MODULATION OF CELL YIELDS AND GENETIC RESPONSES OF *SALMONELLA* FERMENTATION AND COLONIZATION IN THE GASTROINTESTINAL ECOLOGY OF AVIAN SPECIES

A Dissertation

by

KINGSLEY DELROY DUNKLEY

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

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Poultry Science

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ABSTRACT

Modulation of Cell Yields and Genetic Responses of *Salmonella* Fermentation and Colonization in the Gastrointestinal Ecology of Avian Species. (December 2006)

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In these studies we evaluated specific environmental stimuli relevant to *Salmonella* virulence and physiology in the gastrointestinal tract of chickens. Results from *Salmonella* growth in steady state, glucose-limiting continuous culture (CC) indicated that the optimal growth condition was observed between 0.05 h^{-1} and 0.27 h^{-1} dilution rates (D). Cell protein concentrations increased proportionally with an increase in D at each steady state, but after D 0.27 h^{-1} there was a reduction in the cell protein concentrations as the D increased. Genetic responses generally indicated that the lowest D exhibited highest *hilA* relative expression. Relatively higher expression of *hilA* was largely observed at low D (low glucose) (0.0125 h^{-1}, 0.025 h^{-1}, 0.05 h^{-1}). *Salmonella* incubated in CC at different pH shifts demonstrated that cell protein concentration, glucose utilization, Yield ATP and Acetate:Propionate ratios were influenced by an increase in pH (6.14 to 7.41). These parameters increased and decreased consistently with a corresponding increase and decrease in pH.

Polymerase chain reaction-based denaturant gradient gel electrophoresis showed that the overall amplicon band patterns of microbial similarity have demonstrated that

hens molted with Alfalfa (ALC+) diet were similar to the Full-Fed (FF+) treatment group. Additional, FF+ and ALC+ treatment groups exhibited a higher percentage similarity coefficient (>90%) than the feed deprived treatment group. Fermentation response from cecal inocula on feed substrates revealed that alfalfa based samples yielded consistently higher short chain fatty acid levels when compared to other feed substrates. *Salmonella* Enteritidis (SE) colonization in liver, spleen and ovaries was significantly ($P < 0.05$) higher in FW+ hens compared to ALC+ and FF+ treatments groups. A 4-fold ($\log_{10}$ 1.29) reduction in SE colonization for ALC+ hens compared to feed withdrawal hens (FW+) ($\log_{10}$ 5.12) SE colonization was observed. Relative expression of *hilA* in all treatment groups was significantly ($P < 0.05$) higher in FW+ compared to FF+ and ALC+ groups. *hilA* expression in FW+ hens was 3.2-, 4.2-, and 1.9-fold higher for Days 6, 11 and 12 respectively, when compared with to ALC+ hens.

These results suggest that *Salmonella* virulence in the gastrointestinal ecology of chickens could be impacted by a combination of low nutrients availability and pH shifts.

DEDICATION

I dedicate this dissertation to my entire family: my wife Claudia, four sons, Kingsley, Jr., Nicholas, Khori, and Ricardo, and the rest of the family. Without their care, consideration and inspiration, it would have been much more difficult to accomplish this remarkable achievement. To Claudia, who played a multifaceted role in this endeavor, a wife, classmate, lab mate and a shoulder to lean on. My mom, who is fighting cancer, thanks for your relentless encouragement and unwavering support. My favorite aunt, Lucille, thanks for your many phone calls, your financial support and most of all your prayers.

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Also thanks to Dr. Stanley my former professor for providing useful conversation and regular visits at our house during my graduate studies at Texas A&M. Also thanks to the following people for their useful help and assistance; Cassie, Albert, Palmy, Charles, Lynda, Phelue, Rhonda and all my lab mates.

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Salmonella are facultative Gram-negative intracellular bacteria consisting of non-spore forming bacilli of the family *Enterobacteriaceae*. *Salmonella* species continue to be the major causes of gastrointestinal infections, with *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Typhimurium (ST) identified as being responsible for most cases of salmonellosis human infections (Herikstad et al., 2002). It was reported that SE is responsible for 24.7% of non-typhoid illnesses of salmonellosis globally followed by ST (23.5%) (Rabsch et al., 2001). Salmonellosis costs an estimated US$ 2.3 billion annually for medical care and loss of productivity (Frenzen, et al., 1999). Investigation of outbreaks and sporadic cases has indicated that food vehicles identified the most common source of *Salmonella* infections are poultry and poultry by-products including raw and uncooked eggs (Coyle et al., 1988; Harrison et al., 1992; Bryan and Doyle, 1995; Hogue et al., 1997; Humphrey, 2000; Oldfield, 2001).

Salmonella has the capability to survive various environmental stress, nutrient deprivation, osmotic shock, heat stress and pH changes (Foster and Spector, 1995), which significantly determine their survival and virulence potential (Archer, 1996). Stress conditions associated with dietary shifts such as molting elevate the level of *Salmonella* invasion of internal organs of poultry (Humphrey et al., 1989; Thiagarajan et al., 1994; Durant et al., 1999; Holt, 2003; Ricke, 2003a; Woodward et al., 2005). The

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This dissertation follows the style and format of Poultry Science.

penetration of *Salmonella* pathogens through the intestinal epithelium and internal organs is essential for invasion and pathogenesis (Jones et al., 1994; Giannella et al., 1973). Genes required for *Salmonella* pathogenesis are located on *Salmonella* Pathogenicity Island 1 (SPI1) at centisome 63 on the chromosome (Galán and Sansonetti, 1996). HilA, a transcriptional activator encoded by SPI1, coordinately regulate the expression of invasion genes in response to environmental conditions of stress and low nutrients (Bajaj et al., 1996). Additionally, *rpoS* encodes an RNA polymerase sigma factor (δ^S or δ^{38}) known to regulate at least 60 genes in response to environmental signals including nutrients limitation, osmotic challenge, acid shock, heat shock, oxidative damage, redox potential and growth in steady and stationary phase (Hengge-Aronis, 1999; Ishihama, 1997; Loewen et al., 1998; Jishage et al., 2002; Komitopoulou et al., 2004; Hirsch and Elliot, 2005). *RpoS* exhibits several low pH inducible acid defense systems that push the limit of pH tolerance of microorganisms including *Salmonella*; referred to as Acid Tolerance Response (ATR), (Foster, 1995; Lee et al., 1995, 1994).

