The anti-Salmonella and immunostimulatory properties of yeast-based postbiotics in poultry and cattle

Kristina Marie Feye
Iowa State University

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The anti-Salmonella and immunostimulatory properties of yeast-based postbiotics in poultry and cattle

by

Kristina M. Feye

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biomedical Sciences

Program of Study Committee:
Steven Carlson, Major Professor
Timothy Day
(Heather) Mary Greenlee
Ronald Griffith
Michael Kimber

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

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DEDICATION

I would like to dedicate this dissertation to the two people that made this dream a reality—my mentors Dr. Steve Carlson and Dr. Kristi Anderson. Without either of you, I would not have had the opportunity to pursue, let alone finish, a Ph.D. I admire you both more than I can ever put into words, though, admittedly, I try to a few pages from here.

I would also like to expand on the dedication of dissertation to include Nani Cooper and the late Dr. Sarah McIntire. In order to satisfy TWU’s credit requirements for graduation with as little effort as possible, I signed up for a research course in Microbiology. My expectations were an easy “A” and free food at the Undergraduate Symposium. This erroneous decision altered the course of my life both tremendously and unexpectedly. As a result, I found an unexpected passion and here we are.

At both stages of my career, I was lucky to find mentors who both recognized my potential and gave me the tools to rise to the occasion. While the circumstances were unique to each stage of my academic career, the impact was not. Thank you.
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“And now, for something completely different…”

~John Cleese
Saccharomyces cerevisiae fermentation metabolites have been used for over a century as feed additives to improve the feed efficiency of food-producing animals. During the routine microbiological monitoring of poultry houses, reports emerged indicating a significant decrease in Salmonella recovered from houses that recently contained broilers fed a yeast fermentation metabolite product (XPC\textsuperscript{TM}) when compared to houses that recently harbored birds fed a conventional diet. Preliminary studies evaluated the scope and breadth of this discovery, as well as the underlying mechanisms. Based on these studies, we developed the hypothesis (central to this dissertation) that XPC has anti-Salmonella properties stemming from the immunomodulation of the mucosal immune system, possibly at the bridge between the adaptive and innate immune systems. Addressing this hypothesis involved field studies evaluating the anti-Salmonella effects of XPC in cattle and poultry naturally infected with the pathogen. Additional studies investigated the same parameters in experimentally infected animals. These studies revealed that supplementing animals with XPC resulted in a significant reduction in the shedding and colonization of Salmonella, a reduction of integron- and plasmid- associated antibiotic resistance in input and naturally occurring Salmonella isolates, and the attenuation of pathogenicity of isolates obtained in both the field and laboratory settings. Further studies explored the potential mechanism underlying the immunomodulatory effects of XPC after pilot studies revealed that leukocytes recovered from XPC-treated animals demonstrated an enhanced phagocytic clearance of Salmonella. Additionally, gene expression studies revealed that this clearance phenomenon may be linked to enhanced local complement C3 expression observed in XPC-fed birds. Enhanced C3 production will lead to elevated production of complement C5, whose cognate
receptor is a druggable G-protein coupled receptor C5aR1 that plays a central role in enhancing the phagocytic capabilities of innate effector cells. Thus, a series of in vivo experiments were conducted in order to ascertain the specific role that XPC plays in the improvement of innate immune activity via C5aR1. Resulting evidence suggests that C5aR1 may play a novel role in the immunostimulatory efficacy of XPC, which enhances the clearance of Salmonella. Therefore, this thesis provides evidence supporting the anti-Salmonella and immunostimulatory effects of XPC.
CHAPTER 1. INTRODUCTION

Since the formation of agrarian societies, farmers have faced numerous challenges associated with food animal production and management. Ultimately, the goal of animal agriculture is to produce animals capable of yielding high-quality meat (Willems et al., 2013), eggs (Herrero et al., 2013; Willems et al., 2013), and dairy products (Hayes et al., 2013) in a cost-efficient manner. A common metric associated with achieving this aim is termed feed efficiency, which is loosely defined as a reduction in the lifetime feed consumption of an animal while either maintaining or enhancing growth curves or yields (Herd and Arthur, 2009; Herrero et al., 2013). Yeast fermentation metabolite byproducts have been used by producers for nearly a century to improve the production efficiencies of poultry, swine, meat goats, cattle, and aquaculture (Alizadeh et al., 2016; Chaucheyras-Durand and Durand, 2010; Chou et al., 2017; E. Kairie, 2015; Feye et al., 2016; Hippen et al., 2010; Lensing et al., 2012; Mussatto et al., 2006; Poppy et al., 2012; Price et al., 2010; Titi et al., 2008; Xiao et al., 2016).

A novel and unintended effect associated with the addition of yeast fermentation byproducts as a component of animal feed was discovered a few years ago through the routine microbiological surveying of poultry barns. Farms began to use yeast fermentation metabolites, sold under the tradename XPC™, as an in-feed additive to enhance feed efficiency in production poultry. As a matter of standard practice, these barns were routinely monitored for environmental *Salmonella* during the cleaning phase in between production flocks (unpublished). Evidence collected from generations of XPC-treated poultry suggested that within a few generations, there was a reduction of the prevalence of birds naturally infected *Salmonella* (unpublished). Investigations were consequently initiated in order to
understand the potential applications, depth, and the underlying mechanisms associated with the ability of XPC to mitigate *Salmonella*.

The central hypothesis of this dissertation is that XPC has properties that both reduce *Salmonella* virulence and enhance the innate immune clearance of the pathogen. Specifically, the enhanced immunological function converges at the G-protein coupled receptor C5aR1, which is the cognate receptor for the C5a complement protein. This receptor serves not only as an important crossroad for the Host-*Salmonella* interaction, but also the nexus between the innate and adaptive immune system. The resulting dissertation is an important contribution to our understanding of how yeast fermentation byproducts attenuate the pathogen as well as augment the innate immune system to enhance the clearance of pathogens. In order to address this hypothesis, this thesis was executed and organized into the following six chapters:

**Chapter 2:** A literature review documenting our current understanding of the threat of *Salmonella*, the application of XPC, and the immunobiology of the host-pathogen axis.

**Chapter 3:** A ready-for-publication review outlining the history of XPC as well as the potential mechanisms associated with the efficacy of the product.

**Chapter 4:** A published field study in beef cattle examining the reduction of *Salmonella* and *Escherichia coli* H7:O157 fecal shedding and lymph node infiltration of *Salmonella* in animals fed an XPC derivative.

**Chapter 5:** A published study that evaluated the anti-*Salmonella* effects of XPC in broilers.

**Chapter 6:** A series of experiments designed to explore the underlying mechanisms governing the enhanced phagocytic clearance of *Salmonella enterica*. Specifically, this
investigation examined the role of the druggable G-protein coupled receptor C5aR1, which putatively enhances the clearance of *Salmonella* from broilers on an XPC containing diet.

**Chapter 7:** The thesis concludes with a discussion of our findings and the context of our discoveries. The future directions of this research are also outlined in this final section.

Through these investigations, we deliver to the agricultural industry a renovation of an old, proven tool that enhances feed efficiency while simultaneously eliciting changes in *Salmonella* and the immune response. In doing so, we continue the mission to further enhance food animal production and safety.
CHAPTER 2. LITERATURE REVIEW

2.1 The Threat of *Salmonella*

Non-typhoidal *Salmonella* is the leading cause of foodborne illness in the U.S. Direct exposure to raw meat, animal products, or cross-contamination events account for 95% of salmonellosis cases (Finstad et al., 2012; Mead et. al., 1999). The most common *Salmonella* serovars implicated in foodborne illness are Enteritidis, Typhimurium, Heidelberg, Newport, and, in poultry, Kentucky (Finstad et al., 2012). The instances of salmonellosis are consistent despite the implementation of new, data-driven mitigation strategies proposed as a result of advanced surveillance networks such as FoodNet and PulseNet (Besser, 2017; Finstad et al., 2012; Roto, et al., 2016). Likely, the sustained instance of *Salmonella* underscores the pervasive nature of the pathogen (Besser, 2017).

In the United States, poultry and cattle are the main reservoirs for *Salmonella*. In poultry, *Salmonella* is a commensal organism (Chambers and Gong, 2011; Wigley, 2014). *Salmonella* contaminates poultry products by two routes: directly invading the reproductive tract and contaminating eggs, or sulllying meat and skin during de-feathering and evisceration at the plant (Carrasco et al., 2012; Foley et al., 2013). Poultry are recognized as the largest reservoir for *Salmonella* and abating this threat is a significant economic burden for poultry producers (Besser, 2017; Carrasco et al., 2012). Beef cattle are also recognized as an emerging reservoir for *Salmonella* (Brichta-Harhay et al., 2008; Gragg et al., 2013; Terrance M. Arthur, 2008). While some serovars (e.g., Dublin) can cause disease in cattle, other serovars of *Salmonella* are asymptptomatically carried (Gragg et al., 2013, Selander et al., 1992). Peripheral lymph nodes have been found to contain *Salmonella*, even in asymptomatic animals (Gragg et al., 2013; Arthur et al., 2008). These lymph nodes are
commonly incorporated into ground beef and are an emerging reservoir for *Salmonella* (Gragg et al., 2013; Arthur et al., 2008).

Clinical salmonellosis is complicated by the emergence of multiple drug resistant strains and the transference of antibiotic resistance genes to other organisms. Antibiotic-resistant *Salmonella* account for 10-12% of *Salmonella* isolates identified through NARMS (National Antimicrobial Resistance Monitoring System) (Schwarz et al., 2016). This impacts the treatment of elderly, young, and immunocompromised patients who are at risk for bacteremia requiring antimicrobial therapy (Coburn et al., 2007; Kurtz et al., 2017; Tadesse et al., 2016). *Salmonella* belongs to the family *Enterobacteriaceae*, which characteristically propagates antibiotic resistance genes through all known mechanisms of horizontal gene transfer (Huddleston et al., 2014). *Salmonella Typhimurium* phagetype DT104 serves as a notable example of this problem (Leekitcharoenphon et al., 2016). Many isolates of DT104 contain an integron conferring resistance to ampicillin, chloramphenicol, sulfonamides, streptomycin, and tetracycline (Tadesse et al., 2016). The rise in antibiotic-resistant salmonellosis is difficult to mitigate because mobile genetic elements like *SGII* contain an extraordinarily stable toxin-anti-toxin system (Huguet et al., 2016; Michael and Schwarz, 2016). The toxin-anti-toxin system prohibits the loss of the element once it is incorporated into the genome, thus enabling a sustained resistance to antibiotics (Huguet et al., 2016).

Therefore, *Salmonella* poses additional challenges that are difficult to abate. Pre-harvest control measures are ideally pursued as current post-harvest strategies have not curbed the vertical transmission of *Salmonella* (Jones, 2011). Accordingly, new strategies must diminish *Salmonella* from major reservoirs at the farm.
In the early 20th century, yeast fermentation products were discovered to improve production gains (J.J. Dibner, 2005). One of these products, XPC, exhibits strong anti-
Salmonella effects. These anti-Salmonella effects include reducing virulence, shedding, colonization, and antibiotic resistance (Feye et al., 2016). Furthermore, XPC is able to surpass the benefits of antibiotics by improving the immune response and restoring antibiotic susceptibility in foodborne pathogens. Therefore, XPC has the significant potential to be the next keystone in-feed additive in food animal production and management.

2.2 XPC and its Production Benefits

In the early 20th century, Diamond V discovered that dairy cattle fed XPC had increased milk production (Embria Health, 2013). Using XPC has resulted in significant improvement in feed conversion ratios, a stabilization of the gut-microbiome axis, and anti-pathogenic effects in chickens, turkeys, cattle, and pigs. Furthermore, food animals fed XPC showed enhanced production efficiency and robustness in the face of biotic stressors (such as infectious diseases) and abiotic stressors (such as heat stress) (Feye et al., 2016; Gao et al., 2008; Lensing et al., 2012; Osweiler et al., 2010; Price et al., 2010; Roto et al., 2017; S. Kumar, 2017; Xiao et al., 2016). Importantly, recent discoveries indicate that XPC can be used to conciliate the threat of antibiotic resistance (Feye et al., 2016).

Diamond V employs a vertically integrated process, starting with a standard strain of Saccharomyces cerevisiae to homogenously produce XPC. S. cerevisiae are grown aerobically in complete media until the culture is in log phase (Nadler, 2011). At that time, it is mixed with Distiller’s grains and transferred to the anaerobic fermentation vats. Molasses was chosen as the carbohydrate source for anaerobic fermentation because it provides a complete source of monosaccharides required by Saccharomyces cerevisiae (Nadler, 2011).
The culture ferments anaerobically until metabolic activities cease. The media is then collected, desiccated, and packaged (Nadler, 2011). While the components of XPC are unknown, the metabolites produced by *S. cerevisiae* are likely influenced by the composition of the media used during anaerobic fermentation (Tiago et al., 2012; van der Aa Kuhle et al., 2005). All of the available products that Diamond V sells are derivations of the original XPC formulation (Nadler, 2011).

The production benefits of XPC have been assessed in numerous studies. Several studies indicate that, XPC improves meat, milk, and egg yield and quality in cattle and poultry (Alugongo et al., 2017; Brewer et al., 2014; Scott et al., 2015; Wagner et al., 2016; Xiao et al., 2016). Figure 2.1 summarizes how the use of XPC improves food animal health and production. The basis of these improvements stems from improved gut physiology, enhanced nutrient absorption, and beneficial changes to the microbiome. As a result, food animals are healthier, grow more efficiently, and are more resistant to stress.

The production gains are remarkable and beneficial throughout the life of cattle fed XPC (Scott et al., 2015). The offspring of cattle on an XPC-containing diet are profoundly healthier with higher weaning weights (Wagner et al., 2016). XPC-fed calves are also culled less due to weaning or shipping stress and respiratory infections (Scott et al., 2015; Wagner et al., 2016). The continued use of XPC results in higher quality meat in beef cattle and milk in dairy cattle (Wagner et al., 2016). These production benefits stem from improved rumen development with a concordant improvement in nutrient absorption (Alugongo et al., 2017; Brewer et al., 2014; Scott et al., 2015; Wagner et al., 2016; Xiao et al., 2016). Moreover, cattle show greater microbial richness within the rumen, further improving its function (Xiao
et al., 2016). Because cattle acquire 80% of their energy from cellulolytic bacteria in the rumen, this XPC-mediated enhancement is significant (Xiao et al., 2016).

Additionally, the anti-pathogenic effects of XPC in cattle are now emerging in field studies. Cattle on XPC have fewer liver lesions when compared to tylosin-fed controls, resulting in fewer condemnations at the packing plant (Scott et al., 2015). Furthermore, there are anti-Salmonella effects whereby XPC yields an attenuated Salmonella and reduces prominent serovars like Dublin (Brewer et al., 2014; Feye et al., 2016).

The XPC-mediated improvements in physiology and production in cattle have been paralleled in poultry. As with cattle, the superior nutrient absorption of poultry on an XPC-containing diet results from better gastrointestinal physiology (Kidd et al., 2013). Interestingly, the energy demanding metabolism of chickens requires resident microorganisms residing in the ceca to harvest short chain fatty acids. This metabolism accounts for 25% of the nutritionally derived energy for the chicken (Roto et al., 2015). This effect is tied to increased market weights and superior breast quality of broilers (Kidd et al., 2013). In addition to improved feed efficiency, poultry show enhanced reproductive efficiency. Hens fed an XPC-containing diet produce more fertile eggs with reduced contamination, resulting in healthier offspring entering the food supply (Kidd et al., 2013). Further, layers shed less Salmonella which effectively reduces the reservoir potential of eggs (unpublished data). In meat birds, broilers and turkeys show a reduction in shedding and colonization of Salmonella. Taken together, data indicates that there is not a commercial breed or species-specific effect of XPC (unpublished data). The ability of XPC to mitigate the threat of Salmonella is increasingly validated and vital as poultry are the leading source of Salmonella contamination.
While the production benefits of XPC are established, the mechanisms behind its efficacy are emerging. Emerging research indicates that there are beneficial augmentations to host immunobiology that result in improved food animal health. The first layer of the immune system is the barrier functions, and the use of XPC directly enhances gastrointestinal barrier integrity by improving the strength of the gap junctions between enterocytes (Possemiers et al., 2013). This barrier function is further protected by an increased production of non-specific innate proteins, such as lysozymes and IgM (Evans et al., 2012; Jensen et al., 2007; Jensen et al., 2011; Possemiers et al., 2013). Within the innate immune system, phagocytes show more active immunophenotypes (Chou et al., 2017; Jensen et al., 2007; Possemiers et al., 2013). There is also an increase in the production of chemokines and cytokines that assist in recruiting and activating effector cells to clear the invading pathogen (Evans et al., 2012; Jensen et al., 2007; Jensen et al., 2011; Possemiers et al., 2013). Interestingly, while XPC activates innate cells, it also beneficially tunes the inflammatory responses resulting in less non-specific inflammation (Jensen et al., 2007; Jensen et al., 2011). Finally, cell-mediated and humoral immunity are improved in animals on XPC (Chou et al., 2017). Altogether, the tuned immunology of both branches of the immune system likely contribute to a healthier food production animal.

