment (Table 5-3).

Table 5-3 – ANOVA Comparing Phase Average Body Weight (g) Across Days

Treatment	D0 (g) ±SD	D7 (g) ±SD	D10 (g) ±SD	D14 (g) ±SD	D21 (g) ±SD
Control	42.0 ±1.2	118.2 ±17.5	203.3 ±23.1	370.2 ±46.3	809.7 ±88.3
Safmannan	42.2 ±0.8	116.0 ±13.2	204.7 ±15.3	374.6 ±26.6	834.5 ±59.7
Microsaf	41.5 ±1.0	123.8 ±10.1	210.1 ±14.4	378.2 ±25.5	808.4 ±27.0
XPC	41.2 ±1.1	118.2 ±9.0	206.4 ±9.3	377.3 ±16.5	834.3 ±52.2
Bacteriophages	41.8 ±1.2	117.2 ±12.2	199.1 ±14.2	366.6 ±31.0	804.9 ±47.8
LY 26-50a	42.2 ±0.8	118.8 ±7.4	197.3 ±15.1	359.0 ±23.8	815.2 ±106.5
P-Value	0.3394	0.8713	0.6416	0.7957	0.9169
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

In reviewing the phase average weight gain per bird, no significant differences were noticed, and the main nominal changes observed were only between treatments,

rather than between the control groups and a specific treatment. (Table 5-4). However, as noted previously in the average body weight data, both the Safmannan YCW and the XPC YC exhibited a trend towards compensatory growth in the later phases of production.

Table 5-4 – ANOVA Comparing Phase Average Weight Gain per Bird (g) Across Days

Treatment	D0-7(g) ±SD	D7-10 (g) ±SD	D10-14 (g) ±SD	D14-21 (g) ±SD
Control	76.2 ±16.5	85.2 ±9.0	166.9 ±26.1	428.6 ±46.4
Safmannan	73.8 ±13.4	88.7 ±9.4	169.9 ±12.1	445.5 ±38.1
Microsaf	82.4 ±9.6	86.2 ±5.7	168.1 ±12.0	423.3 ±21.9
XPC	77.0 ±8.7	88.2 ±6.4	170.9 ±8.9	446.7 ±38.5
Bacteriophages	75.5 ±12.2	81.8 ±6.5	167.5 ±20.1	429.3 ±28.1
LY 26-50a	76.6 ±7.4	78.5 ±8.7	161.8 ±10.4	446.1 ±94.8
P-Value	0.8151	0.0936	0.9044	0.8900
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

Table 5-5 – ANOVA Comparing Phase and Cumulative Average Feed to Gain Ratio Across Days

Treatment	D0-7 ±SD	D7-10 ±SD	D10-14 ±SD	D14-21 ±SD	Cumulative ±SD
Control	1.22 ±0.08	1.22 ±0.09	1.30 ±0.05	1.39 ±0.08	1.33 ±0.05
Safmannan	1.23 ±0.12	1.20 ±0.14	1.40 ±0.28	1.40 ±0.13	1.35 ±0.12
Microsaf	1.19 ±0.05	1.24 ±0.06	1.41 ±0.12	1.44 ±0.11	1.37 ±0.07
XPC	1.21 ±0.07	1.17 ±0.04	1.31 ±0.05	1.40 ±0.13	1.32 ±0.07
Bacteriophages	1.17 ±0.16	1.21 ±0.08	1.29 ±0.04	1.33 ±0.04	1.29 ±0.03
LY 26-50a	1.17 ±0.05	1.27 ±0.08	1.31 ±0.60	1.32 ±0.14	1.29 ±0.09
P-Value	0.7227	0.3165	0.9391	0.2744	0.2558
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

In reviewing the feed to gain and feed to weight ratios, no significant differences were noticed. However, in the phase feed to gain ratio, cumulatively the bacteriophages

and live yeast treatments resulted in a 4-point (0.04) benefit when compared to the control treatment (Table 5-5). Similar differences were noticed in the feed to weight ratio, except in this case all treatments resulted in slightly improved feed to weight ratios when compared to the control. In this case, the bacteriophages and live yeast treatment mentioned previously resulted in a 12-point (0.12) and 10-point (0.10) benefit when compared to the control treatment (Table 5-6).