Poultry industry management practices, such as feed deprivation employed as a method to induce molt, may be responsible for shifts in the gastrointestinal pH (Durant et al., 1999), which presumably has an effect on *Salmonella* invasion, growth and nutrient uptake. Native intestinal microorganisms such as *Lactobacilli* are known to provide protective mechanisms against *Salmonella* by producing volatile fatty acid (VFA) that maintains pH in the ceca (Barnes et al., 1980; Corrier et al., 1995; Nisbet et al., 1994; Nurmi and Rantala, 1973; Van der Wielen et al., 2001, 2002) and the crop

(Barnes et al., 1980; Durant et al., 1999, 2000). It is well documented that *Salmonella* invasion in the ceca has been negatively correlated with an increase in VFA and decreased pH (Barnes et al., 1979; Corrier et al., 1995; Nisbet et al., 1994). Even though the mode of action has not yet been elucidated, VFA can inhibit *Salmonella* growth when present in undissociated form that is generally higher in concentration at lower pH in cecal ecosystem of chickens.

There is the perception that the role of fermentation of microorganisms in chickens is not as important as in the case of ruminants (Józefiak et al., 2004). The ceca are the major fermentation organs in GI tract of chickens and contain the largest number of bacteria (Annison et al., 1968; Barnes et al., 1972, 1973; Barnes, 1979). It is apparent that the cecal microbial ecology is an important factor in limiting pathogen colonization during dietary stress such as molting. In a previous report Zhu et al. (2002) indicated that only 10-60% of microorganisms in the ceca can be propagated using anaerobic culture techniques. Over 200 different species of bacteria have been isolated and characterized (Barnes et al., 1979), however, the physiology of these bacteria are influenced by various factors including host diet, health and age.

High fiber diets and non-starch polysaccharides have received more interest recently in poultry diets for retaining and promoting beneficial gastrointestinal microbial populations during nutritionally stressful management practices (Seo et al., 2001; Ricke, 2003a; Woodward et al., 2005). Upon ingestion, dietary fiber may affect the GI tract by altering its microbial activities, rate of passage, metabolites and digestive efficacy (Bach Knudsen, 2001; Wenk, 2001). The purpose of the current research was to investigate

Salmonella pathogenicity, growth phases, and competitive niches in the gastrointestinal ecology under natural and simulated conditions. The overall objective of my work is to evaluate specific environmental stimuli encountered by *Salmonella* that regulate their opportunistic behavior. Comparisons of molecular responses, microbial shifts, growth, infectivity rates and fermentative end products have been examined to determine the potential ecological preferences of *Salmonella* during invasion in the gastrointestinal tract of chickens.

CHAPTER II

LITERATURE REVIEW

Salmonella nomenclature

Salmonella are gram-negative bacteria consisting of non-spore forming bacilli and a member of the family *Enterobacteriaceae*. The *Salmonella* nomenclature is quite complex and is based on the names from serotype and subspecies 1; for example, *Salmonella enterica* subspecie *entericae* serotype Enteritidis is shortened to *Salmonella* serotype serovar Enteritidis or *Salmonella* Enteritidis (Brenner et al., 2000). *Salmonella* can be further subdivided into biotype and phage typing; a biotype is a biochemical variation between two microorganisms of the same serotype, whereas the phage type relates to the differences in susceptibilities to a lytic bacteriophage of two microorganisms of the same serotypes (Ward et al., 1987; Varnam and Evans, 1993). *Salmonella* are also classified by three distinct types of antigens and these include the agglutination properties of the somatic O, flagella H, and capsular Vi antigens. Antigens have been used to isolate and identify more than 2500 serotypes of *Salmonella* (Popoff et al., 2003). There are two species of *Salmonella* including, *S. bongori* and *S. enterica.* S. *enterica* is divided into six subspecies including *entericae, salamae, arizonae, diarizonae, houtenae* and *indica*. The most common O-antigen serogroups within *S. enterica* subspecies 1, are A, B, C1, C2, D and E. These serogroups are numerically the most significant and cause approximately 99% of *Salmonella* infections in humans and warm-blooded animals (Uzzau et al., 2000).

Epidemiology

Foodborne salmonellosis is responsible for over 600 deaths and 1.4 million illnesses in the US annually (Mead et al., 1999), and with cost ranging from US$ 464 million to 2.3 billion annually for medical care and loss of productivity (Frenzen, et al., 1999). In 1999, 22% of all culture confirmed *Salmonella* human cases were hospitalized (Kennedy et al., 2000). *Salmonella* have also been commonly associated with foods such as raw meat, poultry, eggs, and dairy products. In Europe the number of human cases reported to the internet was greater than 100,000 in 1997 (O'Brien and de Valk, 2003). Recently, the incidence of salmonellosis has shown a significant decrease across Europe (73,000 cases in 2001) and in the USA since 1996 (CDC, 2003; O'Brien and de Valk, 2003). Approximately 60% of human cases reported to the CDC (in 2001) were caused by four serotypes including *Salmonella* Typhimurium, *S*. Enteritidis, *S*. Newport and *S*. Heidelberg (CDC, 2003). *Salmonella* cases, however increased in 1999 and were accompanied by decreases in *Campylobacter jejuni*, *Shigella*, and *E. coli* O157:H7 (Kennedy et al., 2000). The infectious dose for humans may be as low as 1-10 *Salmonella* cells, although typically a dose of 10^4 to 10^6 *Salmonella* cells is necessary for infection. Low infectious doses are often associated with high fat foods (Portillo, 2000).