The surprising consequence of using XPC is the reduction of antibiotic resistance. There are some clues as to this effect, though they are poorly understood. Inflammation of the gut results in a bloom of Salmonella and other Enterobacteriaceae (Stecher et al., 2012). This bloom results in a favorable environment for horizontal gene transfer (Stecher et al., 2012). Taken with the established production and immune benefits, XPC produces a multifaceted improvement to the health and efficiency of food animals.
2.3 XPC at the Nexus of *Salmonella* Biology and Immunology

*Salmonella*’s pathogenic assault of the gastrointestinal system requires a distinct series of events that not only alter the microenvironment of the lumen, but effectively shut down the immune system. The complement pathway is a significant component of the immune response to *Salmonella*. Interestingly, the complement pathway is optimized in animals on an XPC-containing diet. As complement proteins are upregulated in animals on an XPC-containing diet, perhaps the repeated activation of this pathway may contribute to the anti-*Salmonella* effects.

*Salmonella* gains entry into the gastrointestinal tract through the host’s ingestion of contaminated food or water. From there, the sole goal of the pathogen is to become intracellular, either through phagocytosis or invasion, which occurs within 15 minutes of oral challenge (Kurtz et al., 2017; van Hemert et al., 2006; Wigley, 2014). *Salmonella* hones to its preferential invasion sites using the Aer and Tsr chemotactic receptors (Coburn et al., 2007). These sites include enterocytes, secretory goblet cells, and microfold cells (M-cells) (Coburn et al., 2007; Keestra-Gounder et al., 2015). The initial inflammatory events are driven by one of three events: engagement of the immune system, (*Salmonella* Pathogenicity Island) SPI-1-mediated inflammation or by the *Salmonella* virulence factors (Keestra-Gounder et al., 2015; Kurtz et al., 2017; van Hemert et al., 2006; Zhang et al., 2017). As a result of the inflammatory reactions of infiltrating neutrophils, thiosulfate is oxidized to tetrathionate by *Salmonella* or by the presence of reactive oxygen species in the lumen (Rivera-Chavez and Baumler, 2015). Tetrathionate is the favored terminal electron acceptor of *Salmonella* and enables it to out-compete resident microorganisms and upregulate its invasion machinery (Rivera-Chavez and Baumler, 2015). Typically, in a stable, non-inflammatory gut, *Salmonella* is inert and weakly competitive (Rivera-Chavez and Baumler,
To reach its full pathogenic potential, *Salmonella* requires inflammation (Rivera-Chavez and Baumler, 2015; van Hemert et al., 2006). However, inflammation is a double-edged sword for *Salmonella*. On one hand, the production of inflammation as a result of the T3SS (Type III Secretion System) SPI-1 engagement of the host intracellular machinery produces inflammation. This activity is imperative to the success of the pathogen as inflammation enhances the pro-*Salmonella* metabolite changes in the lumen, resulting in a bloom in *Salmonella* (Ciraci et al., 2010; Coburn et al., 2007; Endt et al., 2010; Rivera-Chavez and Baumler, 2015; van Hemert et al., 2006). On the other hand, without the engagement of the immune system, the host is unable to resolve the infection (Coburn et al., 2007; Hurley et al., 2014). Therefore, *Salmonella* acts to continuously optimize the inflammatory environment through its T3SS and virulence factors.

The invasion of *Salmonella* into the gastrointestinal lumen is mediated by the T3SS, which acts to both activate and circumnavigate the host immune response in both non-phagocytic and phagocytic cells (Golubeva et al., 2016; Golubeva et al., 2012; Hapfelmeier et al., 2005; Keestra-Gounder et al., 2015; Rivera-Chavez and Baumler, 2015; Serhan et al., 2008). Invasion occurs via either the trigger or zipper mechanism (Velge et al., 2012). Both mechanisms are dependent on the upregulation of *hilA*, the master regulator of the T3SS (Bajaj et al., 1995). As shown in Figure 2.2, the trigger mechanism utilizes an injectisome, or molecular needle, to form a 3.5 nm pore in the host cell (Velge et al., 2012). The injectisome is then the conduit to deliver SPI-1 effector molecules into the cytosol. These molecules target the actin/cytoskeleton machinery that lead to a rippling of the phospholipid bilayer (Velge et al., 2012). Consequentially, *Salmonella* is passively engulfed by the host.
cell (Velge et al., 2012). Additionally, the SPI-1 proteins also target NLRP3 and NLRP4 inflammasomes and the activation of NFκB. These actions ultimately promote inflammation (Raymond et al., 2013; Velge et al., 2012). The second mechanism used by Salmonella to invade host cells is termed the zipper mechanism (Velge et al., 2012). This mechanism is initiated by the RCK protein interacting with an unknown host receptor resulting in receptor-mediated endocytosis (Velge et al., 2012).

Regardless of the invasion mechanism undertaken, Salmonella are then subject to one of two fates: cytosolic replication and survival or encasement within the Salmonella containing vacuole (SCV) (Hapfelmeier et al., 2005; Herrero-Fresno and Olsen, 2017; Keestra-Gounder et al., 2015; Knodler, 2015). The inflammation results in apoptotic cell death as an outcome from the detachment of cells from the extracellular matrix. The dead cells release the bacteria back into the gut and contribute the clinical signs of enterocolitis (Knodler, 2015). Together, this massive release of bacteria and the inflammation strengthens the effects of the invasion machinery and feeds forward the pathogenesis of Salmonella.

Because the initial inflammation is beneficial for Salmonella, the pathogen hijacks this process independent of the T3SS. The Salmonella surface protein PgtE is an outer membrane aspartate protease that cleaves C3 to produce C3a and C3b (Ramu et al., 2007). This can initiate the alternative complement pathway producing C5a which instigates inflammation (Ramu et al., 2007; Schraufstatter et al., 2002) Salmonella is then able to circumnavigate its own death by the proteins Rck, TraT, and PagC that prohibit the formation of the membrane attack complex and prevents lysis (Ramu et al., 2007). Moreover, the Vi capsular polysaccharide found in several serovars prevents the deposition of complement
proteins onto the surface (Keestra-Gounder et al., 2015). Thus, *Salmonella* regularly manipulates or evades the complement pathway.

Once the *Salmonella* are intracellular, the pathogen subverts immune surveillance which reduces inflammatory pathway activation and xenophagy. In order to evade the immune system, the intracellular survival of *Salmonella* requires a series of orchestrated and well-timed T3SS-mediated events (Ciraci et al., 2010; Oshota et al., 2017; Razzuoli et al., 2017; van Hemert et al., 2006). There are two main pathogenicity islands required: SPI-1, whose second-stage induction suppresses the intracellular detection of *Salmonella*; and, SPI-2-mediated quiescence of the SCV (Keestra-Gounder et al., 2015). Immune suppression governed by the SPI-1 effector proteins results in evasion of the intracellular NOD receptors, cessation of xenophagy, and the maturation of the SCV via *Salmonella* proteins like SopB and SteA (Keestra-Gounder et al., 2015; Knodler, 2015; Raymond et al., 2013). If successful, this initial phase of invasion enables the SCV to evade intracellular host pattern recognition receptors (Keestra-Gounder et al., 2015). The SCV will then locate itself between the Golgi apparatus and the endoplasmic reticulum in a SPI-2 mediated quiescent state until reactivation via SPI-1 (Herrero-Fresno and Olsen, 2017).

The immune system must effectively engage in order to override the *Salmonella* resistome. The effective activation of the immune system is through one of two routes: through the recognition of *Salmonella* LPS by TLR4 present on resident macrophages and neutrophils; or, via the complement pathway (Kurtz et al., 2017). Ligation of TLR4 on resident neutrophils or macrophages (or heterophils in Aves) causes a massive production of cytokines (Coburn et al., 2007; Janeway and Medzhitov, 2002). This leads to both the activation of innate and adaptive cells, as well as their infiltration into the lamina propria
Additionally, the complement pathway recognizes *Salmonella* which results in opsono-phagocytosis, lysis, and the production of C5a (Ramu et al., 2007). A vital element in overcoming the ability of *Salmonella* to hijack the host is the synergism associated with the actions of the Toll-like receptors and the complement pathway. These synergistic pathways likely converge on MAPK and lead to a successful immune response to *Salmonella* (Sarma, 2012). In host-restricted serovars of *Salmonella*, gene loss events lead to a reduction of inflammation that facilitates the ability of *Salmonella* to successfully evade the immune system (Foley et al., 2013; Golubeva et al., 2012; Klerks et al., 2007; Knodler, 2015; Oshota et al., 2017; Razzuoli et al., 2017; Selander et al., 1992). This is likely due to a highly tuned activation of its T3SS (Diepold and Armitage, 2015; Raymond et al., 2013; Velge et al., 2012).

Eventually in immunocompetent patients the immune response will resolve salmonellosis. The C5aR1 receptor is an important buttress to the clearance of *Salmonella* as its actions are imperative for resolution of the infection. The C5aR1 receptor is ligated by C5a as a consequence of activating the complement pathway (Brodbeck et al., 2008; Karsten et al., 2015; Klos et al., 2009; Lee et al., 2008). The complement pathway is an ancient, evolutionarily conserved set of over 50 proteins that act in concert to kill pathogens and clear damaged cells from the host (Klos et al., 2009; Kolev et al., 2014). The specific pathways of activation are summarized in Figure 2.3 (Kolev et al., 2014). When activated, complement has three effects: to facilitate opsono-phagocytosis, to promote inflammation, and to produce the membrane attack complex leading to cellular lysis (Klos et al., 2009; Kolev et al., 2014; Lee et al., 2008). There are three pathways that feed into complement and include the classical, lectin, and alternative pathways. Kallikren and thrombin can also activate the
alternative pathway by cleaving C3 leading to the formation of C3 convertase (Klos et al., 2009; Kolev et al., 2014; Lee et al., 2008).

The C5a anaphylatoxin is an essential component in producing an inflammatory response to Salmonella and as well as its clearance. Traditionally, complement proteins are produced in the liver (Klos et al., 2009; Kolev et al., 2014; Lee et al., 2008). However, recently it has been demonstrated that antigen-presenting cells can produce complement proteins to stimulate T-cells and enhance their activity (Boothby et al., 2015; Klos et al., 2009; Kolev and Kemper, 2017; Kolev et al., 2014; Lee et al., 2008). As a result, there is significant cross-talk between the adaptive and innate immune system through this receptor (Klos et al., 2009; Kolev et al., 2014; Lee et al., 2008). Antigen-presenting cells secrete C3 and C5, and T-cells activate these proteins via cathepsin L. Both C3a and C5a ligate their cognate receptors and aide in the production of a beneficial inflammatory environment (Klos et al., 2009; Kolev et al., 2014; Lee et al., 2008). The autocrine-driven responses to C3a and C5a are essential for a productive Th1 response to intracellular pathogens (Klos et al., 2009; Kolev et al., 2014; Lee et al., 2008). Therefore, complement has significant effects throughout the entirety of the immune response—both locally and systemically.

The enhancement of complement activation is implicated in the success of XPC in improving the immune response to a Marek’s vaccine challenge (Chou et al., 2017). This up-regulation likely beneficially modulates the immune system and enables animals fed an XPC-containing diet to respond with a more tuned, efficient, and effective response to vaccination (Chou et al., 2017). Other research indicates that feeding animals XPC corresponds to a better phagocytic clearance of pathogens (Chapter 3). There is also a reduction in the dissemination of Salmonella to the peripheral lymph nodes (Feye et al., 2016).
The cellular response of the C5aR1 receptor is well-understood, though its molecular pharmacology remains complicated. C5aR1 is encoded in the mammalian genome as two exons, though that conservation does not necessarily exist in non-mammalian species (Higginbottom et al., 2005; Karsten et al., 2015; Klos et al., 2013; Lagerstrom et al., 2006; Rabiet et al., 2007). The C5aR1 receptor is expressed in all of the leukocyte lineages as well as in the spleen and liver (Klos et al., 2013; Lee et al., 2008). C5aR1 is a pertussis toxin-sensitive G-protein coupled receptor that belongs to the rhodopsin family of receptors (Klos et al., 2013). Activation of this receptor immediately results in the reduction of cAMP, an influx of calcium, and the phosphorylation of AKT/PI-3K as summarized in Figure 2.4. As a consequence of receptor activation, there is enhanced cytokine expression, improved antigen presenting receptor and co-receptor expression, and superior phagocytosis (Bajic et al., 2013; Brodbeck et al., 2008; Higginbottom et al., 2005; Karsten et al., 2015; Klos et al., 2013; Lee et al., 2008; Li et al., 2012; Ricklin et al., 2012; Schraufstatter et al., 2002). In most animals, but not necessarily in poultry, the C5aR1 receptor is a member of the n-formyl peptide family of chemotactic receptors (Klos et al., 2013; Lagerstrom et al., 2006). Thus, the engagement of this receptor is almost universally tied to the infiltration of neutrophils during Salmonella invasion (Coburn et al., 2007; Kurtz et al., 2017). Through numerous studies, it appears that the cellular response as a result of receptor engagement is conserved across taxa (Bajic et al., 2013; Brodbeck et al., 2008; Higginbottom et al., 2005; Karsten et al., 2015; Klos et al., 2013; Lee et al., 2008; Li et al., 2012; Ricklin et al., 2012; Schraufstatter et al., 2002).

Overall, tuning the microbiome and improving the innate effector response may lead to the profound anti-Salmonella effects demonstrated by XPC. Therefore, the probable
mechanisms of XPC intersect at the nexus of *Salmonella* pathogenesis, the microbiome, and immunobiology.

### 2.4 Conclusions

Food animal production will always need to meet the demand of the people with a constant eye towards economics, animal welfare and food safety. The collateral damage associated with the off-label use of antibiotics is austere and unpredictable. Through replacing antibiotics as in-feed additives with postbiotics like XPC, the goals set forth by food animal scientists to feed the world are furthered. XPC improves food safety, feed conversion, and the health of the host. While there is mechanistic variation between species, the relative consistency is promising.

Notably, understanding the underlying mechanisms associated with the efficacy of the product will result in additional discoveries to further improve the product or expand on its utilization. This understanding could lead to enhanced drug targeting strategies and immune based technologies to improve animal health. Thus, the impending food and the antibiotic resistance crisis may have a solution.
Benefits of XPC in Food Animals. XPC is traditionally used in food animal production to improve feed efficiency. XPC has three major effects on the host: improving the host immune system, modulating the microbiota, and augmenting the gastrointestinal physiology to improve nutrient absorption. These changes lead to the improve the immunobiological functioning of the host as well as the microenvironment of the gastrointestinal lumen. As a result, the changes lead to the reduction in Salmonella and other pathogens from food animals.
Figure 2.2  *Salmonella Invasion Mechanisms*. This series of diagrams and photographs show how *Salmonella* invade the host. This figure is taken from Vegle et al., 2012. (A) This is the author’s rendition of the trigger mechanism of invasion. The injectasome forms a pore on the surface of the host cell membrane and injects the SIPA, SIBP, SIPC, SIPE2, and SIPE SPI-1 effector proteins into the cytosol. These proteins engage the host cytoskeleton remodeling machinery and ultimately form a ripple in the host membrane. The *Salmonella* then slips into the host cell. (B) This image is a scanning electron micrograph of this event. (C) This diagram describes the zipper mechanism of invasion. *Salmonella* RCK docks with some unknown host receptor, which initiates receptor mediated endocytosis. (D) This image is a scanning electron micrograph of this event.
Figure 2.3  Complement Pathway. The Complement Pathways is described above and the figure is directly taken from Kloev et al., 2014. (A) This is a diagram of system complement activation includes the regulatory checkpoints and the consequences for activating this pathway. (B) This is a diagram of the lesser known local complement pathway. Antigen presenting cells and T-cells produce local complement proteins whose autocrine and paracrine signaling activates receptors that promote a beneficial inflammatory environment. The tuned inflammation ensures the proper activation of immune effector cells.
Figure 2.4  **C5aR1 Cell Signaling Cascade and Response.** The activities of this receptor vary depending on the cell-type expressing this receptor. However, the end result seems to be conserved. That is, the activation of C5aR1 increases cell survival and proliferation, enhances the response to inflammatory stimuli, and regulates autophagy. (Lagerstrom et al., 2006; Wettschureck and Offermanns, 2005)
CHAPTER 3. ALTERNATIVES TO ANTIBIOTICS AND DISINFECTANTS: FERMENTATION PRODUCTS THAT REDUCE IMPORTANT FOODBORNE PATHOGENS

K. M. Feye\textsuperscript{1}, J. P. Carroll\textsuperscript{2}, K. L. Anderson\textsuperscript{1}, J. H. Whittaker\textsuperscript{2}, G. R. Schmidt-McCormack\textsuperscript{3}, D.R. McIntyre\textsuperscript{4}, H. O. Pavlidis\textsuperscript{4}, and S. A. Carlson\textsuperscript{1}

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1. Department of Biomedical Sciences, Iowa State University, Ames, Iowa
2. Interdepartmental Microbiology, Iowa State University, Ames, Iowa
3. Interdepartmental Neurobiology, Iowa State University, Ames, Iowa
4. Diamond V, Cedar Rapids, Iowa

3.1 Abstract

Due to regulations set forth by the Veterinary Feed Directive, as of January 1, 2017, feed grade antibiotics will be restricted in food animal production. Some defining characteristics associated with feed grade antibiotics are the inhibition of important pathogens, enhanced growth, and improved performance. Recent research reveals that a fermentation product, XPC\textsuperscript{TM} (Diamond V, Cedar Rapids, IA), serves as a viable alternative across multiple food animal species including cattle and poultry. Providing this supplement in the feed of animals leads to reduced prevalence, load, virulence, and antibiotic resistance of foodborne pathogens such as \textit{Salmonella} and \textit{Escherichia coli} O157:H7. These findings are compelling, especially when coupled with the enhanced growth and performance observed with these products. Mechanistically, XPC appears to modulate these effects through the immune system and gut microbiome. Use of fermentates like XPC can lead to an overall reduction of antimicrobial resistance, enhanced herd health, and a reduction in the vertical transmission of important foodborne pathogens. Through continued research and
exploration, the development of XPC as an alternative to feed-grade antibiotics will augment the fight against foodborne pathogens.