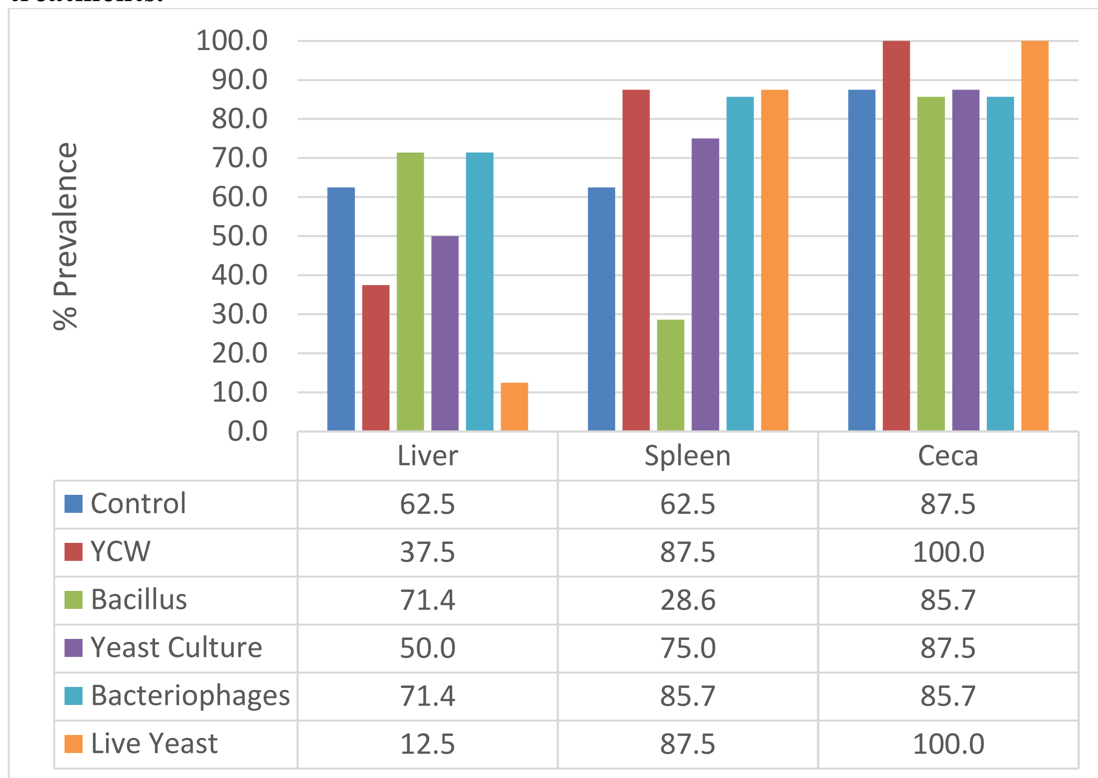
Table 5-6 – ANOVA Comparing Cumulative Average Feed to Weight Ratio Across Days

Treatment	D0-7 ±SD	D0-10 ±SD	D0-14 ±SD	D0-21 ±SD
Control	0.767 ±0.042	0.951 ±0.024	1.109 ±0.023	1.440 ±0.186
Safmannan	0.773 ±0.059	0.948 ±0.055	1.146 ±0.137	1.401 ±0.126
Microsaf	0.789 ±0.019	0.972 ±0.023	1.167 ±0.053	1.433 ±0.077
XPC	0.779 ±0.042	0.943 ±0.027	1.109 ±0.031	1.379 ±0.079
Bacteriophages	0.718 ±0.102	0.916 ±0.09	1.082 ±0.037	1.317 ±0.058
LY 26-50a	0.753 ±0.051	0.957 ±0.032	1.120 ±0.273	1.343 ±0.201
P-Value	0.2532	0.2120	0.8452	0.4058
N	8 pens/trt	8 pens/trt	8 pens/trt	8 pens/trt

5.3.2. Ceca, Liver & Spleen Prevalence

No significant differences were noted in either of the sampling days in which prevalence data was taken. On Day 14, Safmannan showed a reduction in liver prevalence, and Microsaf showed a reduction in spleen prevalence when compared to the control treatments (Figure 5-2). Other treatments showed similar reductions in one or more of the factors observed, however, there was little consistency between all three factors and the treatments regarding prevalence reduction.