Salmonellosis

Foodborne salmonellosis is a gastrointestinal infection that continues to be of tremendous concern in the food animal production and consumption of food animal products (Mead et al., 1999). *Salmonella* species continue to be the major causes of gastrointestinal infections, with *Salmonella* Enteritidis and *Salmonella* Typhimurium

identified as being responsible for most cases of salmonellosis infections (Herikstad et al., 2002). It was reported that SE is responsible for most cases (24.7%) of non typhoid illnesses of salmonellosis globally followed by ST (23.5%) (Rabsch et al., 2001). Salmonellosis costs an estimated US$ 2.3 billion annually in medical care cost and loss of productivity (Frenzen et al., 1999). *Salmonella* cause asymptomatic intestinal infections in a wide range of animal species especially birds (Duchet Suchaux et al., 1995; Suzuki, 1994). A variety of investigations of outbreaks and sporadic cases has indicated that food vehicles identified as the most common source of *Salmonella* infections are poultry and poultry by-products including raw and uncooked eggs (Harrison et al., 1992; Bryan and Doyle, 1995; Hogue et al., 1997; Humphrey, 2000; Coyle et al., 1988; Oldfield, 2001). Egg shells can be contaminated with *Salmonella* as a result of intestinal passage and the ability to penetrate into the avian egg (Parry et al., 2002). *Salmonella* also can be highly invasive in laying hens leading to systemic infections that can potentially be deposited in the internal contents of eggs by transovarian transmission following colonization of the intestinal tract (Humphrey et al., 1989; Thiagarajan et al., 1994; Holt, 1993, 2003; Holt et al., 1995; Durant et al., 1999; Ricke, 2003a; Woodward et al., 2005). Acute outbreaks exhibiting clinical disease along with high levels of mortality occur in chicks younger than 2 weeks old (Lister, 1988). Birds that are asymptomatic carriers may facilitate the spread of disease infections among flocks by environmental contamination of the intestinal contents (Duchet Suchaux et al., 1997; Gast and Holt, 1998). In addition, SE contaminated eggs have

proven to be extraordinarily difficult to detect unless bacterial populations exceed $\log_{10}$ 9.0 per egg (Humphrey, 1994).

Salmonella pathogenic mechanisms

Salmonella are enteric bacteria which colonize the intestinal tract, penetrate the intestinal epithelium and access systemic sites including the spleen and liver (Carter and Collins, 1974). The pathogenesis and invasion mechanism of *Salmonella* is not simply as a result of bacterial contact with epithelial cells, but involve the bacterium and a cascade expression of numerous bacterial virulence genes (Altier, 2005). The culture of mammalian cells *in vitro* has shown that the invasion of ST into epithelial cells is complex and involves genetic loci and host factors (Finlay et al., 1988; Betts and Finlay, 1992). Genes are required for the intestinal phase of ST invasion and are located on *Salmonella* Pathogenicity Island 1 (SPI1) located at centisome 63 on the chromosome (Galán and Sansonetti, 1996). HilA (Hyper Invasive Locus) a member of the *OmpR/ToxR* family of transcriptional activator coordinately regulates the expression of invasion genes in response to environmental conditions including pH, osmolarity, oxygen tension and low concentrations of nutrients (Bajaj et al., 1996).

Salmonella pathogenicity islands

Salmonella Pathogenicity Island 1 is a set of genes encoding for virulence, located on a particular locus in the bacterial genome including virulent strains of *Salmonella* (Donnenberg, 2000). *Salmonella* generally carry five different pathogenicity islands that encode the magnitude of virulence genes needed for invasion and evasion in the host (Wood et al., 1998). Pathogenicity islands constitute a major focused direction in the

evolution of bacterial pathogens, because their acquisition often determines the virulence properties of a microorganism (Blanc-Potard and Groisman, 1997). The most prominent group of genes involved in pathogenicity is *Salmonella* Pathogenicity Island 1 (SPI1), located at the 63 centisome (Wood et al., 1998). SPI1 is present in all phylogenic lineages of the genus *Salmonella*, however it is absent in closely related genera such as *Escherichia* (Bäumler et al., 2000). SPI1 chromosome is inserted between *mutS* and *flhA* genes of ST strains (Ochman and Groisman, 1996) and known to encode multiple genes required for *Salmonella* invasion and survival in the host system. SPI1 encodes three major classes of virulence genes. The first class encodes structural proteins that consist of a type III secretion system (TTSS) (Collazo and Galán, 1997; Hueck, 1998), the second class of SPI1 genes encodes effectors that are secreted by the TTSS as well as accessory secreted proteins that facilitates effector translocation (Hueck, 1998), while the third class is assumed to encode transcriptional factors including HilA, HilD, HilC (SirC and SprA), InvF and SprB (Bajaj, et al., 1995; Eichelberg et al., 1999; Schechter et al., 1999).

Type III secretion system

The Type Three Secretion System (TTSS) is encoded by the *Salmonella* Pathogenicity Island 1 (SPI1) and is required for the invasion of epithelial cells of hosts. Upon oral ingestion, *Salmonella* reach the small intestine where bacterial TTSS enables it to colonize the distal ileum and infect intestinal epithelium (Darwin and Miller 1999b; Wallis and Galyov, 2000). Over 28 genes are responsible for encoding a type III secretion system (*spa, inv, prg, and org*), secretory proteins (*sip*, or *ssp*; *spt*) and

regulatory mechanisms (*invF* and *hilA*), all of which aid in the ability of *Salmonella* to invade the host cell (Deiwick et al., 1998). The SPI1 TTSS creates a needle-like complex that emit effecter proteins, which in turn targets the cytosol of host cells (Kimbrough and Miller, 2000, 2002; Sukhan et al., 2001). The functions of this multi-faceted system and its effector proteins include *Salmonella*-induced necrosis of macrophages (Monack et al., 1996, 2001; Hersh et al., 1999; Brennan and Cookson, 2000), the rearrangement of actin to enhance invasion (Zhou and Galán, 2001), enteropathogenesis (Wallis and Galyov, 2000) as well as the secretion of a pathogen-elicited epithelial chemoattractant (PEEC) that facilitates the migration of polymorphonuclear (PNM's) phagocytes across the epithelial layer (McCormick et al., 1998; Lee et al., 2000). The expression of SPI1 TTSS is dependent on the specific combination of environmental signals that appear to act as sensory cues to the bacteria for the appropriate anatomical location to exhibit virulence behavior (Bajaj et al., 1996; Schechter et al., 1999).