3.2 Introduction

The persistence of important foodborne pathogens continues to prove a formidable challenge to overcome with futile attempts to halt vertical transmission to the food supply outnumbering the successes. Two of the more important foodborne pathogens, *Salmonella enterica* and shiga toxin producing *Escherichia coli* O157:H7 (STEC), are serious concerns in food animal production management. *Salmonella enterica* is a ubiquitous, zoonotic pathogen that is an enduring challenge in food animal production and management (Jones, 2011). Control of *Salmonella* in production animals occurs in pre-harvest and post-harvest settings (Jones, 2011), without universal success. That is, despite a concerted effort to prevent the transmission of *Salmonella* throughout the food production chain, control mechanisms routinely fail.

At animal processing facilities, surveillance is a key for evaluating control mechanisms that halt the perpetuation of enteric pathogens. Harvest facilities heavily rely on routine visual, molecular, and microbiological surveillance of equipment for enteric pathogens with disinfection serving as a mainstay of the abatement process. The implementation and execution of all control and surveillance mechanisms face their own unique set of economic and implementation challenges, and unfortunately are not fail-proof (Jones, 2011). Efficacious pre-harvest abrogation of *Salmonella* and other enteric pathogens will obviously halt transmission to the processing facilities and as a result markedly reduce introduction of pathogens into the food chain (Jones 2011; Tadesse 2016).
On-farm approaches that prevent the transmission of *Salmonella* and other enteric pathogens have two separate branches: the traditional and the alternative. Traditional abatement procedures include vaccination, feed-grade antibiotics (FGAs), chemical treatments of feed and water, and disinfectants used at the facilities housing the animals. Perhaps the most ignoble consequence of FGAs is the correlation between FGAs and the emergence of multiple drug resistant bacteria in veterinary and human clinical settings (Foley et al., 2013). While some controversy still exists as to the outcome of banning FGAs in food animal production, the potential contribution of this approach to the over-utilization of antibiotics should not be ignored (Casewell et al., 2003; Wegener, 2003).

With the advent of the Veterinary Feed Directive (FDA, 2017), alternatives to FGAs must be identified. Alternative methods to prevent the transmission of *Salmonella* include using essential oils, modulation of the microbiota directly through seeding, facilitating healthy microbiota through biotics (prebiotics, postbiotics, and probiotics) and bacteriophage seeding (Brewer et al., 2014; Feye et al., 2016; Feye et al., 2016; Kenneth A. Kudsk, 2002; Torres-Barcelo and Hochberg, 2016). Herein we discuss fermented products as a control measure for enteric pathogens.

There is evidence that the utilization of fermented products, in particular the *Saccharomyces cerevisiae* fermentation product designated as XPC™, impacts enteric pathogens across multiple livestock species through a possible conserved mechanism. A fermented product is a collection of metabolic byproducts produced from commensal microorganisms and/or symbionts, such as *Saccharomyces cerevisiae*, that exert a non-specific yet beneficial biological activity in the host (Patel et al., 2014). Traditionally, XPC is used for its beneficial production effects. However, studies into its anti-pathogenic effects
began after anecdotal observations indicated that there was a decrease in *Salmonella* prevalence in chicken laying barns as part of the National Poultry Improvement Plan environmental monitoring. Questions began to emerge as to the potential use and efficacy of XPC as a pathogen mitigation tool, as well as the mechanisms underlying its effects. Recent and ongoing studies suggest that XPC is a potent intervention with uses in poultry, egg, dairy, and feedlot production. Herein we discuss the evidence and mechanisms regarding XPC as a cost-effective alternative to antibiotics. This review specifically focuses on STEC and *Salmonella enterica*, two important causative agents of world-wide foodborne illnesses.

### 3.3 Current Evidence Revealing the anti-*Salmonella* Effects of XPC and Derivatives

#### 3.3.1 Anti-*Salmonella* Effects of XPC in Dairy Calves

Fermentation products (FPs) have been added to livestock feed as growth promoters whereby these supplements favor sound gut development (Lesmeister et al., 2004; Price et al., 2010). In cattle, FPs apparently improve average daily gain and increase feed efficiency. Immunologically, FPs are mechanistically tied to an increase in overall immune function through the modulation of the host immune surveillance and inflammatory response (Jensen et al., 2008; Jensen et al., 2007; Lesmeister et al., 2004). Furthermore, it is proposed that XPC fosters an increase in intestinal butyrate (Lesmeister et al., 2004)- an important fatty acid in ruminant metabolism. An additional benefit of butyrate is its ability to down-regulate the expression of genes in *Salmonella* pathogenicity island 1 (SPI1), a collection of about 40 genes that facilitate gut epithelial cell invasion and systemic virulence (Foley et al., 2013).

Given the aforementioned potential anti-*Salmonella* effects of XPC in ruminants and the anecdotal reports of XPC-mediated reduction in *Salmonella* in poultry, the anti-
Salmonella effects of XPC were examined in dairy calves. Salmonella was of interest due to its persistence on the farm, the disease-associated retardation of the growth and development of dairy cattle, and the emergence of hypervirulent multi-drug resistant strains (Carlson et al., 2007; Rasmussen et al., 2005). One study addressed the hypothesis that XPC plus SmartCare™, an XPC derivative that is included in milk replacer, reduces the impact of Salmonella in dairy calves on milk (Brewer et al., 2014). Calves were fed SmartCare plus XPC for two weeks prior to experimental infection with multi-resistant Salmonella Typhimurium DT104. When compared to calves fed the Control diet, XPC plus SmartCare lead to beneficial anti-Salmonella effect resulting in a decrease in rectal temperature, diarrhea scores, and fecal shedding, as well as an with an overall improvement in rumen development and body weight gain (Brewer et al., 2014). Interestingly, and important for long-term control of Salmonella at the farm, there was a decrease in the ileal colonization of Salmonella in the calves fed XPC plus SmartCare. Therefore, the study suggests that the consequences of feeding calves XPC plus SmartCare is an overall improvement of gastrointestinal health and development as well as a profound reduction in Salmonella load and virulence (Brewer et al., 2014).

3.3.2 Anti-Salmonella Effects of XPC in Beef Cattle

The positive results from the dairy calf studies prompted analogous studies in beef cattle using an XPC derivative designated as NaturSafe™. This study examined the effects of NaturSafe on fecal shedding, lymph node infiltration, virulence, and antibiotic resistance of Salmonella as well as the prevalence and load of E. coli O157:H7 (Feye et al., 2016). Cattle were fed either a standard industry diet containing monensin, tylosin, and bovamine (Control group) or a diet lacking these three constituents but containing NaturSafe. Results
indicated that NaturSafe mediated an approximate 50% decrease in *Salmonella* load across pens, a four-fold decrease in *Salmonella* shedding, and a seven-fold decrease in *Salmonella* infiltration to the lymph nodes when compared to the Control diet (Feye et al., 2016).

Regarding virulence, there was about a 65% reduction in *Salmonella* virulence as measured by decrease in both *hilA* expression and tissue culture invasiveness (Feye et al., 2016). This is extrapolated to an approximate five-fold increase in the dose of *Salmonella* needed to infect and cause salmonellosis in a non-ruminant (Carlson et al., 2000). Furthermore, there was a significant reduction of antibiotic resistant phenotypes of *Salmonella* and a reduction in more aggressive and epidemiologically relevant *Salmonella* serotypes such as Typhimurium, Dublin, and Newport. Regarding *E. coli* O157:H7, there was over a 50% reduction in fecal load and a 20% reduction in prevalence. These data suggest that there is an overall decrease in virulence, antibiotic resistance, load, and prevalence of *Salmonella* in cattle fed NaturSafe verses those fed a standard industry diet (Feye et al., 2016) which contains an ingredient (tylosin) that will be restricted 2017. Although tylosin is included in the diet for controlling liver abscesses and is not intended for control of *Salmonella*, its absence may increase the incidence of liver abscesses. However, there is evidence that the NaturSafe-fed cattle had indistinguishable incidences of liver abscesses when compared to cattle fed the Control diet containing tylosin (unpublished).

### 3.3.3 Anti- *Salmonella* Effects of XPC in Poultry

*Salmonella* is not typically a pathogen in poultry since avian salmonellosis is restricted to a few serotypes that are mostly absent from the U.S. The central concern in poultry involves the vertical transmission of the zoonotic pathogen to the human population. A study was conducted to look at the anti-*Salmonella* properties of XPC in regards to the
load, prevalence, virulence, and antibiotic resistance of *Salmonella* in a controlled experiment with broilers. One day-old chicks were repeatedly infected with multiple antibiotic resistant *Salmonella* Typhimurium DT014 from days 2 to 20 (Feye et al., 2016). Uninfected chicks were removed on day 21 and remaining chicks were fed either a diet containing XPC or a Control diet lacking XPC. Fecal and cecal samples were taken from the birds and selective culture for *Salmonella* revealed a reduction of load and prevalence of *Salmonella* as well as a reduction in virulence and antibiotic resistance (Feye et al., 2016).

Evidence for a reduction in virulence came from a reduction in the expression of hilA, a gene whose expression is central to *Salmonella* invasion through the Type III Secretion System (Foley et al., 2013), concordant with a decrease in *Salmonella* invasiveness as measured by a tissue culture-based invasion assay (Gianella et al., 1973). The reduction in antibiotic resistance correlated with the loss of the SGI1 integron from the input strain. Studies are ongoing regarding the potential mechanism associated with the anti-*Salmonella* activities of XPC in poultry.

### 3.4 Additional Evidence Supporting the anti-*Salmonella* Effects of XPC and Derivatives in Calves

#### 3.4.1 Immune Clearance of *Salmonella* in Dairy Calves

The study with SmartCare and XPC revealed diminished *Salmonella* shedding and carriage in calves fed the products, when compared to calves fed the Control diet. Both groups were infected with the same dose of *Salmonella* yet the Treated group appeared to be clearing the pathogen (Brewer et al., 2014). Two possible explanations are that the XPC-fed calves were either capable of killing the *Salmonella* or that these calves were more efficient at translocating the *Salmonella* to lymph nodes.
To address the first possibility, we examined peripheral blood cells for the ability of the leukocytes to kill the *Salmonella*. Specifically, whole blood was taken and incubated with *Salmonella* for 1 hour. After 1 hour, a portion of leukocytes were removed and exposed to gentamicin in order to kill the non-engulfed/non-invasive *Salmonella*. Leukocytes were then lysed with Triton and lysates were plated on XLD that incubated overnight at 37°C for colony enumeration the next day. This process was repeated at multiple time points in order to determine the ability of the leukocytes to temporally kill *Salmonella*. As shown in Figure 3.1, the number of *Salmonella* was equivalent in both groups of calves (n=5 calves/group) at the first time point but the numbers of *Salmonella* dropped dramatically at each successive time point for leukocytes obtained from XPC-fed calves. Importantly, this mechanism seems to be conserved across two divergent *Salmonella enterica* serovars—Typhimurium and Dublin.

To address the second possibility, peripheral lymph nodes (superficial cervical) were excised at euthanasia and subjected to selective culture for *Salmonella*. An increase in *Salmonella* recovered from the lymph nodes would indicate the pathogen was able to evade host immune responses, and arrive at the lymph nodes in a viable state. Such viability is not possible if there is sufficient phagocytic killing of the *Salmonella*, and subsequent processing for antigen presentation (Levy et al., 2015; Vieira et al., 2016). As shown in Figure 3.2, *Salmonella* was significantly less abundant in lymph nodes from XPC-fed calves. Thus it appears that leukocytes from XPC-fed calves are more efficient at killing *Salmonella* while lymph nodes from these calves are less likely to harbor *Salmonella*. 
3.4.2 Diminished Virulence and Antibiotic Resistance of *Salmonella* in Dairy Calves

Since the input strain used in the poultry studies (Feye et al., 2016) was also used in the calf studies (Brewer et al., 2014), similar virulence and antibiotic resistance assays were performed using *Salmonella* recovered from the calves. As shown in Figure 3.2, tissue culture invasiveness, hilA expression, antibiotic resistance, and prevalence of the antibiotic resistance element (SGII) were all diminished in *Salmonella* recovered from XPC-fed calves.

3.4.3 Restoration of *Salmonella* Invasion after the Pathogen Exits an XPC-fed Host

To investigate the possibility that the hypo-invasive *Salmonella* recovered from animals fed XPC have the potential to quickly return to the fully invasive state after exiting an XPC-fed animal, a series of temporal invasion assays were performed on isolates recovered from XPC-fed poultry and cattle. As shown in Figure 3.3, invasiveness returns after three successive incursions into tissue culture cells and the invasion does not go above that observed for *Salmonella* recovered from Control-fed animals. Thus it appears that, after exiting an XPC-fed animal, the *Salmonella* need to invade three hosts before returning to the fully virulent state. These data include *Salmonella* obtained from the experimentally infected dairy calves (Brewer et al., 2014a), naturally infected beef cattle (Feye et al., 2016), and from poultry that were experimentally infected (Feye et al., 2016) and naturally infected. Thus the data represent a variety of *Salmonella* serotypes in a variety of hosts.

3.5 Conclusions

Through the implementation of XPC as a feed additive, there is a clear advantage for the reduction of important foodborne illnesses in food animal production. Evidence
continues to show that XPC leads to a reduction in *Salmonella* antibiotic resistance, load, virulence, prevalence, and shedding across multiple species. The mechanism of the reduced invasiveness, and deduced virulence, converges in a reduction in SPI-1 and *hilA* in *Salmonella* recovered from both cattle and chickens. Further, the *Salmonella* recovered from treated animals have a sustained increase in dose required to cause disease. Not only is a reduction in prevalence important, but XPC directly decreases antimicrobial resistance which is the focus of the VFD. The anti-resistance benefit is two-fold. The first benefit is a reduction in SGI1 in MDR *Salmonella* DT104, which reduces the presence of genes associated with drug resistance (Feye et al., 2016). The second is likely beneficial in years to come following the cessation of unnecessary antibiotics in feed animals, thus reducing the evolutionary pressure to maintain resistant genotypes within a population.

The conserved mechanism for XPC efficacy relating to its anti-*Salmonella* effects is largely unknown. Research has been initiated to elucidate this interesting mechanism, though the literature is highly suggestive of two mechanisms: augmenting gut immunophysiology and enhancing microbial competition. Sustained inflammation resulting from any number of sources ultimately leads to a reduction in gut-barrier integrity, increases circulating endogenous endotoxin, and is linked to decreased residual feed intake and susceptibility to disease in food animals (Gaggìa et al., 2010; Mani et al., 2012; Turner, 2009). The utilization of fermented products specifically addresses that issue, leading to enhanced gut physiology resulting in reduced pathogenesis of important foodborne pathogens across multiple species (Brewer et al., 2014; Feye et al., 2016; Feye et al., 2016; Vieira et al., 2016).
Further mechanistic potentials for XPC exist in the enhancement of commensal microbial populations directly. This enhancement improves competition, gastrointestinal physiology, and nutrient utilization. The use of prebiotics enhances gut villi morphology through a manipulation of the gut microbiome in poultry (Pan and Yu, 2014) and cattle (Brewer et al., 2014). Further studies into beneficial exudates from microbial populations categorized as prebiotics include: short chain fatty acids, aromatic amino acid derivatives, vitamins, enzymes that catalyze the de-hydralization and de-conjugation of bile acids to enhance vitamin absorption, and choline (Krishnan et al., 2015). The product XPC potentially goes beyond that, though the specific components and mechanisms are unknown. Nonetheless, the underlying mechanism governing the success of XPC may also be pertinent to the reduction of other pathogens that extend beyond zoonoses identified in this review. Therefore, XPC has the potential to be a significant and economically viable solution for curtailing foodborne pathogens and further studies will continue to enhance our understanding of postbiotics on physiology.
3.6 Figures