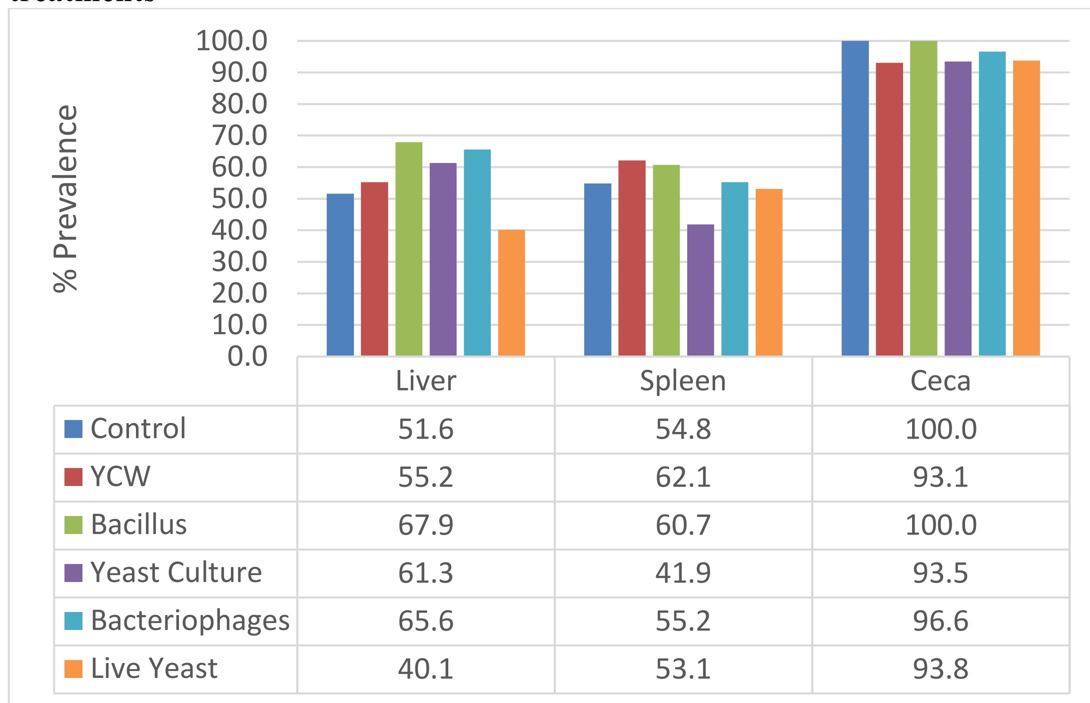
Figure 5-2 - Day 14 *Salmonella* Infantis Prevalence (%) across sample types and treatments.



	Liver	Spleen	Ceca
Control	62.5	62.5	87.5
YCW	37.5	87.5	100.0
Bacillus	71.4	28.6	85.7
Yeast Culture	50.0	75.0	87.5
Bacteriophages	71.4	85.7	85.7
Live Yeast	12.5	87.5	100.0

On Day 21, while not significant, Safmannan showed a reduction in ceca prevalence compared to the control group. Microsaf showed no reductions in colonization across all three of the factors observed at Day 21. Only the live yeast showed consistent prevalence reductions across all three factors analyzed at Day 21 (Figure 5-3).

Figure 5-3 - Day 21 *Salmonella* Infantis Prevalence (%) across sample types and treatments



	Liver	Spleen	Ceca
Control	51.6	54.8	100.0
YCW	55.2	62.1	93.1
Bacillus	67.9	60.7	100.0
Yeast Culture	61.3	41.9	93.5
Bacteriophages	65.6	55.2	96.6
Live Yeast	40.1	53.1	93.8

5.3.3. Ceca Enumeration

Although not significant, at Day 14 Safmannan, resulted in a reduction of >0.5 logs in *Salmonella* Infantis load in the ceca compared to the control treatment. All other treatment groups exhibited higher levels of colonization compared to the control treatment at Day 14 (Table 5-7). Similar reductions were noted at Day 21 in which the Safmannan, XPC, Bacteriophages and Live yeast treatments resulted in reductions in *Salmonella* Infantis colonziation in the ceca when compared to the control group (Table 5-8).