Gene regulation in SPI1

HilA (Hyper Invasive Locus) is a member of the OmpR/ToxR family of transcriptional activator encoded on SPI1 and directly activates the TTSS genes (Lee et al., 1992; Bajaj, et al., 1995; Ahmer et al., 1999; Lostroh and Lee, 2001). The *hilA* encoding protein is believed to be approximately 531 to 553 amino acids in length (Bajaj, et al., 1995; Bajaj et al., 1996; Darwin and Miller, 1999a; Lucas and Lee, 2000; Lostroh and Lee, 2001; Lucas and Lee, 2001; Baxter et al., 2003; Boddicker et al., 2003) and is thought to be a requirement for *Salmonella* invasion due to its transcriptional properties. HilA, directly activates the expression of the *invF* and *prgH* operons that

encode the inv, spa, prg and Org protein components of the type III secretion system (Bajaj et al., 1995; Lostroh et al., 2000). *InvF*, a member of the AraC/XylS family, activates the expression of SPI1 and non-SPI1 effector genes including *sipBCDA*, *sptP*, *sopB* and *sopE* (Darwin and Miller, 1999b; Eichelberg and Galán, 1999). Even though *hilA* are a central regulator of invasion, expression is determined by two additional regulators; HilC (SirC and SprA) and HilD (Johnston et al., 1996; Eichelberg et al., 1999; Schechter et al. 1999). HilC and HilD function by binding to a region upstream of the *hilA* promoter required for both environmental and genetic control of *hilA* which eventually induce *hilA* expression (Lucas and Lee, 2001; Schechter and Lee, 2001; Boddicker et al., 2003; Olekhnovich and Kander, 2004).

The expression of *hilA* and other invasion genes is dependent on environmental signals, oxygen levels, osmolarity, pH, low carbon and growth phase (Galán and Curtiss, 1989, 1990; Ernst et al., 1990; Lee and Falkow, 1990; Schiemann and Shope, 1991; Behlau and Miller, 1993; MacBeth and Lee, 1993; Jones et al., 1994; Pegues et al., 1995; Rakeman et al., 1999; Fahlen et al., 2000, 2001; Boddicker et al., 2003). *hilA* expression can be inhibited by high oxygen, osmolarity, *pho-24*, and disturbance in the *sirA* or *barA* genes (Bajaj et al., 1996, Johnston et al., 1996, and Lundberg et al., 1999). This inhibition requires the specific region of 39 to 314 upstream of the *hilA* start site (Schechter et al. 1999). However, Mg^{+2}, Ca^{+2} and inorganic phosphate also regulate *hilA* as observed when certain mutations were introduced into the *phoPQ* and *phoRB* two-component regulator systems, a subsequent reduction in *hilA* expression occurred (Lucas and Lee, 2000).

Site of *Salmonella* invasion

Salmonella invasion occurs in the Peyer's patches of the small intestine and is marked by the penetration of *Salmonella* through the mucosal epithelium, associating preferentially with columnar epithelial and microfold cells (M cells) overlaying the Peyer's patches (Carter and Collins, 1974). Peyer's patches are made up of specialized lymphoid tissue (Slauch et al., 1997), which consists of approximately twenty-eight percent T cells including CD4: CD8 with a 3.7:1 ratio (Hathaway and Kraehenbuhl, 2000). M cells form a cover over the Peyer's patches and are responsible for the consumption of antigens found in the lumen (Hathaway and Kraehenbuhl, 2000; Slauch et al., 1997). The invasion of M cells is also associated with their destruction as well as the destruction of follicle-associated epithelium (Slauch et al., 1997). Contact of an antigen by M cells allows it to eventually pass on to the immune system (Hathaway and Kraehenbuhl, 2000). Penetration of the M cells results in the migration of *Salmonella* to macrophages residing in the lymphoid follicles. *Salmonella* have been shown to survive and replicate within macrophages from the host including mice and chickens (Abshire and Neidhardt, 1993). After passage through the Peyer's patch, *Salmonella* enters the follicle dome, which is home to the host lymphocytes and macrophages (Jones and Falkow, 1996). Interaction between *Salmonella* and the epithelial cells triggers the chemotaxis of phagocytic cells to the infected site (Ruitenberg et al. 1971).

***Salmonella* pathogenesis in chickens**

Even though *Salmonella* pathogenesis has been well characterized in the mammalian model, it is surprising to know that there is limited information on this

subject in the avian species. This could be attributed to the fact that except for infection which caused mortality in chickens in in less than 2 weeks old (Lister, 1988) *Salmonella* generally remained asymtomatic in adult birds. The utility of the light and electron microscopic examinations of intestines taken from chickens experimentally infected with various *Salmonella* species demonstrated similar cellular responses to these organisms, including the influx of heterophils and macrophages to the luminal surface of the intestine (Barrow et al., 1987; Turnbull and Snoeyenbos, 1973). Heterophils are considered to be the avian counterpart to mammalian neutrophils in their action as tissue phagocytes and their importance to host defense against bacterial infections (Brune et al., 1972). The capabilities of the heterophils and avian macrophages to kill *Salmonella* have been demonstrated through bactericidal assays performed *in vitro* (Stabler et al., 1994). If *Salmonella* evade the immune system, colonization and invasion of the intestinal epithelial occurs. *Salmonella* is able to move through and colonize other cells by inducing them to take up the bacteria (Lucas and Lee, 2000). Studies have shown that *Salmonella* in experimentally infected birds will migrate from the intestine to the liver, spleen and ovaries (Turnbull and Snoeyenbos, 1973; Thiagarajan, 1994; Holt et al., 1995; Durant et al., 1999; Ricke, 2003a; Woodward et al., 2005). This indicates that the invasion of avian *Salmonella* involves a sequential dissemination of the organ that is similar to what has been established in the mammalian model.