Figure 3.1  Assessment of *Salmonella* obtained from lymph nodes of calves fed XPC plus SmartCare compared to the control. Newborn dairy calves were fed daily doses of XPC plus SmartCare for five weeks (Brewer et al., 2014). At the end of the study, calves were euthanized and *Salmonella* were cultured from the superficial cervical lymph nodes. *Salmonella* were enumerated and subjected to the virulence and antibiotic resistance assays described in other studies (Feye et al., 2016; Feye et al., 2016). Data presented are the mean ± SEM from data obtained from 10 calves from each group on at least two separate occasions. (P<0.05 for all asterisks)
Figure 3.2  Assessment of *Salmonella* lysis by leukocytes obtained from calves fed XPC compared to the control. Newborn dairy calves were fed daily doses of XPC® for five weeks (Brewer et al., 2014a). At various points during the five weeks of the study, approximately 4mL of whole blood was collected into an EDTA tube, of which 3mL was transferred into a microfuge tube and subjected to density gradient centrifugation (the other 1mL was submitted for CBC analysis). The erythrocyte fraction was then removed and 120μL of the buffy coat interface was collected and aliquoted into six separate tubes, to which 10^7 colony-forming units of *Salmonella* (approximate multiplicity of infection=100) were added and the tubes were incubated at 37°C. After 1h, extracellular (i.e., non-invasive) bacteria were killed by the addition of 50μg/mL gentamicin. At 0, 1, 2, 4, 8, and 12 hrs post-killing, leukocytes were centrifuged and the gentamicin-containing media was removed and replaced with 50μL of phosphate-buffered saline containing 1% Triton which lyses the leukocytes. Lysates were then plated on XLD agar that was incubated overnight at 37°C. The following day, black-centered colonies were enumerated and *Salmonella* survival/cell was calculated as number of recovered colonies/number of leukocytes per 20μL blood (derived from the CBC analyses). The strains used were *S. Dublin* SGI1 (Xiong et al., 2010) and *S. Typhimurium* LNWI (Wu et al., 2002). Data presented are the mean ± SEM from leukocytes obtained from 10 calves from each group on at least two separate occasions. (p<0.05 for all asterisks).
Figure 3.3  Persistence of the effect of XPC on hilA expression and invasiveness of *Salmonella* recovered from feces of naturally infected chickens fed XPC (or a Control diet) for ≥ 28 days. *Salmonella* were recovered from treatment-specific birds and then serially subjected to the invasion assays examining cell penetration and virulence gene expression. Generations 1-4 represent *Salmonella* serially recovered from tissue culture studies while Generation 0 represents *Salmonella* directly recovered from birds.
CHAPTER 4. REDUCTION OF SALMONELLA AND E. COLI O157:H7 IN FEEDLOT CATTLE FED A PROPRIETARY SACCHAROMYCES CEREVISIAE FERMENTATION PROTOTYPE

Kristina M. Feye¹, Kristi L. Anderson¹, Mark F. Scott², Darin L. Henry², Kristy L. Dorton², Brandon E. Depenbusch³, and Steve A. Carlson¹

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1. Department of Biomedical Sciences, Iowa State University, Ames, Iowa, USA
2. Diamond V, Cedar Rapids, Iowa, USA
3. Innovative Livestock Services, Great Bend, Kansas, USA

4.1 Abstract

*Salmonella* and *E. coli* O157:H7 are insidious problems for the beef industry. Asymptomatic fecal shedding of these pathogens contaminates the hide and carcass. Furthermore, *Salmonella* are unique in their ability to infiltrate lymph nodes leading to the post-harvest contamination of ground beef. These contaminations yield the two most important food safety hazards associated with the consumption of beef. Herein, we report the anti-*Salmonella* and anti-*E. coli* O157:H7 effects of a novel *Saccharomyces cerevisiae* fermentation prototype (PRT; NaturSafe™) fed to feedlot cattle. Cattle fed PRT were compared to those fed a combination of monensin, tylosin, and a direct-fed microbial- a standard conventional practice in the U.S. beef industry. In this investigator-blinded study, 1,495 feedlot heifers (300-400 kg) were fed PRT (n=747 heifers) or the standard industry diet (PC; n=748 heifers) without PRT for 125-146 days prior to slaughter. At the abattoir, fecal swabs were obtained from 400 animals (n=200/group) and subjected to selective culture for enumerating *Salmonella* and *E. coli* O157:H7. Additionally, subiliac lymph nodes were obtained from 400 carcasses for enumeration of *Salmonella* spp. *Salmonella* isolated from the feces and lymph nodes were subjected to a virulence assay and some antibiotic
susceptibility and *Salmonella* serovar testing. When compared to cattle that received PC, *Salmonella* fecal shedding, lymph node infiltration, virulence, and antibiotic resistances were significantly decreased in cattle fed PRT. Additionally, PRT-fed cattle had a lower prevalence of certain *Salmonella* serovars (Newport, Typhimurium, and Dublin) and shed fewer *E. coli* O157:H7. The decrease in *Salmonella* virulence was associated with a decreased expression of *hilA*, a genetic regulator of *Salmonella* invasion into eukaryotic cells. This study revealed that a proprietary *Saccharomyces cerevisiae* fermentation prototype inhibits the shedding, lymph node carriage, downstream virulence, and antibiotic resistance of *Salmonella* residing in cattle beyond the standard conventional practice that includes monensin, tylosin, and a direct-fed microbial. Key words: *Salmonella*, cattle, *Saccharomyces cerevisiae* fermentation product

### 4.2 Introduction

Bovine-associated *Salmonella* are capable of causing clinical disease, but these *Salmonella* can also asymptomatically reside in the intestinal tract and lymph nodes of cattle. Both of these reservoirs create food safety hazards, especially the latter situation where lymph nodes serve as a protective conduit for *Salmonella* passage into ground beef (Bricha-Harhay et al., 2012). The intestines also serve as a reservoir for *E. coli* O157:H7, which is non-pathogenic in cattle but highly pathogenic in humans (Smith et al., 2014).

Intestinal *Salmonella* and *E. coli* O157:H7 lead to surface contamination of beef, which can be mitigated by a number of different strategies at the abattoir (Soon et al., 2011). Lymph node infiltration, on the other hand, is problematic given the number of lymph nodes unavoidably co-harvested with ground beef and the covertness of the *Salmonella* within the
lymph nodes (Brichta-Harhay et al., 2012). Concerns with *Salmonella* in the lymph nodes underscores the need for improving *Salmonella* prophylaxis either in the pre-harvest or post-harvest setting. Recent studies have demonstrated that soluble components present in *Saccharomyces cerevisiae* fermentation products (SCFP; SmartCare® and Original XPC™, Diamond V) eliminate *Salmonella* from the gastrointestinal tract of experimentally-infected calves (Brewer et al., 2014). Specifically, SCFP (SmartCare and Original XPC) significantly reduced the intestinal burden and pathogenic effects of *Salmonella* in calves (Brewer et al., 2014). Because of these benefits, the anti-*Salmonella* effects of SCFP were examined in feedlot cattle naturally infected with *Salmonella*. The aims of this study were to determine a broad range of effects of a proprietary *S. cerevisiae* fermentation prototype (PRT; NaturSafe™) on *Salmonella* when fed to finishing cattle. Specifically, fecal shedding, lymph node infiltration, downstream virulence, antibiotic resistance, and the prevalence of virulent *Salmonella* serotypes with a broad host range were determined. The virulence aspect was examined, in part, because SCFP (EpiCor®, Embria Health Sciences) can increase intestinal butyrate production (Possemiers et al., 2013), and this short-chain fatty acid has been shown to inhibit *Salmonella* virulence mechanisms *in vitro* (Gantois et al., 2006). The antibiotic resistances were examined since SCFP (Original XPC) can modulate the microbiome (Mullins et al., 2013; Price et al., 2010), which can alter the antibiograms of resident bacteria (Kirkup et al., 2004). Additionally, we examined the effects of the SCFP on the shedding of *E. coli* O157:H7 since this microbe shares mammalian cell adhesion strategies with *Salmonella* (Rossez et al., 2015). Comparisons were made to cattle fed a combination of monensin, tylosin, and a direct-fed microbial, which is a standard conventional practice in the U.S. beef industry.
4.3 Materials and Methods

4.3.1 Animal Care and Study Design

Heifers (n = 1,495; 300 – 400kg) were obtained from two sale barns (n = 438) and one backgrounding facility (n = 1,057) in Kansas in May of 2015. Cattle were shipped to a commercial feedlot in Central Kansas and were provided water and hay *ad libitum*. On day 1 post-arrival, heifers were individually weighed, identified, implanted, and vaccinated using standard procedures at the feedlot. Heifers were then randomly assigned into pens in groups of five until each pen reached its optimal capacity (~75 animals) based on bunk space and the area of the pen (14.4 inches of bunk space and 231 square feet of pen space per animal).

Two adjacent pens were designated as a single block and 10 blocks were created within the feedlot. Pens of heifers in each block were provided either a diet that contained a combination of standard industry technologies (PC), including monensin (Rumensin, Elanco Animal Health, 300 mg/head/day), tylosin (Tylovet, Huvepharma, 90 mg/head/day) and a direct-fed microbial (Bovamine Defend, Nutrition Physiology Company, 50 mg/head/day); or a diet containing 18 gm/head/day of a *S. cerevisiae* fermentation prototype (PRT; NaturSafe™, Diamond V) without monensin, tylosin, or the direct-fed microbial. Heifers received three step-up diets prior to their final finishing diet (Table 4.1). All treatment feed additives were stored under manufacturer-recommend conditions and added to the final ration using a micro-ingredient weight machine (Micro Beef Technologies, Amarillo, TX).

During the study, heifers were monitored for illness and treated as per recommendations by a veterinarian. Heifers that responded to treatment were returned to the
study while non-responders were removed from the study. Morbidities and mortalities were indistinct between the two groups (data not shown).

At the conclusion of the study, heifers were shipped 145 miles to a commercial abattoir on two separate dates that were three weeks apart. These shipping dates corresponded to 125 and 146 days on study for the first and second groups, respectively. An equal number of pens per treatment group were shipped on each date (n = 5 per treatment). Shipments and carcass processing occurred on a pen-by-pen basis.

4.3.2 Sample Collection at the Abattoir

Individual animal numbers were not carried through the entire harvest process. Only the first and last animal within a pen was tagged with a lot identification tag that corresponded back to the pen number. Since individual identification was not maintained, it was not possible to keep track of which animals were sampled. As a result, lymph node and fecal samples may or may not have been collected from the same animals.

Fecal swabs were collected on the rail from 20 animals per pen (replicate). Samples were collected from every third or fourth animal within a replicate. Fecal samples were collected using a 3M-sponge stick pre-saturated with buffered peptone water. Sponge sticks were inserted into the rectum (recto-anal junction) to collect the sample. After the sample was collected, the sponge was placed into a pre-labeled bag containing buffered peptone water. The bag was closed and placed into a cooler.

Subiliac lymph nodes and the surrounding tissue were collected post-evisceration. Sample collection began with the first carcass in each replicate and continued with every third or fourth carcass within that replicate. Lymph nodes were placed into pre-labeled Whirlpak bags. The bags were closed and placed in a cooler. Fecal swabs and lymph node samples were then immediately shipped on ice to Iowa State University College of
Veterinary Medicine (Ames, Iowa, USA) for microbiological analyses.

**4.3.3 Assessment of *Salmonella* Fecal Load and Lymph Node Infiltration**

*Salmonella* spp. were enumerated from every fecal swab sample and lymph node collected (20 per pen; 200 per treatment) using selective agar (XLD) methods described by Brewer *et al.* (Brewer et al., 2014). Approximately 0.3 gm of feces or lymph node were collected on a sterile cotton swab and then aseptically transferred into 10mL Lennox broth and an aliquot of the broth was immediately plated on XLD agar, incubated overnight at 37°C, and subjected to enumeration by manual counting of black-centered colonies the next day. Load was then determined as (colonies recovered) x (the dilution factor) / gm of feces or lymph node. Prevalence was calculated as percent of heifers harboring any *Salmonella* and was compiled across pens within a treatment group.

**4.3.4 Assessment of *E. coli* O157:H7 in the Feces**

*E. coli* O157:H7 was enumerated in 100 of the swab samples (five per pen; 50 swab samples per treatment) using selective media (Sorbitol-MacConkey agar) and a PCR targeting *E. coli* O157:H7 virulence genes as per Sharma and Casey (Sharma and Casey, 2014). Approximately 0.3 gm of feces were transferred into enrichment broth (Sharma and Casey et al., 2014) and an aliquot of the broth was plated on sorbitol-MacConkey agar, incubated overnight at 37°C, and subjected to enumeration by manual counting of non-fermenting colonies the next day. From each pen-specific set of agar plates, 96 colonies were selected and subjected to the PCR targeting *E. coli* O157:H7 virulence genes. Load was then determined as (colonies recovered x the dilution factor x the percent of colonies yielding an *E. coli* O157H7-specific amplicon within the pen) / gm of feces. Prevalence was calculated as percent of heifers harboring any *E. coli* O157:H7 within a pen, and was compiled across pens within a treatment group.
4.3.5 Assessment of Tissue Culture Invasiveness by *Salmonella* Recovered from Cattle

Approximately 50% of the recovered *Salmonella* were subjected to a standard antibiotic protection-based tissue culture invasion assay (Giannella et al., 1973) adapted for use with both antibiotic-susceptible and antibiotic-resistant *Salmonella* (Carlson et al., 2000). Colonies were collected *en masse*, on a pen-by-pen basis, into nutrient broth and then immediately incubated for 1 hr with HEp-2 tissue culture cells at 37°C. Bacteria were recovered from inside tissue culture cells via cell lysis, incubated on XLD agar overnight at 37°C, and enumerated the next day. Percent invasion was determined as (number of black-centered colonies recovered from inside cells / number of colonies added to cells) x 100.

4.3.6 Assessment of Virulence Gene Expression in *Salmonella* Isolated from Cattle

In order to correlate the virulence of *Salmonella* recovered from cattle with gene expression events in the pathogen, approximately 10% of the recovered *Salmonella* isolates were subjected to an assay that quantitates the expression of *hilA* (a key regulator of *Salmonella* invasion genes; (Bajaj et al., 1995). RNA was extracted from the isolates that were collected *en masse* on a pen-by-pen basis, and then subjected to a semi-quantitative RT-PCR targeting the *hilA* transcript (Carlson et al., 2007)

4.3.7 Assessment of Antibiotic Resistance in *Salmonella* Recovered from Cattle

Approximately 20% of the recovered *Salmonella* were individually subjected to micro-broth assays with individual antibiotics (florfenicol, ceftiofur, and enrofloxacin) at breakpoint concentrations (Institute, 2015). Colonies that grew in the breakpoint concentrations were deemed to be resistant. Percent resistant were then determined as (number of resistant colonies / number of colonies examined) x 100. Data were compared across pens and between groups.
4.3.8 Assessment of the Presence of Three Important Salmonella Serotypes in the Samples

Nearly 20% of the recovered Salmonella were individually subjected to PCR assays that detect the presence of genes related to Dublin (Akiba et al., 2011), Typhimurium (Akiba et al., 2011), and Newport (Cao et al., 2013) serotypes. Colonies yielding a specific PCR amplicon(s) were deemed to belong to the ascribed serotype. Percent belonging to the serotype were then determined as (number of colonies yielding a specific amplicon / number of colonies examined) x 100. Data were compared across pens and between groups.

4.3.9 Statistical Analyses

Statistical comparisons were made using an analysis of variance with Tukey’s ad hoc test for multiple comparisons (GraphPad Prism, Version 6, La Jolla, CA). Significant differences were defined at $P \leq 0.05$.

4.4 Results

4.4.1 Assessment of Salmonella Fecal Shedding in Cattle

Salmonella can be a potential pathogen source for humans who consume improperly cooked beef. Carcasses can become exposed to Salmonella through fecal contamination during processing. In this study, fecal shedding of Salmonella was evaluated in 200 heifers postmortem from each treatment group. As shown in Figure 4.1a, fecal shedding of Salmonella was significantly less ($P < 0.05$) in cattle fed PRT (105 versus 405 CFU/gm of feces, respectively). The relative prevalence of fecal shedding was significantly less ($P < 0.05$) in heifers fed PRT (6 versus 13%, respectively) as per Figure 4.1b.
4.4.2 Assessment of Lymph Node Infiltration by *Salmonella*

Since *Salmonella* lymph node carriage can be a source of contamination of ground beef (Brichta-Harhay et al., 2008), *Salmonella* load was determined in the subiliac lymph nodes of 200 carcasses from each treatment group. As shown in Figure 4.2a, lymph node infiltration was significantly less (*P* < 0.05) in carcasses from heifers fed PRT (902 versus 6,642 CFU/gm of lymph node, respectively). The percent of *Salmonella*-bearing lymph nodes was significantly less (*P* < 0.05) in carcasses from heifers fed PRT (4 versus 14%, respectively) as per Figure 4.2b.

4.4.3 Determination of *E. coli* O157:H7 in the Feces of Cattle

To determine if PRT had an effect on the presence of *E. coli* O157:H7 in the feces of the heifers, fecal samples (100 per treatment group) were quantitatively examined for the presence of this critical foodborne pathogen. As shown in Figure 4.3a, heifers fed PRT had a statistically lower (*P* < 0.05) *E. coli* O157:H7 fecal load than heifers fed PC (52 versus 122 CFU/gm of feces, respectively). Figure 4.3b reveals a decreased prevalence (*P* < 0.05) of *E. coli* O157:H7 in heifers fed PRT when compared to those receiving the PC diet (37 versus 57%, respectively).

4.4.4 Assessment of the Virulence of *Salmonella* Recovered from Cattle

In order to compare the virulence of *Salmonella* recovered from cattle, the isolates were subjected to an assay that predicts the ability of *Salmonella* to invade gut epithelial cells, which is a hallmark of *Salmonella* virulence (Millemann, 1998). The effects of PRT on virulence were examined due to the ability of SCFP to increase butyrate in the intestine (Possemiers et al., 2013). Research has shown that butyrate can decrease the *Salmonella* invasion gene (*hilA*) expression *in vitro* (Gantois et al., 2006), which results in the decreased ability of *Salmonella* to invade cells. In the current study, invasiveness of *Salmonella* was
significantly less ($P < 0.05$) in *Salmonella* recovered from the feces and lymph nodes of
cattle fed PRT (Figure 4.4). This decrease in invasiveness coincided with a decrease in the
expression of *hilA* (Figure 4.5), a major regulator of *Salmonella* virulence for mammalian
hosts (Bajaj et al., 1995).