Table 5-7 – ANOVA Comparing Day 14 *Salmonella* Infantis Log_{10} CFU/g of Cecal Content Across Treatments

Treatment	Log_{10} CFU/g	Std. Err.	95% Conf. Interval
Control	1.73	.8251	[0.011, 3.455]
Safmannan	1.17	.8251	[-0.546, 2.898]
Microsaf	2.30	.9109	[0.462, 4.144]
XPC	1.88	.8521	[0.160, 3.605]
Bacteriophages	2.82	.9109	[0.982, 4.664]
LY 26-50a	1.93	.8521	[0.206, 3.650]
	P=0.8506		

Table 5-8 – ANOVA Comparing Day 21 *Salmonella* Infantis Log_{10} CFU/g of Cecal Content Across Treatments

Treatment	Log_{10} CFU/g	Std. Err.	95% Conf. Interval
Control	2.79	0.205	[2.388, 3.199]
Safmannan	2.06	0.212	[1.645, 2.483]
Microsaf	2.86	0.216	[2.435, 3.287]
XPC	2.16	0.205	[1.755, 2.566]
Bacteriophages	2.33	0.212	[1.913, 2.751]
LY 26-50a	2.74	0.202	[2.344, 3.142]
	P=0.0194		

Treatment Comparison	Day 21 Bonferroni P>|t|
Microsaf vs Control	0.216
Safmannan vs Control	1.000
Microsaf 125 + Safmanann 125 vs Control	0.457
Microsaf 125 + Safmannan 250 vs Control	1.000
Bacteriophages vs Control	1.000
Safmannan vs Microsaf	0.138
Microsaf 125 + Safmannan 125 vs Microsaf	1.000
Microsaf 125 + Safmannan 250 vs Microsaf	1.000
Bacteriophages vs Microsaf	0.325
Microsaf 125 + Safmannan 125 vs Safmannan	0.298
Microsaf 125 + Safmannan 250 vs Safmannan	1.000
Bacteriophages vs Safmannan	1.000
Microsaf 125+Safmannan 125 vs Microsaf 125+Safmannan 250	1.000
Microsaf 125+Safmannan 125 vs Bacteriophages	0.670
Microsaf 125 + Safmannan 250 vs Bacteriophages	1.000

5.3.4. Discussion

Yeast components such as yeast fractions and yeast cell wall have a proven affinity for pathogen binding within the intestinal tract of poultry, which ultimately results in their removal instead of colonization (Posadas *et al.,* 2017, Akhtar *et al.,* 2016, Kiros *et al.,* 2019). However, not all yeast products are created equal, which is where the larges differentiation can be found between yeast cell wall (Safmannan) and yeast cultures (XPC). The primary difference between the two stems from the content of mannans and β-glucans; Safmannan contains a minimum 20% of each guaranteed, while XPC does not. Since these are the portions of the yeast fraction that initiate both immune responses and pathogen binding, a higher content will likely result in a greater reduction in *Salmonella* load. In this case, that is seen, with ceca enumeration being decreased by over 0.7 logs at Day 21 in Safmannan compared to the control. While these values were not statistically significant, they are a biological indicator that products such as Safmannan have an ability to reduce *Salmonella* colonization.

The other treatments such as the *Bacillus* product Microsaf, bacteriophages, and the live yeast LY26-50a did not perform as well and only showed marginal benefit. Each one possessed separate issues which likely led to less efficacy in reducing the negative effects of the *Salmonella* Infantis challenge. Regarding Microsaf, the largest issue with the product came from product dispersibility due to its fine powder carrier. The carrier on the MicroSaf used in this experiment as well as the other broiler experiment was extremely fine, with particle size ranging from 12-20 microns. Because of this, the

powder tended to clump during the mixing process, likely leading birds to receive inadequate or incorrect amounts of product based on its intended use.

The bacteriophages and the live yeast (LY26-50a) possessed similar issues related to product efficacy. The bacteriophages used in this experiment as well as the other experiments are sensitive to high temperatures and must be stored in a cold environment. In the early growth phases of the broilers, especially around the time that the birds were challenged with *Salmonella* Infantis it was hot, typically around 90°F, which causes the product to degrade, thus losing efficacy. It is likely that this, combined with the low inclusion rate to start, resulted in a loss of efficacy. The live yeast on the other hand suffered from the exact opposite problem. Due to the high pelleting temperatures that broiler feeds are subjected to, the live yeast was protected by being formed in a microspherule form. During the pelleting process, the exterior yeast would be killed, and the interior would survive. However, due to the short time in the intestinal tract, it is unlikely that the exterior yeast would be removed, and the interior live yeast would be able to exhibit any sort of effect. In short, it's form that was meant to assist it in surviving to the intestinal tract was too great of a protectant and reduced its efficacy.