Post exponential growth phase of *Salmonella*

When nutrients become limiting in the natural environment, the growth of bacteria is frequently defined as stationary phase (Kolter, 1993). In this feature bacterial

replication ceases and cell density begins to decrease. Historically, research has shown that transition into survival during stationary phase is a more physiologically controlled event in bacteria than previously thought (Siegele and Kolter, 1992; Kolter, 1993). Cessation of growth can be caused by many environmental factors, including acid pH, osmotic stress, heat shock and redox potential (Almirón et al., 1992; Foster and Spector, 1986; Gentry et al., 1993; Lange and Hengge-Aronis, 1991). Considerable attention has been given to nutrient starvation with a primary focus on carbon, nitrogen or phosphorous source because these are identifiable with already highly characterized genetic and phylogenic changes that occur in bacteria including *Salmonella* (O'Neal et al., 1994). A major observation by Ševčík et al. (2001) demonstrated that under anaerobic conditions when electron acceptors are scarce the stationary phase of growth may be reached not only by nutrient deprivation, but due to a limited availability of electron acceptors such as oxygen. When *Salmonella* become exposed to such conditions upon colonization of a susceptible host, they quickly multiply and reach a density of 10^8 cfu/g as in cecal contents (Ševčík et al., 2001).

Genetics of *Salmonella* post exponential growth

The central regulator of stationary phase is expressed by *rpoS* (Lange and Hengge-Aronis, 1991; Hengge-Aronis, 1993), which is responsible for induction from a specific subset of bacterial genes expressed under stress condition. *RpoS* is known to be positively regulated by a starvation-specific molecule ppGpp (Gentry et al., 1993) accumulated as part of the stringent response. In addition, induction of sigma factor can influence the signal of alteration in the efficiency of metabolism including reduction in

cellular concentration of cAMP (Lange et al., 1993) and UDP-glucose. *RpoS* is an alternative sigma factor (σ^s) (katF) which has been demonstrated to be essential for stationary phase stress response in *Salmonella* and *E. coli* and an essential gene regulator for virulence in *Salmonella* Typhimurium (Kowarz et al., 1994; O'Neal et al., 1994; Robbe-Saule et al., 1995). *RpoS* encodes an RNA polymerase sigma factor (δ^S or δ^{38}) known to regulate at least 60 genes in response to environmental signals including various stress conditions, nutrients limitation, osmotic challenge, acid shock, heat shock, oxidative damage, redox potential and growth in steady and stationary phase (Hengge-Aronis, 1999; Ishihama, 1997; Loewen et al., 1998; Jishage et al., 2002; Komitopoulou et al., 2004; Hirsch and Elliot, 2005).

ST is an intracellular pathogen, which during the course of an infection are capable of residing in the macrophages, however, ST become exposed to a wide range of antimicrobial effectors including NAD(P)H oxidase (Phox) contained in the phagocytes. An initial oxidative bactericidal phase associated with the production of superoxide anion and hydrogen peroxide is followed by bacteriostatic phase where nitric oxide is produced (Robey et al., 2001). The combination of nutrient limitation and stress condition in the intracellular environment is probably a stimulus for *rpoS* induction (Fierer et al., 1992). Starvation also increases the intracellular levels of ppGpp, whereas the expression of *rpoS* is positively regulated by ppGpp (Ibanez-Ruiz et al., 2000). *Salmonella* strains difficient in ppGpp fail to synthesize *rpoS* as cells enter into stationary phase in a rich medium and under starvation (Ibanez-Ruiz et al., 2000). The

major effect of ppGpp induction is not exerted on *rpoS* mRNA abundance or on protein turnover but instead affects translational efficiency (Coynault et al., 1992)

Acid tolerance response of *Salmonella*

In *Salmonella, rpoS* is also integrally involved in the development of several low-pH inducible acid defense systems collectively referred to as Acid Tolerance Response (ATR) that, as the name implies, expands the range of pH tolerance (Foster, 1995; Hall et al., 1995; Foster and Hall, 1991; Lee et al., 1994, 1995). The pH regulation mechanisms involve a series of proton antiport systems that have been identified as critical features for the survival of acid condition at least among *Salmonella* and *E. coli* (Booth, 1985; Kroll and Booth, 1983; Zilberstein et al., 1982). These systems appear to be inducible by acid to protect the cell in certain pH limits (Foster and Hall, 1991; Zilberstein et al., 1982). Bearson et al. (1997) demonstrated that *rpoS* in ST is also an acid shock protein (ASP) that is expressed by 4-fold by transition from normal to acid condition (pH < 4.5). The importance of this induction has been demonstrated for ST to mount and sustain induction of the ATR (Lee et al., 1995). It is a complex adaptive response that induces both an σ^s-independent transient which is maximally induced by 20 min of pH 4.4 acid shock but progressively lost during longer adaptation (Foster, 1993) and a σ^s-dependent sustained ATR which can been seen during a longer period of acid environment of 60 to 90 min (Lee et al., 1995).