**4.4.5 Assessment of the Antibiotic Resistance of *Salmonella* Recovered from
Cattle**

The antibiotic resistance of *Salmonella* in lymph nodes and fecal samples were
examined since SCFP can modulate the microbiome (Mullins et al., 2013; Price et al., 2010),
which can alter the antibiograms of resident bacteria (Benjamin C. Kirkup, 2004). To assess
the possibility that PRT inhibits antibiotic resistant *Salmonella* or induces the expulsion of
antibiotic resistance elements from *Salmonella*, isolates recovered from cattle were subjected
to an antibiotic susceptibility assay that utilized three individual antibiotics (ceftiofur,
enrofloxacin, and florfenicol). These three antibiotics were chosen given their extended
spectra and importance in bovine therapeutics. Additionally, two of the three antibiotics
tested (ceftiofur and enrofloxacin) have counterparts important for human therapeutics
(ceftriaxone and ciprofloxacin, respectively).

Figure 4.6 reveals a decrease in the prevalence of resistant ($P < 0.05$) *Salmonella*
recovered from heifers fed PRT for all three antibiotics. This figure represents isolates from
both feces and lymph nodes. It is of note that resistance of these antibiotics was, in general,
more prevalent in the fecal isolates when compared to the lymph node isolates.

**4.4.6 Assessment of the Presence of Three Important *Salmonella* Serotypes in the
Samples**

*Salmonella* isolates recovered from the feces or lymph nodes of cattle were subjected
to PCR assays targeting three serotypes (Dublin, Newport, and Typhimurium). As shown in
Figure 4.7, the prevalence of two of these serovars was diminished in heifers fed PRT,
regardless of the source of the isolates. No *S. Dublin* were isolated from feces and only one colony of *S. Dublin* was isolated from lymph nodes. Thus, statistical evaluations are not presented for this minor subsection of the study.

4.5 Discussion

*Salmonella* and *E. coli* O157:H7 are insidious problems for the beef industry and represent critical food safety hazards. *Salmonella* and *E. coli* O157:H7 can be shed in fecal material that can contaminate the carcass during processing. *Salmonella* is also harbored in the lymph nodes, which can lead to contamination of ground beef. Therefore, identifying mitigation strategies for both pathogens is needed especially considering the covert nature of *Salmonella* lymph node infiltration (Brichta-Harhay et al., 2008).

In this study, the anti-*Salmonella* and anti-*E. coli* O157:H7 effects of NaturSafe™ (PRT) were examined and two critical indicators of *Salmonella* contamination (fecal shedding and lymph node infiltration) were significantly reduced by NaturSafe™. In this study, heifers fed PC shed a higher number of *Salmonella* and *E. coli* O157:H7 and had more *Salmonella* present in the lymph nodes, which ultimately increases the risk of pathogen transmission to humans that ingest beef. There are currently no other research studies looking at the effects of NaturSafe™ on pathogens in beef cattle but there are studies examining the effects of other SCFP products on pathogen load. These results described herein are consistent with a previous study in which SCFP (SmartCare and Original XPC) reduced intestinal colonization in pre-ruminant calves (Brewer et al., 2014a), which is possibly due to macrophage-associated clearance of the pathogen. In another study, cannulated beef heifers supplemented with SCFP (Original XP®, Diamond V) had less *E. coli*
O157:H7 colonizing the recto-anal junction after being challenged with *E. coli* O157:H7 (Liou et al., 2009). Therefore, SCFP products seem to minimize the levels of *Salmonella* and *E. coli* O157:H7 in cattle.

Other significant and unique findings in this study were the reduction in virulence and antibiotic resistance in *Salmonella* recovered from heifers fed PRT. Reduced virulence was detected by diminished tissue culture invasion with a concomitant reduction in the expression of *hilA* (Bajaj et al., 1995). It is unclear how virulence was mitigated by feeding this SCFP prototype. However, previous studies revealed that SCFP (EpiCor) increases the gastrointestinal production of butyrate (Possemiers et al., 2013), a putative repressor of *hilA* expression (Durant et al., 2000). Regardless of the mechanism underlying this effect, the observed magnitude of decreased invasiveness is likely to increase the infectious dose of *Salmonella* for a human as evidenced by our prior study, in which this level of diminished invasiveness altered the murine LD<sub>50</sub> approximately 5-fold (Carlson et al., 2000).

### 4.6 Conclusions

In summary, NaturSafe™-fed feedlot heifers were significantly less likely to shed *Salmonella* and harbor this pathogen in the lymph nodes. The anti-shedding effect of NaturSafe™ was also observed for *E. coli* O157:H7. Additionally, NaturSafe™ reduced the virulence and antibiotic resistance of recovered *Salmonella*. Ultimately, these beneficial effects will have a marked positive effect on food safety in the beef industry. Future mechanistic studies are warranted to uncover the molecular bases for these effects.
Conflict of interest

This work was funded by Diamond V. Salary support was provided to K.L.A. in exchange for her time devoted to the project, but no other financial compensation was or will be awarded for this study. Thus the authors declare no conflict of interest.

Acknowledgments

The authors thank Dr. Katelyn Malin for her assistance in sample collection and feedlot personnel for animal husbandry.
### 4.7 Tables and Figures

#### Table 4.1 Composition of Diets

<table>
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<th>Ingredient</th>
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<th>Ration 3</th>
<th>Finisher</th>
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<td>-</td>
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<td>2.6</td>
<td>2.7</td>
<td>3.9</td>
<td>5.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Control rations were formulated to provide 300 mg of monensin (Elanco Animal Health, Greenfield, IN), 90 mg of tylosin (Zoetis Animal Health, Florham, NJ), 0.5 mg of melengestrol acetate (Zoetis Animal Health), and 50 gm Bovamine Defend (Nutrition Physiology Company, Overland Park, KS) per heifer daily throughout the study, and 250 mg of ractopamine hydrochloride (Zoetis Animal Health) per heifer daily during the last 28 days on feed.

<sup>2</sup>Rations containing PRT were formulated to provide 18 gm of a *Saccharomyces cerevisiae* fermentation prototype (NaturSafe<sup>TM</sup>; Diamond V, Cedar Rapids, IA) and 0.5 mg of melengestrol acetate (Zoetis Animal Health) per heifer daily throughout the study, and 250 mg of ractopamine hydrochloride (Zoetis Animal Health) per heifer daily during the last 28 days on feed.
Figure 4.1  *Salmonella* fecal load in heifers fed PRT (NaturSafe\textsuperscript{TM}) or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe\textsuperscript{TM}; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for measurements on 200 heifers in each group. *P* < 0.05 versus Control.
Figure 4.2  Prevalence of *Salmonella* fecal shedding in heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for the qualitative prevalence (％ of heifers shedding Salmonella) across pens in each group. *P < 0.05 versus Control.
Figure 4.3  
Assessment of Lymph node infiltration by *Salmonella* in heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for measurements on 200 heifers in each group. *P* < 0.05 versus Control.
Assessment of the Prevalence of *Salmonella* lymph node infiltration in heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for the qualitative prevalence (% of heifers shedding *Salmonella*) across pens in each group. *P < 0.05 versus Control
Figure 4.5  *E. coli* O157:H7 fecal load in heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for measurements on 50 heifers in each group. *P* < 0.05 versus Control.
Figure 4.6  Prevalence of *E. coli* O157:H7 fecal shedding in heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for the qualitative prevalence (% of heifers shedding *E. coli* O157:H7) across pens in each group. *P < 0.05 versus Control.
Figure 4.7 Tissue culture invasiveness of *Salmonella* recovered from the feces or lymph nodes of heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for group measurements on *Salmonella* recovered from 200 heifers in each group. *P < 0.05 versus Control.
Figure 4.8  Semi-quantitation of *hilA* expression of *Salmonella* recovered from the feces and lymph nodes of heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for pooled group measurements on *Salmonella* recovered from feces or lymph nodes. *P* < 0.05 versus Control.
Figure 4.9 Prevalence of antibiotic resistant *Salmonella* recovered from the feces and lymph nodes of heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for pooled group measurements on Salmonella recovered from feces and lymph nodes. *P* < 0.05 versus Control.
Figure 4.10 Prevalence of certain *Salmonella* serotypes recovered from the feces and lymph nodes of heifers fed PRT or PC. The PC diet contained monensin (Elanco Animal Health), tylosin (Huvepharma) and a direct-fed microbial (Nutrition Physiology Company). The PRT diet contained *Saccharomyces cerevisiae* fermentation prototype (NaturSafe™; Diamond V) and no monensin, tylosin, or a direct-fed microbial. Data represent the mean ± SEM for group measurements on *Salmonella* recovered from feces and lymph nodes. *P* < 0.05 versus Control.
CHAPTER 5. INHIBITION OF THE VIRULENCE, ANTIBIOTIC RESISTANCE, AND FECAL SHEDDING OF MULTIPLE ANTIBIOTIC-RESISTANT SALMONELLA TYPHIMURIUM IN BROILERS FED ORIGINAL XPC™

K. M. Feye1, K. L. Anderson1, M. F. Scott2, D. R. McIntyre2, and S. A. Carlson1

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1. Department of Biomedical Sciences, Iowa State University, Ames, Iowa, USA
2. Diamond V, Cedar Rapids, Iowa, USA

5.1 Abstract

Salmonella carriage is an insidious problem for the poultry industry. While most Salmonella serotypes are avirulent in poultry, this bacterium can contaminate chicken meat during processing and leads to one of the most important food safety hazards. In this study, we examined the anti-Salmonella effects of Diamond V Original XPC™ (XPC) included in the finisher diet fed to commercial broilers. On three occasions between D1 and D20, broilers were experimentally infected with multiple antibiotic-resistant Salmonella Typhimurium. After confirming that the chicks were shedding Salmonella in the feces on D21, broiler chicks were fed a diet containing XPC (n=57 birds; 1.25kg/MT) or an XPC-free Control diet (CON) (n=57 birds) to Day 49. Fecal samples were obtained weekly and subjected to selective culture for enumerating and determining the antibiotic resistance of the Salmonella. Salmonella isolates were then subjected to an in vitro virulence assay, which predicts the ability of Salmonella to cause illness in a mammalian host. Broilers were euthanized on D49 and a segment of the large intestine was removed and subjected to the same assays used for the fecal samples. When compared to the birds fed the CON diet, Salmonella fecal shedding, virulence, and antibiotic resistance were significantly decreased in birds fed XPC (5-fold, 7.5-fold, 6-fold, and 5.3-fold decreases, respectively). Birds fed XPC exhibited heavier body weight (BW) and greater BW gains than those fed the CON diet.
The decrease in virulence was associated with a decreased expression of a genetic regulator of *Salmonella* invasion into eukaryotic cells (*hilA*), while the decrease in antibiotic resistance was due to a loss of an integron (SGI1) from the input strain. This study revealed that Original XPC™ inhibits the shedding, downstream virulence, and antibiotic resistance of *Salmonella* residing in broilers.

### 5.2 Introduction

Most *Salmonella*, except for serovars Pullorum and Gallinarum (Wilson et al., 2000) and possibly other strains [e.g., S. Kentucky (Ogunleye et al., 2015)], are capable of asymptptomatically residing in the intestinal tracts of poultry. Many *Salmonella* serotypes can be acquired by the fecal-oral route and then be shed into the feces (Traub-Dargatz et al., 2006). Many birds can be infected since the ingestion, colonization, and shedding events typically cause no harm to the bird and since *Salmonella* is ubiquitous in the environment. *Salmonella* can therefore contaminate poultry meat prior to (from fecal shedding) or during processing (from intestinal leakage), resulting in one of the leading causes of *Salmonella* infections in humans (CDC, 2016).

Recent studies have shown that the fermentation metabolites of Original XPC™ (XPC, Diamond V, Cedar Rapids, IA), and earlier product derivatives, enhance poultry health and performance (Jensen et al., 2008) by promoting immune functions such as inducing the production of anti-viral antibodies, enhancing serum lysozyme activity, increasing IgM, increasing T-lymphocytes, and increasing secretory IgA (Gao et al., 2009; Gao et al., 2008). Additional studies revealed that an XPC-like technology, available for use
in humans (EpiCor®, Embria Health Sciences), increases NK cell activity and serum antioxidant protection (Jensen et al., 2011).

Furthermore, *Salmonella* suppressing effects have been shown in the gastrointestinal tract of the bird (Al-Homidan and Fahmy, 2007; Ibukic et al., 2012). Specifically, XPC fostered a significant reduction in both numbers and prevalence of *Salmonella* Heidelberg in broilers (Horface, 2015). These anti-*Salmonella* effects (decreased load and prevalence) have also been observed in calves fed XPC, where the product also protected the animal from various pathophysiological consequences of the pathogen (Brewer et al., 2014).

Because of these potential food safety benefits, a study was designed to further examine the anti-*Salmonella* Typhimurium effects associated with XPC in chickens. The objectives of this study were to determine the effects of XPC on a multiple antibiotic resistant *Salmonella* Typhimurium in broiler chickens experimentally infected with the microbe: specifically, fecal shedding, large intestinal carriage, downstream virulence, and antibiotic resistance. The virulence aspect was examined since an XPC-like technology (EpiCor®, Embria Health Sciences) has been shown to increase intestinal butyrate production (Possemiers et al., 2013), and this short-chain fatty acid has been shown to inhibit *Salmonella* virulence mechanisms *in vitro* by down regulating 17 genes (including *hilA*) in the pathogenicity island of *Salmonella* (Gantois et al., 2006). This repression in *hilA* was most evident in *S*. Enteriditis where a six-fold decrease in expression was observed through an unknown mechanism (Gantois et al., 2006). We also pursued the virulence studies since XPC can apparently suppress *Salmonella* virulence in cattle (Brewer et al., 2014).

Antibiotic resistance was examined since XPC can modulate the microbiome (Mullins et al., 2006; Price et al., 2010), which can alter the antibiograms of resident bacteria
(Kirkup and Riley et al., 2004). To assess the parameters, birds were experimentally infected with integron-bearing multi-resistant *Salmonella* and fed XPC after which quantity, prevalence, virulence, and antibiotic resistance of *Salmonella* was examined. Integron-mediated resistance was chosen since our previous studies revealed that intestinal factors facilitate the expulsion of integrons from *Salmonella* (Brewer, 2013). Comparisons were made to *Salmonella* recovered from experimentally-infected broilers fed a standard commercial diet.

### 5.3 Materials and Methods

#### 5.3.1 Experimental Design and Husbandry

An experiment was conducted at Iowa State University (Ames, IA) using day-old Cobb broiler chicks that were obtained from Welp Hatchery (Bancroft, Iowa). Three separate and independent replications of this experiment were conducted using a total of 50 chicks per experiment (25 per treatment group), resulting in a total of 75 chicks per treatment. On D0, birds were housed in a BL-2 facility in pens (0.09 m²; 10 birds/pen) within rooms that were both humidity (~40 %) and temperature controlled (35°C for three days then 28-31°C for the remainder of the study). On D14, birds were moved to elevated Tenderfoot-type decks (13.4 m² per treatment group) for the remainder of each experiment. Feed was provided in a metal feed trough and water through a bell drinker.

All birds were fed a non-medicated starter diet (24 % crude protein; Tractor Supply Company, Brentwood, TN) from D0 to 21. Birds were then randomly assigned on D21 to one of two feed treatment groups: 1) finisher Control diet only (CON), or 2) finisher diet that contained 1.25 kg/MT Original XPC™ (XPC; Diamond V, Cedar Rapids, IA). From D21 to
the basal diet was a non-medicated finisher diet (18-19 % crude protein; Solon Feed Mill, Solon, IA) and birds were allowed *ad libitum* access to feed and water. Photoperiod consisted of 12 h light and 12 h dark. All birds were individually weighed on D21 and then again at the end of the study on D49.

Each room held one treatment group to avoid inadvertently administering the wrong treatment within a room. Throughout the three consecutive studies, treatment groups were alternated in the two different rooms to avoid a potential room effect. The investigators at Iowa State University were blinded as to which birds received the CON or XPC diet during the entire study. The protocol used in these experiments was approved by the Institutional Animal Care and Use Committee at Iowa State University.

### 5.3.2 *Salmonella* Challenge

All birds were confirmed to be *Salmonella*-free by fecal culture upon arrival. Specifically, 1 to 5 g of freshly voided feces from each chick was diluted in 10 mL of Lennox L broth (Invitrogen, Carlsbad, CA). After settling for 1-2 hrs at room temperature, an aliquot (100 μL) of this mixture was streaked onto and then incubated overnight at 37°C on Xylose Lysine Deoxycholate (XLD) agar (Fisher Scientific, Pittsburgh, PA) that is selective for *Salmonella* which appear as white colonies with black centers (Anderson et al., 2015). All pre-infection fecal samples were free of *Salmonella*.