With *Salmonella* Infantis continuing to grow annually in outbreaks and prevalence, it is important that we pay attention to methods that are proven to reduce its prevalence and load in broilers. As more serovars are controlled through alternative methods, such as vaccination for *Salmonella* Enteritidis, serovars such as *Salmonella* Infantis will move in to fill the niche left empty through their removal. In order to reduce the possibility of this occurring, research on how to control this serovar, as well as many

others is necessary. Overall, this study is in agreement with previous studies showing that Safmannan yeast fraction supplementation results in reduction of *Salmonella* load in the ceca (Price *et al.,* 2020, Price *et al.,* 2020, Kiros *et al.*, 2019). While reductions in this case were not statistically significant, Safmannan did show a biological indication towards its ability to reduce *Salmonella* load in the ceca, as well as reductions in systemic prevalence. This in turn resulted in slightly higher body weights, and improved feed to weight ratios at the end of the study. Cumulatively, these results show again that supplementation of the Safmannan yeast fraction possesses an excellent potential as a food safety intervention in the commercial broiler industry.

6. CONCLUSION

For decades, the use of antibiotics in commercial poultry production for improved growth performance and control of infectious diseases was common (Gadde *et al.,* 2018). However, in the current marketplace, it is more common for prebiotics and probiotics to take their place, with antibiotics being used sparingly and only in specific situations under advisement of a veterinarian. With a growing consumer movement shifting the market towards antibiotic-free production, alternatives are necessary to help continue to meet the needs of a growing population.

In order to effectively implement prebiotics and probiotics into the commercial production pipeline, several factors must be addressed. Those include product efficacy across a wide range of challenge situations, product stability, and in-vitro compatibility in birds. Not only do they have to meet all of these characteristics, but they also must be affordable as in commercial poultry production feed ingredients and manufacturing account for roughly 75% of the production costs. Therefore, the objective of this project was to evaluate the efficacy of two commercially available pre- and probiotic products at mitigating *Salmonella* in broilers and laying hens. We evaluated how these products were able to affect *Salmonella* colonization and translocation in both broilers and laying hens across 5 different *Salmonella* serovars which are prevalent within commercial poultry production.

From the experiment in chapter II, it can be concluded that the use of YCW at 500ppm in early production laying hens is effective at reducing *Salmonella* Typhimurium colonization in the ceca, as it was reduced by over 1 log when compared

to the control treatment. This reduction represents both a statistically significant ($p=0.033$) and biologically relevant reduction in *Salmonella* load in the ceca. The same YCW treatment achieved similar results in chapter III, in which it affected *Salmonella* Enteritidis colonization in the ceca of late production laying hens, effectively reducing colonization by over 1 log in an individual room and approached a 1 log reduction cumulatively. The *Bacillus* treatment showed similar reductions in a single room, reducing *Salmonella* Enteritidis colonization by 1 log.

Minimal benefits were noted in chapter III with the *Salmonella* Typhimurium challenge, in which none of the treatments were able to reduce *Salmonella* load in the ceca by a statistically significant amount. At Day 14, the YCW treatment resulted in a biologically relevant reduction of *Salmonella* Typhimurium load, effectively reducing cecal load by over 1.3 logs. In chapter IV the YCW treatment was able to achieve slightly smaller nominal reductions in *Salmonella* Infantis colonization, with approximately 0.6 and 0.7 log reductions achieved at Days 14 and 21 respectively. This is especially important due to *Salmonella* Infantis' rising prevalence in commercial poultry production. As more *Salmonella* serovars are controlled through vaccination and other methods, serovars such as *Salmonella* Infantis will proliferate to fill the niche left empty.

While these results have varied by experiment, much of this variability can be attributed to the serovar of *Salmonella* that was used. This is especially important to know for product development and implementation. Not all products will control each serovar the same, and not all serovars can be controlled in the same method.

With *Salmonella* infections and outbreaks related to poultry continuing to be an issue, and no sure-fire blanket control method in sight, it is obvious that more work will be needed in the coming years. However, for this dissertation both yeast fraction/yeast cell wall prebiotics and *Bacillus* probiotics are a potential option for use in a multi-step approach towards controlling *Salmonella*. The use of yeast fractions and *Bacillus* showed positive benefits, though not always significant, across multiple strains of *Salmonella* in challenge situations, both in broilers and laying hens. The data has shown positive benefits including reductions in prevalence and cecal *Salmonella* loads, which is being mirrored at this time in large-scale industry field trials. However, further research will be needed as both the serovars of *Salmonella* and poultry genetics continue to evolve in order to feed a hungry world.

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