Response to short chain fatty acids (SCFA)

Short chain fatty acids are end-products of microbial fermentation (including *Salmonella*) in GI tract of humans and animals and include acetate, propionate, butyrate,

valerate, isovalerate, isobutyrate (Hungate, 1966; Annison et al., 1968; Kass et al., 1980; Jamroz et al.1998). Young chickens do not contain a wide diversity of anaerobic bacteria as a dominant fraction of the microflora (Van der Wielen et al., 2000), and as expected the concentration of acetate, propionate and butyrate are low during the first week of life (Barnes et al., 1979; Corrier et al., 1990; Van der Wielen et al., 2000). In the first 15 days of a young chickens' life, concentrations of SCFA in the ceca varies, this may contribute to their inability for protection against pathogen colonization (Nurmi and Rantala, 1973). After 15 days, however, SCFA concentrations increased and stabilized (acetate at 70 μmol g^{-1}; propionate 8 μmol g^{-1}; butyrate 24 μmol g^{-1}) in young chicks and are suggested to provide optimum concentration for pathogen exclusion (Van der Wielen et al., 2000). Increased in acetate, propionate and butyrate in ceca have been assumed to cause a decrease in viable population of *Enterobacteriaceae* in chickens (Van der Wielen et al., 2001). However, prior exposure of *Salmonella* to high levels of SCFA at neutral pH, may enhance survivability by increasing acid resistance (Kwon and Ricke, 1998).

The production of SCFA is assumed to be correlated with dietary composition. It has been concluded that concentrations of SCFA in cecal-chyme were decreased when chickens were fed a diet that contained high-methylated citrus pectin, while low methylated citrus pectin had no effect (Langhout and Schutte, 1996). However, studies by Tsukahara and Ushida (2000) showed that feeding a plant protein-based diet to chickens generates a higher concentration of SCFA than a diet based on animal protein.

It was assumed that the difference in SCFA concentration was due to a higher concentration of dietary fiber component in the plant diet.

SCFA can inhibit *Salmonella* growth when present in the undissociated form. Van der Wielen et al. (2001) demonstrated in a batch fed competitive exclusion co-culture that acetate, propionate and lactate inhibited *Salmonella* growth at pH 5.8, but did not at neutral pH. At pH 5.8 the total undissociated SCFA were significantly higher compared to the dissociated form at neutral pH (Van der Wielen et al., 2002). At low pH (5.8), it is thought that SCFA promote bacteriostatic action by increasing the concentration of undissociated acid, which can permeate the cell membrane of bacteria (Cherrington et al., 1991a) and cause bacteria to lose energy generating capacity in the form of ATP, hence, replication is compromised. While a bacteriostatic activity was observed on *Enterobacteriaceae* the SCFA did not inhibit beneficial GI tract bacteria such a *Lactobacillus* (Van der Wielen et al., 2000). McHan and Shotts (1993) observed toxic effects of SCFA on some *Entrobacteriaceae* and in an *in vitro* study showed a 50-80% reduction in ST in presence of SCFA.

In addition, it has been demonstrated that certain microorganisms indigenous to the GI tract of poultry have the potential to hydrolyze dietary fiber into oligosaccharides and other low molecular weight carbohydrates which leads to production of SCFA (Kass et al., 1980; Ricke, 2003b; Ricke et al., 2004a) needed to retain beneficial microorganisms such as lactic acid producing bacteria (Van der Wielen et al., 2000). Furthermore, studies with four species of birds indicated that metabolizable energy obtained from total SCFA

production was equivalent to 5 to15% of daily requirement for maintenance (Annison et al., 1968; Gasaway, 1976a, b).

Estimating microbial response with continuous culture

The growth of bacteria in a chemostat is controlled by the availability and concentration of the limiting nutrients (glucose) in the chemostat reservoir. As an infusion of fresh medium enters the growth chamber, the growth-limiting nutrient is immediately utilized with a comparative increase in cell concentration (White, 1995). However, cell growth will not continue until fresh medium with limiting nutrient is infused into the chemostat (Monod, 1950; Novick and Szilard, 1950; Pirt, 1965). Based on this mechanism bacterial growth rate in continuous culture chemostat is also dependent on the the flow rate (F, mL per h^{-1}) at which fresh medium is infused into the chemostat. This is referred to as dilution rate (D), which is determined by dividing the flow rate by the volume (V) of medium in the chemostat vessel (F/V). In batch cultures, in which fresh medium is not added, four phases of growth are observed (lag, exponential, stationary and death phase), however, steady states are rarely seen because the composition of the growth medium is always changing. In contrast, bacteria at steady state growth in a continuous culture (reached after 4-5 turnover of V), and occurred when growth rate constant (k) becomes equivalent to dilution rate (D) (Novick and Szilard, 1950). In addition, steady state is reached in a chemostat growth vessel when the rate of formation of new cells is equal to the rate of cells lost from the outflow of the chemostat chamber. Consequently, in a continuous culture chemostat growth vessel the growth rate of the bacterial culture can be externally changed by adjusting the flow rate,

which is technically the dilution rate (D) (Monod, 1950; Novick and Szilard, 1950; Pirt, 1965).

Microbial growth physiology and measurement

Y_{ATP} is the relationship between the formation of ATP and microbial cell biosynthesis; therefore it is the amount of dry cell weight an organism produced per g-mole of ATP (Bauchop and Elsden, 1960). Monod (1942) demonstrated that the growth of three microorganisms (*Bacillus subtilis*, *S.* Typhimurium and *E. coli*) in a batch culture yielded dry weights that were proportional to the source of substrate, however, the estimation of Y_{ATP} of carbohydrates fermentation was not determined. It was observed that *E. faecalis* produced a higher cell yield from glucose fermentation than *Lactotobacillus mesenteroides* (DeMoss et al., 1951). Further investigation indicated that each microorganism used a different fermentative pathway that produced different amounts of ATP per glucose (Heath et al. 1958). Based on this result it was initially accepted that cell yield was relatively equivalent to Y_{ATP} (Senez, 1962; Sokatch and Gunsalus 1957).

Originally, it was thought that Y_{ATP} was a biological constant and no more than 10.5 g of cell could be obtained per g-mole ATP (Forrest and Walker, 1971). However, based on a variety of studies Y_{ATP} value relative to g-cell appears to differ by approximately 50%, therefore, it could not be confirmed that Y_{ATP} was generally a biological constant (Stouthamer and Bettenhaussen, 1973). It was later concluded that there was at least a five-fold range in Y_{ATP} value (Stouthamer, 1979) and that theoretically Y_{ATP} should be

three times higher (32 versus 10.5 g of cells per mole of ATP) than the initial value proposed by Bauchop and Elsden (1960).