On D2, 9, and 16, birds were orally inoculated with *Salmonella* Typhimurium strain LNWI (Wu et al., 2002; Anderson et al., 2015). The dose increased from 2 x 10⁸ CFU/bird on D2 (Anderson et al., 2015) to 4 x 10⁸ CFU/bird on D9 to 8 x 10⁸ CFU/bird on D16 and this procedure was done to maximize the likelihood of large intestinal carriage. The *Salmonella* inoculum was prepared and dosed as previously reported (Anderson et al., 2015;
Xiong et al., 2010; Xiong et al., 2013; Xiong et al., 2012). The inoculum was slowly introduced into the mouth of each bird using a pipette tip. Previous studies revealed that *Salmonella* is viable after incubation with either XPC (at the concentration equivalent to the dose used in this study) or the CON treatment (Anderson et al., 2015).

5.3.3 Assessment of *Salmonella* Fecal Shedding Prior to Treatments

On D6, 13, and 20, 1 to 5 g of freshly voided feces from each bird was diluted in 10 mL of Lennox L broth (Invitrogen, Carlsbad, CA). After settling for 1-2 h at room temperature, an aliquot (100 μL) of this mixture was streaked onto and then incubated overnight at 37°C on XLD agar. On D7 and 14, fecal samples were examined for the qualitative presence of *Salmonella* colonies on XLD agar. On D21, *Salmonella* were enumerated on XLD agar and shedding was determined quantitatively as number of colonies x 100 (i.e., the dilution factor) divided by the grams of feces in the sample. Any non-shedding individual birds (as determined by fecal culture, n=6 per group per each of the three separate trials) were euthanized and removed from the study on D21. The remaining birds were assigned to either treatment group based on body weight and *Salmonella* shedding, using a serpentine assignment format that mathematically redistributes birds in order to prevent a weight bias between groups. Specifically, each bird was ranked based on weight and the bird with the lowest weight was grouped (e.g., Treatment Group A) with the bird with the highest weight; the bird with the second lowest weight was placed in the other group (Treatment Group B) along with the bird with the second highest weight; the bird with the third lowest weight was placed in Group A along with the bird with the third highest weight; the bird with the fourth lowest weight was placed in Group B along with the bird with the fourth highest weight; *etc*. As an illustrative example using 36 birds segregated into two
treatment groups (either XPC or CON), the following body weight-based rankings would be used in each group: Treatment Group A, birds 1, 36, 3, 34, 5, 32, 7, 30, 9, 28, 11, 26, 13, 24, 15, 22, 17, and 21; Treatment Group B, birds 2, 35, 4, 33, 6, 31, 8, 29, 10, 27, 12, 25, 14, 23, 16, 21, 18, and 19. Fecal shedding was also factored into the assignments for birds with identical weights or when an odd number of birds were available for segregation into the two groups. That is, the fecal shedding data was considered, when necessary, in order to make the average fecal shedding equivalent between the groups.

5.3.4 Assessment of *Salmonella* Shedding During Treatments

On D21, treatments began for each group of birds (n=19 to 22 per group after removing non-shedders in each experiment). On D28, 35, and 42, approximately 0.5 g of freshly voided feces (from each bird) was briefly vortexed in 10 mL of Lennox L broth (Invitrogen, Carlsbad, CA). An aliquot of this mixture (100 μL) was incubated overnight at 37°C on XLD agar. The following day white colonies with black centers were enumerated and CFU/g of feces was calculated based on a dilution factor equal to 100.

5.3.5 Assessment of Large Intestinal Carriage by *Salmonella*

On D49, all remaining birds were euthanized and a 5 cm section (approximately 3g) of distal intestine (between the cloaca and ceca) was aseptically removed from each bird and cut longitudinally. Each section was placed in 10 mL Lennox L broth (Invitrogen, Carlsbad, CA) and briefly vortexed to dislodge the *Salmonella*. An aliquot (100 μL) of this mixture was then dispersed onto XLD agar plates that were incubated overnight at 37°C. The following day, white colonies with black centers were enumerated and CFU/g of intestine was calculated based on a dilution factor equal to 100.
5.3.6 Assessment of the Invasiveness of *Salmonella* Recovered from Broiler Chickens

On D21, 28, 35, 42, and 49, *Salmonella* recovered from broiler chickens were subjected to a mammalian tissue culture invasion assay. After enumeration of colonies on XLD agar plates, approximately 30% of recovered colonies were immediately inoculated *en masse* into LB broth that was used in a standard gentamicin protection-based invasion assay using Human Epithelial Type 2 cells (Carlson et al., 2007; Carlson et al., 2000), with a multiplicity of infection equal to at least one. Bacteria were allowed to adhere and invade tissue culture cells for 1 h, after which the extracellular (i.e., non-invasive) were killed with 50μg/ml gentamicin. Tissue culture cells were then lysed with 1% Triton and the lysates were plated on XLD agar and grown overnight at 37°C. The next day, colonies were counted and percent invasion was calculated as 100 x (number of *Salmonella* recovered from tissue culture wells/number of *Salmonella* incubated with tissue culture wells). Invasion assays were performed in triplicate for both groups (XPC and CON) in each of the three separate experiments.

5.3.7 Assessment of Invasion Gene Expression in *Salmonella* Recovered from Broiler Chickens

Approximately 20% of *Salmonella* recovered from the birds were subjected to a semi-quantitative RT-PCR that assesses the expression of *hilA* (Carlson et al., 2007), the global regulator of *Salmonella* invasion (Bajaj et al., 1995). RNA was isolated and subjected to the semi-quantitative RT-PCR assay in which the number of PCR cycles (5 to 40) required to visualize an amplicon on agarose gel electrophoresis is documented (Carlson et al., 2007).

5.3.7.1 RNA Isolation

RNA was isolated from a group of colonies (n>40 colonies) picked directly from XLD plates and placed into PBS. RNA was isolated using the RNEasy kit (Qiagen) as per
the manufacturer’s protocol.

**5.3.7.2 Semi-Quantitative RT-PCR**

RNA (50ng/assay) was subjected to the semi-quantitative RT-PCR assay in which the number of PCR cycles (5 to 40) required to visualize an amplicon on agarose gel electrophoresis is documented (Carlson et al., 2007). PCR conditions and the *hilA* primers are described previously (Carlson et al., 2007). The *rpoS* primers are 5’-ATGAGTCAGAATACGCTGAA-3’ and 5’-TTACTCGCGGAACAGCGCTT-3’, representing the forward and reverse primers, respectively.

**5.3.7.3 Gene Analysis and Expression**

Subsets of reactions are removed every five cycles and resolved on 2% agarose gels run for 30 min., and amplicons are visualized under UV light. Expression is then calculated as percent of CON, *i.e.*, 100 x (lowest number of cycles required to visualize an amplicon for CON samples/lowest number of cycles required to visualize and amplicon for XPC samples). Invasion gene expression assays were performed in triplicate for both groups (XPC and CON) in each of the three separate experiments, with *rpoS* used as the housekeeping gene control whose expression does not change significantly. That is, *rpoS* amplicons are typically observed at 25-30 cycles whereas *hilA* amplicons were typically observed at a wider range (10-35) of cycles. Data were pooled in order to calculate the Mean ± SEM for three experiments performed separately.

**5.3.8 Assessment of the Antibiotic Resistance of Salmonella Recovered from Broiler Chickens**

On D21, 28, 35, 42, and 49, approximately 20% of *Salmonella* recovered from broiler chickens were assessed for resistance to chloramphenicol at the breakpoint concentration (32 μg/mL; (Institute, 2015). Chloramphenicol was chosen since resistance to this antibiotic is
encoded by the SGI1 integron present in the input *Salmonella* isolate (Wu et al., 2002). Individual black-centered colonies from XLD plates (n=96/treatment group) were inoculated into an individual well of a 96-well dish containing 200 µL of LB broth. Bacteria were grown statically overnight at 37°C to an OD$_{600}$ equal to approximately 0.3, which corresponds to a concentration of 3 x 10$^8$ CFU/mL. Approximately 3 µL of the growth was pin-replicated into a fresh 96-well dish in which each well contained 32 µg/mL of chloramphenicol in 200 µL of LB broth. Percent chloramphenicol resistance was calculated as 100 x (number of wells in which *Salmonella* grew in the presence of chloramphenicol/96). Chloramphenicol susceptibility assays were performed for both groups (XPC and CON) in each of the three separate experiments.

5.3.9 Assessment of the Presence of the Antibiotic Resistance-Encoding Integron in *Salmonella* Recovered from Broiler Chickens

To determine if the changes in chloramphenicol resistance were due to loss of the SGI1 (*Salmonella* genomic island 1) integron from the input strain, a PCR assay was performed to determine the qualitative presence of the SGI1 integron. Recovered *Salmonella* colonies were individually inoculated into LB broth in 96-well dishes in the absence of chloramphenicol. Bacterial growth was then subjected to a qualitative PCR assay developed and previously described by Carlson et al. (Carlson, 1999). Percent SGI1(+) was calculated as 100 x (number of wells in which *Salmonella* yielded an SGI1-specific amplicon visualized using agarose gel electrophoresis/96). SGI1 prevalences were determined for both groups (XPC and CON) in each of the three separate experiments, with the input strain used as a positive control.
5.3.10 Statistical Analysis

For data in which assessments were performed on multiple days (antibiotic resistance, invasion, and fecal shedding), statistical comparisons were made using a repeated measures analysis of variance with Tukey’s *ad hoc* test for multiple comparisons (GraphPad Prism, Version 6, La Jolla, CA). For data involving single measurements from each group (large intestinal carriage), statistical comparisons were performed using a student’s t-test (GraphPad). Body weight data were analyzed by the general linear model procedure of SAS software (Version 8.02, SAS Institute, Cary, NC) with Treatment (CON or XPC), Trial, and the interaction Treatment x Trial considered as the main effects. Absolute weight data were transformed to common logarithms prior to analysis. Mean separations were accomplished using LSMEANS with Tukey’s correction. For all variables under analysis, significant differences were defined at *P* ≤ 0.05. Statistical trends were consistent when the three sets of experiments were examined independently (data not shown).

5.4 Results

Three individual and independent replications of this experiment were conducted and no significant Trial*Treatment or Trial effects were observed, so data were combined for analysis. Data presented in Table 5.1 and Figures 5.1A, 5.1B, 5.2A, 5.2B, 5.3, 5.4, 5.5, and 5.6 represent the Mean ± SEM for three experiments performed separately.

Fecal shedding of *Salmonella* is a potential source of this pathogen for humans who consume poultry products. On D2, 9, and 16, birds were orally inoculated with *Salmonella* Typhimurium and on D21 they were assigned to either the CON or XPC dietary treatment. As expected, no significant differences were observed for fecal shedding of *Salmonella*
between chicks assigned to CON and XPC on D21 (6.5x10^5 versus 6.9x10^5 CFU/g, respectively), as dietary treatments did not start until D21 (Figure 5.1A). Significant differences (P <0.05) in fecal shedding were observed between CON and XPC on D28, 35, and 42 with lower fecal shedding of *Salmonella* in birds fed XPC when compared to the CON-fed birds (Figure 5.1A). On D28, the XPC-fed group had a 2.4-fold decrease (0.38 on a log_{10} scale) in shedding compared to CON-fed birds (2.7x10^5 versus 6.9x10^5 CFU/g, respectively). The greatest difference was observed on D42, with an approximately 7.5-fold decrease (0.88 on a log_{10} scale) in shedding for chicks fed XPC (7.7x10^5 versus 1.2x10^5 CFU/g, respectively). The relative prevalence of fecal shedding D49 (as indirectly measured by large intestinal carriage) was significantly less (P < 0.05) in birds fed XPC than CON (76% vs. 100%, respectively) (Figure 5.1B).

Since *Salmonella* carriage in the large intestine can be a source of contamination for poultry meat, the *Salmonella* load in each bird was determined at the end of the study. On D49, large intestinal sections were excised and subjected to *Salmonella* culture and enumeration that quantifies the intestinal load of *Salmonella*. As shown in Figure 5.2A, large intestinal carriage was significantly less (P < 0.05) in birds fed XPC compared to CON (3,875 vs. 29,023 CFU/g of intestine, respectively; 0.88 log_{10} reduction). The relative prevalence of large intestinal carriage was significantly less (P < 0.05) in birds fed XPC versus CON (71% vs. 100%, respectively) (Figure 2B).

To determine if the treatment had an effect on broiler performance as previously reported after challenge with a different *Salmonella* serovar (McIntyre, 2013), broilers in each group were individually weighed on D21 and 49 and these data are presented in Table 5.1. No significant two-way interaction between Treatment and Trial were observed for any
of the measurements, therefore only Treatment effects are presented in Table 5.1. Because of the serpentine assignment format, no significant differences in BW (body weight) were observed between birds assigned to XPC and CON groups on D21. By D49, XPC-fed birds were significantly heavier than CON-fed birds (3.504 vs. 3.243 kg, respectively). Birds fed XPC from D21 to 49 exhibited significantly heavier weight gain than CON-fed birds (2.613 vs. 2.343 kg, respectively).

At all five time points in which *Salmonella* were recovered and quantitated from the birds (D21, 28, 35, 42 and 49), presumptive colonies were collected by group and then subjected to a tissue culture invasion assay. This approach was taken since a similar technology (EpiCor®, Embria Health Sciences) has been reported to increase butyrate levels in the intestine (Possemiers et al., 2013) and butyrate can decrease *Salmonella* invasion gene expression *in vitro* (Gantois et al., 2006). No significant differences were observed for invasiveness of *Salmonella* on D21 (1.06 vs. 1.03%), as dietary treatments did not start until D21. Significant differences ($P < 0.05$) in invasiveness were observed between CON and XPC (1.08% vs. 0.18%, respectively; *i.e.*, a 0.78 reduction on a log$_{10}$ scale) on D49 with *Salmonella* exhibiting decreased invasiveness following isolation from birds fed XPC when compared to the CON-fed birds (Figure 5.3). This decrease in invasiveness of *Salmonella* coincided with a decrease in the expression of *hilA* (Figure 5.4), a major regulator of *Salmonella* virulence for mammalian hosts (Bajaj et al., 1995).

*Salmonella* recovered from birds were subjected to a chloramphenicol susceptibility assay. This line of study was pursued since the input strain bears a chloramphenicol resistance-encoding genetic structure (SGI1) that can be dislodged from *Salmonella* based on previous studies (Brewer et al., 2013). Figure 5.5 reveals a similar prevalence of resistant
Salmonella on D21 in both groups (96 vs. 94%) followed by a decrease in the prevalence of chloramphenicol resistance ($P < 0.05$) in Salmonella recovered from broilers fed XPC on D28, 35, 42 and 49, as compared to Salmonella recovered from CON fed birds (57 vs. 88%, 33 vs. 81%, 15 vs. 78%, and 15 vs. 75%, respectively). This reduction in chloramphenicol resistance was likely due to egress of the SGI1 integron, as presented in Figure 5.6, where about 80% of the isolates from the CON-fed birds retained SGI1 yet only 10-20% of isolates retained SGI1 in birds fed XPC.

5.5 Discussion

Salmonella is an insidious problem for the poultry industry, and this problem represents a critical food safety hazard. In addition, there are reports of non-typhoidal serovars causing salmonellosis in chickens (Ogunleye et al., 2012; Gong et al., 2016). Therefore, identifying mitigation strategies is of paramount importance to the poultry industry.

In this study, the anti-Salmonella effects of XPC were examined and two critical indicators of Salmonella contamination (shedding and large intestinal carriage) were significantly reduced by XPC. Both shedding and large intestinal carriage are dependent upon pathogen burden and it appears that Salmonella may be less efficient at maintaining an attachment to the intestinal tract in the presence of XPC. The diminished shedding was observed at all three time points including just seven days following the initiation of feeding XPC. In the CON-fed broilers, a higher number of Salmonella were present in the large intestinal tract, which ultimately increases the risk of pathogen transmission to humans that ingest the poultry meat. These results are consistent with a previous study in which XPC
reduced intestinal colonization (Ibukic et al., 2012). Additionally, the current studies revealed a performance benefit for feeding XPC, which is consistent with previous reports (Nsereko, 2013)

McIntyre (2013) reported that broilers, which were orally gavaged on Day 0 with a nalidixic acid resistant strain of *Salmonella* Heidelberg, and fed a diet with and without XPC, at an inclusion rate of 1.25 kg/MT, did not differ in BW Gain from D0 to 35 or D0 to 42. However, birds fed XPC did exhibit statistically lower cumulative feed conversions to D35 and 42 and better livability as compared to CON-fed birds (McIntyre, 2013). In the present study, birds were fed the CON diet until they were sorted into dietary treatment groups on D21, which was based on BW and *Salmonella* shedding using a serpentine assignment format. Therefore, it was not unexpected to find no significant differences for BW on D21 between the XPC- and CON-fed birds. However, the addition of XPC in the diet, starting on D21, resulted in improved BW on D49 and for BW gain from D21 to 49, suggesting that the addition of XPC can result in an improvement in a commercially important trait such as BW.

Other significant and unique findings in this study are the reduction of tissue culture invasiveness and SGI1-based antibiotic resistance in the *Salmonella* recovered from broilers fed XPC. The reduced virulence was manifested by diminished tissue culture invasion concordant with a reduction in the expression of *hilA*, the master regulator of *Salmonella* invasion (Bajaj et al., 1995). It is unclear how these virulence parameters were improved by feeding XPC, but previous studies revealed that XPC increases the gastrointestinal production of butyrate (Broomhead, 2012), which is an established repressor of *hilA* expression (Durant et al., 2000). Nonetheless, the observed magnitude of decreased invasiveness is likely to increase the infectious dose for a human as evidenced by a prior
study in which this level of diminished invasiveness altered the murine LD$_{50}$ by 10-fold (Carlson et al., 2000).