Bacterial cell yields are usually calculated on the basis of the depletion of energy, however, the estimation of energy source utilization does not take into consideration the energy source that is incorporated into cell materials (Pirt, 1965, 1982). If the ATP production from catabolism is minimal most of the energy source will be used for energy. Conversely, if the ATP production is high, the fraction of energy source used for carbon can be of a significant proportion (Russell and Cook, 1995). Cell growth in a minimal medium where Y_{ATP} value of 32 g of cell per mol of ATP, it is possible that 90% of the energy source could be used as carbon (Russell and Cook, 1995). Contribution of energy source to carbon can be relatively low. Bauchop and Elsden, (1960) reported that *Enterobacter faecalis* contributed as little as 4% of its energy source to cell carbon.

Maintenance energy

The growth of bacteria using continuous culture techniques has demonstrated that cell yields were generally reduced at slow growth rates (Russell, 1986). It was historically assumed that energy could be used for functions that are not directly related to growth (Herbert et al., 1956), this was identified as maintenance energy or energy related to non-growth functions. Based on experimental observations, Marr et al. (1962) concluded that maintenance energy could be described as a negative growth rate constant that would allow the prediction of the number of additional cells that could be produced in a scheme where maintenance energy was absent. Maintenance energy has

become defined as the difference between the theoretical maximum growth yield (if no maintenance was present) and the observed yield (Pirt, 1965). There are several cellular functions that are identifiable with maintenance energy including motility, maintaining ion gradient, actively transporting molecules, regulation of internal concentrations, heat production and turnover of enzyme and macromolecules (Turner et al., 1989; Tempest and Neijssel, 1984; Russell, 1986).

Marr et al. (1962) and Pirt (1965), in a mathematical model further explain maintenance energy by the use of a double-reciprocal plot of growth and cell mass. The data analysis in a reciprocal format demonstrated a statistical bias. The plot using the specific rate of substrate utilization versus growth rate has shown to provide a more even distribution of data points and possible deviations from linearity were more easily identified (Neijssel and Tempest, 1976; Stouthamer, 1979; Tempest and Neijssel, 1984). Maintenance was assumed to be a time-dependent function that was proportional to cell mass, but was not related to growth rate. Whenever energy source is sufficient in a continuous culture chemostat a disproportionately lower cell yield is usually observed (Neijssel and Tempest, 1976; Stouthamer, 1979; Tempest, 1978; Tempest and Neijssel, 1984), this may suggest that maintenance energy has a growth rate-dependent component (Pirt, 1982). At high bacterial growth rate in continuous culture, maintenance energy constitutes a relatively small proportion of total energy used while at low growth maintenance become significantly higher. At low dilution rates over 55% of energy derived from glucose goes to maintenance energy compared to approximately 15% for high dilution rates (Isaacson et al., 1975). In a previous study conducted by Ricke and

Schaefer (1996), the maintenance energy was determined from the growth of *Selenomonas ruminantium* in limiting and non-limiting concentration of NH_4Cl. The maintenance energy for *Selenomonas ruminantium* was somewhat higher for concentration of NH_4Cl below 100 mM. The $Y_{ATP\ MAX}$ increased by 22% from 0.05 mM to 5 mM NH_4Cl and by 59.2% 0.5 mM to 25 mM NH_4Cl. In addition, the maintenance energy was also somewhat higher for concentrations below 50 mM NH_4Cl (Ricke and Schaefer, 1996).

Methods for studying gastrointestinal microbial ecology

The concepts of the continuous culture (CC) techniques as described in the previous sections provide an *in vitro* model to study simulated GI tract metabolism and fermentation (Isaacson et al., 1975; Russell, 1986; Russell and Cook, 1995; Ushijima and Seto, 1991; Coleman et al., 1996; Hume et al., 1997; Nisbet et al., 2000;). A typical continuous culture experiment consists of a chemostat vessel that simulates specific GI tract physical and chemical properties and consists of an afferent inlet which inputs substrates and buffer and an efferent outlet that facilitates outflow port of a homogenous mixture of microorganisms, fermentative metabolites and substrates (Hoover et al., 1976). The growth of bacteria in CC in a chemostat can provide a more accurate reflection and control conditions that are closely related to the natural ecosystem. Steady state can be attained in which significant parameters can be quantified including rate of growth manipulation of nutrient source, pH status and evaluation of maintenance energy (Isaacson et al., 1975). This however, depends on how continuous the nutrient flows are in the particular GI tract system.

Experimentally CC techniques have been used to simulate GI tract microenvironment of humans (Coleman et al., 1996; Ushijima and Seto, 1991), and various animal species including chickens (Nisbet et al., 2000; Hume et al., 1997) and ruminants (Isaacson et al., 1975). Studies which model the human colonic ecology (Ushijima and Seto, 1991) demonstrated antagonism of indigenous microflora against enteropathogen from crude human fecal culture in anaerobic culture system. These studies observed that five human fecal microorganisms which provide levels of antagonism that mimic the crude fecal flora in GI tract in the presence of carbon sources such as lactose, sucrose and starch that were fermentable only by antagonistic bacteria. The enteropathogen (ST) was suppressed to 10^4 CFU/mL by 5 to 7 days after post challenged (Ushijima and Seto, 1991). Parameters such as competition for growth-limiting amino acid and microfloral density contributed to the superior competitiveness for normal microflora to antagonize pathogen. In another study, mixed culture consisting of 5 microorganisms with 6 substrates exhibited microbial antagonism to reduce *Salmonella* Typhimurium population (Coleman et al., 1996). This occurs when the density of indigenous microflora were equivalent.