For the reduction in antibiotic resistance, this effect was correlated with a loss of the SGI1 integron from the input *Salmonella* strain. While we have no hypothesis for the mechanism by which XPC mediates this expulsion event, previous work revealed that SGI1 can be lost from *Salmonella* while in the intestinal tract (Brewer et al., 2013).

In summary, broilers were significantly less likely to harbor large intestinal *Salmonella* and subsequently shed the pathogen in birds that were fed XPC. It is unclear if this effect is due to decreased attachment or increased clearance of the microbe. Additionally, feeding XPC reduced the virulence and antibiotic resistance of the input *Salmonella* strain. Ultimately, these varying yet beneficial effects will have a marked positive effect on food safety in the poultry industry and future mechanistic studies will uncover the molecular bases for these effects.
### 5.6 Tables and Figures

#### Table 5.1 Feed Efficiency

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>CON&lt;sup&gt;3&lt;/sup&gt; ±</th>
<th>XPC&lt;sup&gt;3,4,5&lt;/sup&gt; ±</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW 21d</td>
<td></td>
<td>0.896 ±</td>
<td>0.881 ±</td>
<td>0.5077</td>
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<tr>
<td></td>
<td></td>
<td>0.018 ±</td>
<td>0.017 ±</td>
<td></td>
</tr>
<tr>
<td>BW 49d</td>
<td></td>
<td>3.243 ±</td>
<td>3.504 ±</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.045&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.044&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>BW Gain (21-49d)</td>
<td></td>
<td>2.343 ±</td>
<td>2.613 ±</td>
<td>0.0122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.045&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.043&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

1. Means ± SEM.

2. Means across rows within the same variable column with no common superscript differ significantly (P < 0.05).

3. Sample size: CON (n=57) and XPC (n=57)

4. Diamond V Original XPC™ inclusion rate was 1.25 kg/MT for all diets in the XPC treatment group.

5. Dietary treatment was applied on Day 21.

Body weights (kg), body weight gains (kg), and statistical probabilities for broilers fed with and without Diamond V Original XPC™ and challenged with *Salmonella* Typhimurium.
Salmonella fecal shedding (CFU/g) in broilers. Birds were orally inoculated with multiple antibiotic-resistant Salmonella Typhimurium on D2, 9, and 16. Dietary treatments, CON (n=57) or XPC (n=57), were applied on Day 21 and Salmonella were isolated from feces (using XLD agar) on D21, 28, 35, and 42. Data represent the Mean ± SEM for three experiments performed separately. \( a^b P < 0.01 \).
Prevalence of *Salmonella* fecal shedding in broilers. Birds were orally inoculated with multiple antibiotic-resistant *Salmonella* Typhimurium on D2, 9, and 16. Dietary treatments, CON (n=57) or XPC (n=57), were applied on D21 and *Salmonella* were isolated from feces (using XLD agar) on D21, 28, 35, and 42. Data represent the Mean ± SEM for three experiments performed separately. \(^{a,b}P < 0.01, ^{a,c}0.01 < P < 0.05.\)
Figure 5.3  Large intestinal colonization by *Salmonella* on Day 49 in broilers. Dietary treatments, CON (n=57) or XPC (n=57), were applied on D21. On D49, all birds were euthanized and a section of the large intestine was removed and selectively and quantitatively cultured for *Salmonella* using XLD agar. Data represent the Mean ± SEM for three experiments performed separately. $^{a,b}P < 0.01$.
Figure 5.4  Prevalence of large intestinal colonization by *Salmonella* on Day 49 in broilers. Dietary treatments, CON (n=57) or XPC (n=57), were applied on D21. On D49, all birds were euthanized and a section of the large intestine was removed and selectively and quantitatively cultured for *Salmonella* using XLD agar. Data represent the Mean ± SEM for three experiments performed separately. *a,b*P < 0.01.
Figure 5.5  Tissue culture invasiveness of *Salmonella* recovered from broilers challenged with *Salmonella Typhimurium*. Dietary treatments, CON (n=57) or XPC (n=57), were applied on D21 and continued until D49 when intestinal samples were taken and approximately 30% of the *Salmonella* recovered (approximately $10^5$ CFU) were subjected to the invasion assay using mammalian tissue culture cells and a multiplicity of infection equal to at least one. Percent invasion is calculated as $100 \times \frac{\text{number of } *Salmonella\text{ recovered from within the tissue culture wells}}{\text{number of } *Salmonella\text{ added to the tissue culture wells}}$. Data represent the Mean ± SEM for three experiments performed separately. $^{a,b}P < 0.01$. 
Figure 5.6  Expression of *hilA* in *Salmonella* recovered from feces of broilers challenged with *Salmonella Typhimurium*. Dietary treatments, CON (n=57) or XPC (n=57), were applied on D21 and continued until D49 when intestinal samples were taken and approximately 20% of the *Salmonella* recovered were subjected to the RNA isolation and semi-quantitative RT-PCR as previously described (Carlson et al., 2007). Expression was then calculated as percent of CON, i.e., 100 x (number of cycles required to visualize an amplicon for CON samples/number of cycles required to visualize an amplicon for XPC samples). Data represent the Mean ± SEM for three experiments performed separately. a,bP < 0.01
Figure 5.7  Chloramphenicol resistance of *Salmonella* recovered from the feces (D21, 28, 35, and 42) or intestines (D49) of broilers. Dietary treatments, CON (n=57) or XPC (n=57), were applied on D21. On D21, 28, 35, 42, and 49, approximately 20% *Salmonella* recovered from broiler chickens were assessed for resistance to chloramphenicol at the breakpoint concentration (32 μg/mL; CLSI, 2008). Data represent the Mean ± SEM for three experiments performed separately. \(^{a,b}P < 0.01\)
Figure 5.8  Presence of *SGII* in *Salmonella* recovered from the feces (D21, 28, 35, and 42) or intestines (D49) of broilers. Dietary treatments, CON (n=57) or XPC (n=57), were applied on D21. Recovered *Salmonella* colonies were individually inoculated into LB broth in 96-well dishes in the absence of chloramphenicol. Bacterial growth was then subjected to a PCR assay developed and previously described by Carlson et al. (1999). Percent SGI1(+) was calculated as 100 x (number of wells in which *Salmonella* yielded an SGI1-specific amplicon/96). Data represent the Mean ± SEM for three experiments performed separately. 

a,b P < 0.01, a,c 0.01 < P < 0.05
CHAPTER 6. THE C5AR1 ANTAGONIST NDT9513727 BLOCKS THE ANTI-SALMONELLA EFFECTS OBSERVED IN BROILERS FED XPC™, A SACCHAROMYCES CEREVISIAE FERMENTATION PRODUCT

K.M. Feye¹, K.L. Anderson¹, H.O. Pavlidis², G. R. Schmidt-McCormack³, N. C. Eischen¹, J. P. Carroll⁴, S.A. Carlson¹

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1. Department of Biomedical Sciences, Iowa State University, Ames, Iowa, USA
2. Diamond V, Cedar Rapids, Iowa, USA
3. Interdepartmental Neurobiology, Iowa State University, Ames, Iowa, USA
4. Interdepartmental Microbiology, Iowa State University, Ames, Iowa, USA

6.1 Abstract

The ability of the functional metabolites of Diamond V Original XPC™ (XPC) to reduce *Salmonella enterica* has been established across multiple food animal species. Previous gene expression studies suggested that the effects of XPC may be based on an upregulation of part of the complement cascade. The C5a complement protein, and its cognate receptor (C5aR1), contributes to the clearance of *Salmonella* in mammals and it may have the same properties in poultry. In order to evaluate the role that C5aR1 plays in the anti-*Salmonella* effects of XPC, we used a putative C5aR1 antagonist to block the effects of XPC. The drug NDT9513727 is an established bioavailable C5aR1 antagonist that was used for this study. Chicks were infected with *Salmonella enterica* Typhimurium DT104 on Days 2, 8, and 15 post-hatch. *Salmonella*-negative chicks were removed on Day 21, and the remaining chicks were redistributed to equalize body weights and *Salmonella* shedding between groups. The broilers were then placed on either the control diets (CON or XPC only) or experimental diets (NDT9513727 alone or XPC + NDT9513727). Fecal samples were collected on Days 21, 28, 35, and 42 followed by a terminal ceca collection on Day 49. Broilers were monitored for differences in *Salmonella* fecal shedding and colonization, as
well as feed efficiency. Significant differences in feed efficiency as well as *Salmonella* shedding and colonization (p< 0.05) were observed in chicks that were treated with XPC versus the three other groups. That is, NDT9513727 reduced the positive effects of XPC. Therefore, it is likely that C5a and C5aR1 are central, yet possibly indirect, to the anti-*Salmonella* effects of XPC.

### 6.2 Introduction

*Salmonella* is the leading etiological agent of foodborne illness and death in the United States (Besser, 2017; Finstad et al., 2012). While *Salmonella* are not overt pathogens in poultry in the U.S., mollifying the vertical transmission of *Salmonella* through the food chain is necessary to protect human health (Besser, 2017). As a result of this threat, regulations meant to safeguard consumers are an encumbrance for the poultry industry. Many mitigation products are available to poultry producers, including XPC™ (Diamond V, Cedar Rapids, IA), which has shown significant potential to abate *Salmonella* in food animal production (Feye et al., 2016; Feye et al., 2016). The specific chemical composition of XPC is not available but it is known that XPC is comprised of dried *Saccharomyces cerevisiae* fermentates containing over 300 distinct compounds (Jensen et al., 2008; Jensen et al., 2007; Jensen et al., 2011; Possemiers et al., 2013). When XPC is used as feed additive, it apparently generates changes in the microbiome that impact *Salmonella* biology and the host immune system (Park et al., 2017; Roto et al., 2015; Rubinelli et al., 2016).

The anti-*Salmonella* effects of XPC in poultry have been well-documented in field and laboratory experiments. XPC has three major anti-*Salmonella* effects: the reduction of antibiotic resistance, the attenuation of virulence mechanisms, and the reduction of
Salmonella shedding and colonization (Feye et al., 2016). That is, XPC-fed animals harbor Salmonella that are less likely to cause human illness and isolates are more likely to be susceptible to antibiotics. Moreover, the dampened virulence is sustained in the pathogen and does not return to baseline until it apparently passes through at least two additional hosts (Chapter 3).

A growing body of evidence suggests that the anti-Salmonella effects of XPC are a result of changes to the microbiome (Park et al., 2017; Roto et al., 2015; Rubinelli et al., 2016). This microbiome shift enhances the production of butyrate which is an established transcriptional down-regulator of hilA, the master regulator of mammalian cell invasion for Salmonella (Bajaj et al., 1995; Durant et al., 2000). This effect results in a less invasive pathogen, but it does not explain the reduction of Salmonella load and in shedding from animals fed XPC. Additional evidence suggests that XPC beneficially modulates the immune system resulting in the improved clearance of pathogens, which is likely downstream of the microbiome changes initiated by XPC (Blacher et al., 2017; Chou et al., 2017; Elooe-Fadrosh and Rasko et al., 2013; Jensen et al., 2007a; Pan and Yu, 2014; Park et al., 2017). This theory is termed the Salmonella clearance hypothesis, whereby XPC beneficially stimulates the immune system and improves innate effector function resulting. As a result, there is an improved clearance of Salmonella exhibited by broilers fed an XPC-containing diet. There are three pieces of evidence that lend credence to the enhanced Salmonella clearance hypothesis: gene expression studies (Chou et al., 2017), in vivo results from cattle on an XPC-containing diet (Feye et al., 2016), and ex vivo experiments evaluating the phagocytosis of Salmonella by innate effector cells (Chapter 3).
In the gene expression studies, Chou et al. demonstrated that complement protein C3 gene expression was upregulated 7.36-fold above the background in the spleens of broilers fed an XPC-containing diet (Chou et al., 2017). C5a acts as a chemoattractant for neutrophils and interacts with its cognate G-protein coupled receptor, C5aR1, to improve the innate effector response and perpetuate an appropriate inflammatory response (Kolev et al., 2014; Lee et al., 2008; Li et al., 2012). Central to the Salmonella clearance hypothesis, the C5aR1 actions lead to neutrophil infiltration into the lamina propria, the perpetuation of the response of adaptive effector cells, and the resolution of the infection (Coburn et al., 2007; Kurtz et al., 2017).

The second and third pieces of evidence to support the enhanced Salmonella clearance hypothesis arise from two studies that evaluated the ability of XPC to abrogate Salmonella in cattle. Feye et al. (2016) reported a reduction of Salmonella recovered from the peripheral lymph nodes of cattle fed an XPC-like product (Feye et al., 2016). This could be due to one of two possibilities: (1) XPC fortifies gut barrier integrity and Salmonella are unable to invade the lamina propria; or, (2) the Salmonella cannot shut down host surveillance strategies and as a result are successfully cleared. The third piece of evidence was provided by an ex vivo experiment that evaluated the difference in the clearance of Salmonella from the whole blood of cattle either fed XPC or a Control diet lacking XPC (Chapter 3). These data revealed that enhanced Salmonella killing in whole blood cells recovered from animals fed XPC. Taken together, these data support the enhanced Salmonella clearance hypothesis, which we further theorize to include C5aR1.

As a result of preliminary evidence and the established role of C5aR1 for clearing Salmonella, it became prudent to determine if C5aR1 activity underlies the anti-Salmonella
effects of XPC. The working hypothesis of this study is that blocking the C5aR1 receptor will reduce the XPC-mediated effects on *Salmonella*. To inhibit C5aR1, we used the well-established and bioavailable antagonist designated as NDT9513727 (Brodbeck et al., 2008; Klos et al., 2009; Klos et al., 2013).

### 6.3 Materials and Methods

#### 6.3.1 Experimental Design and Husbandry

Three independent cohorts were conducted at Iowa State University in a Biosafety Level-2 facility. Each study started with one-day old standard industry Cobb 100 chicks that were purchased from Welp Hatchery (Bancroft, Iowa). The protocol for these experiments was approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. The animal protocol is very similar to those executed by Anderson, *et al.* and Feye, *et al.* (Anderson et al., 2015; Feye et al., 2016)

Each of three experiments started with 25 chicks per treatment group, *i.e.*, 75 chicks in total per group. At 1-day post hatch, broilers were placed in brooder pens (0.09 m² with 10 broilers per pen) with *ad libidum* access to chick feed and water. The conditions of the brooder pens were established and maintained by the protocol delineated in Feye *et al.*, 2016 (Feye et al., 2016). Starting at two weeks post-hatch and throughout the remainder of the study, the broilers were moved to elevated Tenderfoot Decks (13.4 m² per treatment group) with consistent and easy access to feed and fresh water. The rooms were cleaned and monitored daily as to ensure a consistent environment and access to fresh water and food.

All of the broilers were fed a standard starter diet consisting of 24% crude protein (Tractor Supply Company, Brentwood, TN) from arrival to D21. At D21, *Salmonella*
shedding from the broilers was quantitatively assessed on a CFU/gram of feces basis. The broilers were then segregated into one of four treatment groups as per Feye et al., (2016) and fed a grower/finisher diet (Feye et al., 2016). The XPC group received 1.25kg/MT of XPC (Diamond V, Cedar Rapids, IA). The NDT9513727 group received the C5aR1 antagonist NDT9513727 (Tocris Bioscience, Pittsburg, PA) at 33 ng/kg of grower/finisher feed. This dose was chosen based on allometric scaling and the established IC_{50} of the antagonist (Brodbeck et al., 2008). The NDT9513727+XPC group contained NDT9513727 and XPC at the aforementioned respective concentrations. The CON diet contained neither XPC nor NDT9513727.

The investigators were blinded as to the identity of groups throughout the course of study, and the designation of each group changed with each cohort. Each group was simply known as A, B, C, or D to the researchers. The identities of each treatment group were matched to a different alphabetic designation for each study. After the data was collected and at the end of each study, a third party revealed the identity of the groups to the researchers.

**6.3.2 Salmonella Challenge**

Upon arrival to Iowa State University, broilers were qualitatively evaluated for the presence of *Salmonella*. In order to evaluate the broilers for *Salmonella*, approximately 0.1-0.3 grams of freshly voided feces were collected into sterile conical tubes containing 10 mL of Luria Broth (LB) (Invitrogen, Carlsbad, CA). The samples were vortexed and 100 μL aliquots were distributed onto Xylose Lysine Deoxycholate (XLD) agar (Fischer Scientific, Lenexa, KS). The XLD agar was chosen because it is selective for *Enterobacteriaceae* and differential for *Salmonella* (Feye et al., 2016). The individual aliquots were spread evenly
onto individual XLD plates using sterile plastic spreaders and allowed to absorb into the media. The plates were then inverted and incubated overnight at 37°C. The XLD agar was evaluated for *Salmonella* 24 hours post-inoculation. Colonies were considered to be *Salmonella* if they were round with even white or yellow margins and black centers.

On D2, 8, and 15, broilers were orally inoculated with *Salmonella enterica* Typhimurium phagetype DT104 as per Feye *et al.*, 2016 (Feye *et al.*, 2016). Starting on D20, the broilers were banded with identification numbers and individually analyzed for quantitative shedding on a gram of feces/CFU basis as per Feye *et al.*, 2016 (Feye *et al.*, 2016). The *Salmonella* was quantified on a CFU/gm of feces and was calculated for each broiler.

Any broilers that were not shedding *Salmonella* were removed from the study on D21. In order to distribute the bird weights and *Salmonella* shedding evenly across treatment groups, a serpentine block method was utilized to ensure an equal distribution of both factors as described in Feye *et al.*, 2016 (Feye *et al.*, 2016). The means of each factor within each group were taken to ensure that each treatment group had on average the same distribution of weights and shedding prior to the start of the feed trial phase (D21 to D49).

**6.3.3 Assessment of *Salmonella* shedding and Cecal Colonization During Treatment**

Quantitative analysis of the shedding of each bird was performed throughout the remainder of the study as previously described in Feye *et al.*, 2016 (Feye *et al.*, 2016). Quantitative analysis of the cecal colonization of each bird was performed on Day 49 as previously described in Feye *et al.*, 2016 (Feye *et al.*, 2016).
6.3.4 Assessment of Feed Efficiency

Feed efficiency was determined based on the protocol from Feye et al., 2016 (Feye et al., 2016d). The broilers were weighed on D21 on D49. The amount of feed consumed was also monitored. The feed:gain ratio was calculated using the following formula: (total feed consumed)/(start weight in kg – end weight in kg). Any bird that died between D21 and D49 was weighed and included in the calculations.

6.3.5 Statistical Analyses

For data with multiple sampling time points (prevalence, shedding), a repeated measures analysis was used with the Bonferroni ad hoc test for multiple comparisons (Prism Graph Pad 7.0). Since the level of shedding at D21 was different between experiments, all shedding was normalized to that observed on D21 for a given experiment. For feed efficiency and cecal colonization, an analysis of variance (ANOVA) was conducted with the Tukey’s ad hoc correction for multiple comparisons.

6.4 Results

Figures 1 through 5 are representative of the combination of three independent experiments. There was an inter-experiment difference regarding Salmonella shedding on D21. In an effort to address this issue, D21 served as the normalization point for each individual experiment. That is, shedding is reported as % of Day21 where Day 21 equals 100%.

6.4.1 Assessment of Feed Efficiency

Previous studies indicated that XPC boosts the feed efficiency of poultry (Feye et al., 2016) and thus C5aR1 antagonism should abrogate this production benefit if C5 is involved in the mechanism of XPC. To address this hypothesis, we utilized the feed:gain (F:G) ratio
as it is a global and non-specific indicator of feed conversion. As seen in Figure 6.1, the inclusion of NDT9513727 negated the XPC-mediated enhancement of feed conversion. That is, the NDT9513727 + XPC results were similar to that observed with the Control or NDT9513727 diets.

6.4.2 **Assessment of *Salmonella* Shedding from D21 to D42**

As shown in previous experiments, XPC reduces *Salmonella* shedding in broilers (Feye et al., 2016). As shown in Figure 6.2, which represents the amount of *Salmonella* shed relative to that observed on D21, NDT9513727 dissipated the XPC-mediated reduction of *Salmonella* shedding. Differences began to emerge on D28 and were fully evident on D35 and D42.

6.4.3 **Assessment of Prevalence of *Salmonella* Shedding from D21 to D42**

Previous studies revealed that XPC will reduce the prevalence of *Salmonella* shedding in broilers (Feye et al., 2016). That is, XPC increases the number of birds that shed no *Salmonella*. As shown in Figure 6.3, NDT9513727 eliminated the XPC-mediated effect on the prevalence of *Salmonella* shedding. This effect was observed at all three time points.

6.4.4 ***Salmonella* Cecal Colonization on D49**

Evisceration and de-feathering are important steps in the vertical transmission of *Salmonella* from poultry to the food supply. The ability of XPC to reduce colonization and prevalence is essential in mitigating that threat. In order to determine if NDT951372795 negates the effects of XPC, we quantitatively assessed the broilers for *Salmonella* colonization of the ceca. As shown in Figure 6.4, NDT9513727 inhibited the ability of XPC to prevent *Salmonella* colonization of the ceca. This trend was also noted for the prevalence of cecal colonization, as shown in Figure 6.5.
6.5 Discussion

The studies presented herein were designed to address the hypothesis that the C5aR1 is involved in the beneficial effects of XPC on *Salmonella* in broilers. This hypothesis was formulated based on gene expression studies revealing an upregulation of the expression of C3, a complement protein that is closely upstream of C5aR1. Based on data presented herein, the C5aR1 receptor is a likely component of the effects of XPC on chicken immunobiology as some of the activities of XPC are blocked by the putative C5aR1 antagonist designated as NDT9513727. Not all of the benefits of XPC were negated by NDT9513727. XPC can reduce *Salmonella* virulence and antibiotic resistance (Feye et al., 2016), but NDT9513727 had no effect on these parameters (data not shown).

While we have not directly shown that NDT9513727 inhibits C5aR1 in broilers, we have demonstrated that NDT9513727 is bioavailable and exhibits biological activity in the chicken by increasing *Salmonella* shedding, colonization, prevalence, and reducing feed efficiency in the presence of XPC. Future studies will ascertain the ability of NDT9513727 to perturb C5a binding to the C5aR1. Nonetheless, our results are consistent with the established ability of C5a to act as a chemoattractant for neutrophils. This complement protein interacts with C5aR1 to improve the innate effector response and perpetuate an appropriate inflammatory response (Klos et al., 2009). Moreover, our studies bolster the *Salmonella* clearance hypothesis whereby the actions of C5aR1 lead to neutrophil infiltration into the lamina propria, the perpetuation of the response of adaptive effector cells, and the resolution of the infection (Boothby et al., 2015; Klos et al., 2009; Kolev et al., 2014; Lee et al., 2008; Li et al., 2012; Ricklin et al., 2010; Schraufstatter et al., 2002).

The use of XPC as a production strategy in food animals to improve feed efficiency is evolving to be an important tool in improving food safety. The mechanisms behind the
efficacy of XPC are rapidly being uncovered. By understanding these pathways further, potentially new mitigation tools can be created and will expand upon the beneficial utility of products like XPC.
6.6 Figures

Figure 6.1  Effects of NDT9513727 on XPC-mediated enhancement of feed efficiency. Broilers were fed the Control diet, XPC, NDT9513727, or NDT9513727 + XPC. Feed efficiency was monitored from D21 through the terminal endpoint on D49. The data represents the mean ± SEM of the three independent experiments. For the XPC group, all pairwise comparisons were statistically significant at p<0.05 (i.e., * p<0.05 versus XPC).
Figure 6.2  Effects of NDT9513727 on XPC-mediated reduction in *Salmonella* shedding. The broilers were fed the four diets starting on D21 and were monitored for *Salmonella* shedding from D21 through D42. Shedding was normalized to D21 as the initial level of *Salmonella* shedding varied per experiment, though the pattern maintained itself. The data represents the mean ± SEM of the three independent experiments. * p<0.05, ** p<0.005, and *** p<0.0001 versus XPC.
Figure 6.3  Effects of NDT9513727 on XPC-mediated reduction in the prevalence of *Salmonella* shedding. The broilers were fed the four diets starting on D21 and were monitored for *Salmonella* shedding from D21 through D42. The data represents the mean ± SEM of the three independent experiments.  *p*<0.05 versus XPC.
Figure 6.4  Effects of NDT9513727 on XPC-mediated reduction in the colonization of *Salmonella* in the ceca. The broilers were fed the four diets starting on D21 and were monitored for *Salmonella* cecal colonization on D49. The data represents the mean ± SEM of the three independent experiments. For the XPC group, all pairwise comparisons were statistically significant at p<0.05 (i.e., * p<0.05 versus XPC).
Figure 6.5  Effects of NDT9513727 on XPC-mediated reduction in the prevalence of *Salmonella* in the ceca. The broilers were fed the four diets starting on D21 and were monitored for *Salmonella* cecal colonization on D49. The data represents the mean ± SEM of the three independent experiments. For the XPC group, all pairwise comparisons were statistically significant at p<0.05 (i.e., * p<0.05 versus XPC).
CHAPTER 7. DISCUSSION

The evidence presented herein supports the central hypothesis of this dissertation. That is, XPC exhibits anti-Salmonella properties resulting in the reduction of Salmonella prevalence, shedding, colonization, virulence, and antibiotic resistance in poultry and cattle. Further, XPC stimulates the immune system to efficiently phagocytize Salmonella, likely through the actions of C5aR1. As a result of our data, XPC becomes valuable tool to abate Salmonella and improve food safety.

7.1 XPC Reduces Salmonella Shedding and Colonization and Future Directions

In every study conducted by our group, there was a reduction in Salmonella prevalence, shedding, and colonization. Therefore, XPC is successful in mitigating the threat of Salmonella in poultry and cattle production. As a result of the data, Diamond V successfully validated the anecdotal reports that spurred on these investigations.

An unexpected result of XPC in cattle was the disproportionate reduction in the prevalence of naturally acquired Dublin, Typhimurium, and Newport. Interestingly, there may be serovar-specific metabolite preferences that dictate the emergence or disappearance of a particular serovar (Hayward et al., 2015; Rubinelli et al., 2016; Stecher et al., 2012; Steeb et al., 2013). XPC affects changes to the microbiome and alter the metabolites available to Salmonella (Park et al., 2017; Roto et al., 2015; Rubinelli et al., 2016). Therefore, in order to explore this phenomenon further, metabolomics studies targeting the interaction between the microbiome and various Salmonella serovars (e.g. Dublin, Typhimurium and Newport) need to be conducted in cattle fed XPC.
7.2 XPC Attenuates *Salmonella* and Future Directions

Despite what would appear to be an optimal paradigm, the abrogation of *Salmonella* to improve food safety does not necessarily require the complete removal of the pathogen from food animals. XPC attenuates the virulence of *Salmonella* while minimizing its burden in cattle and poultry (Feye et al., 2016; Feye et al., 2016). In doing so, XPC showed the capacity to succeed where antibiotics have failed, producing a weaker pathogen without spurring a resistance response. Part of this attenuation may come from the butyrate-driven reduction of the expression of the T3SS master regulator, *hilA* (Bajaj et al., 1995; Golubeva et al., 2016; Golubeva et al., 2012).

It is currently unknown as to how the changes in the microbiome alter *Salmonella* biology. In order to fully investigate the scope of how XPC affects *Salmonella*, regulatory networks and transcriptional responses must be queried through next-generation sequencing. Whole Genome Correlation Network Analysis can then be employed to mathematically correlate the transcriptional response of *Salmonella* with any known quantifiable phenotype, such as virulence factors or antibiotic susceptibility (Langfelder and Horvath et al., 2008). These clusters of phenotypically correlated genes, termed eigengene groups, can then be exported into a network analysis software program and evaluated for novel transcriptional regulatory relationships (Langfelder and Horvath et al., 2008). By utilizing this approach, we can identify currently unknown pathways or gene relationships whose XPC-mediated vulnerabilities may be exploited for the development of novel mitigation tools.

Altogether, investigations into the XPC-mediated reduction in virulence were productive and important for food safety. A diminution of *Salmonella* transmitted through the food chain may profoundly reduce the overall threat of *Salmonella*, resulting in less
morbidity and mortality worldwide. Thus, XPC has succeeded where antibiotics have failed by mitigating *Salmonella* without collateral effects.

### 7.3 XPC Reduces Antibiotic Resistant Genotypes and Phenotypes of *Salmonella* and Future Directions

In an era where antibiotic resistance renders modern therapeutics ineffective, a re-establishment of antibiotic susceptibility in foodborne pathogens like *Salmonella* is powerful. When evaluating potential tools to replace antibiotics, the topic of antibiotic resistance should be considered paramount as new tools should not worsen an already pervasive problem. Crucially, XPC directly combats antibiotic resistance in *Salmonella*. It can even be argued that, while anti-shedding and anti-colonization effects are important, the successful reduction in multi-modal mechanisms of antibiotic resistance are pivotal to the future of XPC and its derivatives.

The discovery of a total reduction in antibiotic resistance, including human clinical antibiotics (Feye et al., 2016; Feye et al., 2016), is provocative by itself. However, from a basic science perspective, additional questions arise as to whether or not there is a broader scope that may be explored by expanding upon the data presented. The maintenance of antibiotic resistance-conferring plasmids and transposable elements are energetically expensive for bacteria. In the controlled experiment in poultry (Chapter 4), the SGI-1 integron was undetectable in the majority of animals fed XPC. The probable loss of the integron is interesting when compared to other groups studying SGI-1 stability in *Salmonella*. These groups support the argument that the loss of the penta-resistant phenotype does not necessarily correlate with a loss of the integron after multiple passages (Huguet et al., 2016). In fact, experiments produced by these groups go even further and demonstrate that the loss of the integron is rare as it is nearly impossible to remove from the genome once
it is inserted (Kiss, 2012). The use of XPC data not only demonstrates a reduction in antibiotic resistance phenotypes, but a complete and uncharacteristic loss of a selfish and stable integron due to the use of XPC.

For this reason, our data evaluating the genotypic and phenotypic changes in antibiotic resistance of *Salmonella* are particularly powerful because XPC is able to destabilize an extraordinarily stable integron. Therefore, it will be necessary to further understand the DT104 integron and the changes that result to the biology of *Salmonella* that governs its loss. It is theorized that in order to destabilize the DT104 integron, one of two events need to occur: (1) the bacteria harboring this integron die; or, (2) a stable mutation or methylation of the genomic sequence for the toxin occurs, which uncouples the system and the bacteria evade suicide.

We can directly address this question in future work using both bioengineered tracer strains of *Salmonella* DT104 to examine integron mediated changes within the *Salmonella* population as well as COLD-PCR to evaluate the methylation of the integron. Scientists have started to bioengineer tracer strains with sequence tags inserted into inert regions of the genome with hopes of evaluating the spread of *Salmonella* between poultry flocks (Yang et al., 2017). Herein it is proposed that a second, highly specific tag could easily be inserted into an inert region of the *Salmonella* SGI-1 that is protected by the toxin-anti-toxin system, sgiTA (Huguet et al., 2016). We could then directly determine if the *Salmonella* population that persists within a flock has the integron but loses the phenotype, dies, or if the *Salmonella* completely loses the integron all together and persists. If this data are coupled with COLD-PCR, which exploits the T<sub>m</sub> differences between methylated and un-methylated regions of DNA, we could then address whether or not the loss of antibiotic resistance was due to
methylation (Kurdyukov and Bullock, 2016). It is important to understand this mechanism to try to predict if evasion by the selfish elements are possible as a result of uncoupling the toxin-anti-toxin system. It is also crucial to grasp whether or not the population of *Salmonella* that reside within a poultry flock still have, but do not express the integron on a much larger scale.

Obviously, antibiotic resistance is impactful worldwide. XPC is able to produce a multi-modal reduction in antibiotic resistance resulting in pan-sensitivity in *Salmonella* recovered from animals fed an XPC-containing diet. Since *Salmonella* and other *Enterobacteriaceae* serve as reservoirs for antibiotic resistant genes, the loss of these elements from those populations may result in a restoration of antibiotic susceptibility to the commensal *Enterobacteriaceae* over time. More research is needed to fully support that notion, but if proven, would be significant.

### 7.4 XPC, C5aR1, and Future Directions

Throughout the execution of this dissertation, we discovered that phagocytes recovered from cattle on XPC-containing diets were able to clear *Salmonella* more efficiently when compared to the controls. This data corresponds with a reduction in *Salmonella* recovered from peripheral lymph nodes, which are an emerging source for beef contamination. The mechanism behind this superior clearance of *Salmonella* by phagocytes likely lies within the complement system, specifically the C5aR1 pathway. Evidence supports this theory as the immunostimulatory effects of XPC were reversed by treating animals with NDT9513727, which is a putative antagonist for C5aR1. Furthermore, unpublished evidence also suggests that there is no difference in *hilA* expression and
antibiotic resistance between NDT9513727, NDT9613727+XPC, and XPC treatment groups when compared to the Control. Thus, the direct anti-virulence and anti-antibiotic resistance effects mediated by XPC are independent of the activation of the C5aR1 pathway.

The ability of XPC to modulate the immune system is likely global and is even demonstrated in the establishment of the immune system of poultry (Chou et al., 2017), the host-microbiome axis, and the ability to combat pathogenic assaults. As a result, XPC exhibits a multi-modal approach that is anti-Salmonella, through directly weakening Salmonella and indirectly improving the clearance of the pathogen.

7.5 Conclusions

Likely, the anti-Salmonella effects demonstrated by this dissertation are multi-modal and interconnected. These interrelated relationships are governed by the modulation of the microenvironment of the gut. The tuned immune system stabilizes the gut microenvironment and barrier functions, and results in improving animal health and feed efficiency (Blacher et al., 2017; Cho and Blaser, 2012; Eloé-Fadrosh and Rasko, 2013; Pan and Yu, 2014; Yan et al., 2017).

Importantly, XPC serves as an invaluable tool to improve feed efficiency in food animal production. As antibiotics are slowly phased out world-wide, the niche will need to be filled by a product like XPC that has demonstrated efficacy across species. Because XPC is immunostimulatory and is anti-Salmonella, it directly satisfies the void left by antibiotics in food animal production.
CHAPTER 8. REFERENCES


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