In ruminants, the CC techniques have been used to extensively define the complexity and uniqueness of microorganisms and how they interact to successfully convert low nutrient diets to needed energy for metabolism (Isaacson et al., 1975; Russell, 1986; Russell and Cook, 1995). Parameters including passage rates, dilution rates, fermentative metabolites and enzymes kinetics are better understood with the use of these concepts. Increasing dilution rates and changing microbial efficiency (Crawford

et al., 1980; Hoover et al., 1984) resulted in a decrease in digestibility of ingesta (Kennedy and Milligan, 1978; Meng et al., 1995). In a single-effluent continuous culture system (Meng et al., 1999) the effect of dilution rates on fibrous and non-fibrous carbohydrates and protein fermentation by ruminal microorganisms was studied. An increase in D resulted in a decrease in digestion of all diets (Kennedy and Milligan 1978; Hoover et al., 1984). A reduction in digestibility with increased dilution rates could be associated with a decrease in retention time of the solid fractions which reflects a faster passage rate in GI tract microenvironment. Dilution was a determinant factor of ruminal microbial fermentation of fibrous and non-fibrous carbohydrates and protein fermentation and was positively related to microbial cell yield and growth efficiency.

Probiotics

Probiotics are generally referred to as any live microbial feed supplements that benefit the host animals by largely improving intestinal microbial balance (Fuller, 1989). The effects of probiotics in poultry includes maintaining normal intestinal microflora by competitive exclusion (CE), increasing metabolism, decreasing enzymic activity and ammonia production, as well as increasing feed intake and neutralizing digestive enterotoxins (Jin et al., 1997; Guillot, 2000). Microorganisms of the intestine that are recognized with probiotic properties include *Lactobacilli* and *Bifidobacteria* spp. which have been shown to exhibit beneficial effects for host such as promotion of gut maturation, gut integrity, antagonism against pathogen (*Salmonella*) and immune modulation (Carter and Pollard, 1971; Berge and Savage, 1975; Tlaskalova-Hogenova et al. 1994).

The use of probiotics in the food animal industry is synonymous to the use of a competitive exclusion culture. A competitive exclusion culture in which the bacterial composition is unknown is termed undefined CE culture while known bacterial composition is termed defined CE culture (Nisbet, 2002). Competitive exclusion as a pathogen-reduction strategy involves the addition of a (non-pathogenic) bacterial culture to the intestinal tract of food animals in order to reduce colonization or decrease populations of pathogenic bacteria in the gastrointestinal tract (Fuller, 1989; Nisbet et al., 1993, 1994; Steer et al., 2000). Nurmi and Rantala (1973) were the first to utilize CE as a viable pathogen-reduction strategy demonstrated and that *Salmonella* colonization in juvenile chickens was reduced by the administration of a preparation of gut bacteria originally isolated from healthy adult chickens. There have been numerous preparations desirous of improving their microbial complexity and resistant capabilities (Stavric, 1987; Stavric and D'Aoust, 1993). An established, mature, gastrointestinal microbial population fills all available environmental niches nutritionally, metabolically and physically, making an animal more resistant to colonization by opportunistic pathogen infections (Fuller, 1989).

Based on molecular assessment (Van der Wielen et al., 2002) results indicate that in adult chickens the microbial population becomes more complex, stable and better able to resist enteropathogens than their younger counterparts. In the 1990's, there was considerable progress achieved in developing cultures maintained in continuous flow culture, which were shown to effectively control *Salmonella* colonization when administered to chickens (Nisbet et al., 1993, 1994, 1996; Corrier et al., 1990, 1991)

The inhibitory mechanism against *Salmonella* colonization has been associated with a reduction in cecal pH, increase in cecal lactic acid and short chain fatty acid (SCFA), competition for attachment sites, competition for growth limiting nutrients, production of antimicrobial compounds, immunomodulation, and synergistic and antagonistic interaction (Stavric et al., 1987; Hinton et al., 1990, 1991; Ushijima and Seto, 1991; Ha et al., 1994; Van der Wielen et al., 2002).

The safety and efficacy of CE products have been proven to be quite successful (Salvat et al., 1992). CE should not be used arbitrarily; products have to be derived from a suspension or anaerobic culture of intestinal content of healthy birds as a prophylactic treatment rather than a therapeutic agent (Watkins and Miller, 1983). A CE culture can be derived from the animal of interest. For instance, a CE culture for use in chickens must be derived from healthy chickens, and likewise for pigs. Administration of a bacterial community to newly hatched chickens can lead to an early colonization of adherent bacteria on the intestinal mucosal surface forming a mat of microorganism occupying and environmental niches (Soerjadi et al., 1982; Stavric et al., 1991).

Prebiotics

Prebiotics can be defined as non-digestible carbohydrate fractions fed in diets that are beneficial to host by stimulating the growth of one or more bacteria in the GI tract (Gibson and Roberfroid, 1995). Prebiotics (dietary fibers) are predominantly a constituent of plant cell walls and also consists of non-starch polysaccharides (NSPs) along with non-carbohydrate compounds including lignin, protein, fatty acid, and wax (Bach Knudsen, 2001). Upon ingestion, dietary fiber may affect the GI tract by altering

its microbial activities, rate of passage, metabolites and digestive efficacy (Bach Knudsen, 2001; Wenk, 2001). Recently, certain dietary fractions including polysaccharides were identified as having the potential to be utilized as prebiotics (Verstegen and Schaafsma, 1999; Cummings and MacFarlane, 2002).

The particle size of feed structure is also known to influence *Salmonella* numbers (Mikkelsen et al., 2004). It has been demonstrated that pigs fed a coarse non-pelleted diet exhibited a significant increased in numbers of anaerobic bacteria, along with increased concentrations of organic acids and reduced pH in stomach compared to fine pelleted diets (Mikkelsen et al., 2004). Furthermore, changes in these parameters as well as a significantly higher concentration of undissociated lactic acid were presumably influential in reducing *Salmonella* population. Numerous studies have been carried out to evaluate the effects of feed structure on performance of poultry (Engberg et al., 2002, 2004; Jones and Taylor, 2001; Nir et al., 1995, 1994a; Svihus et al., 1997, 2001). In previous reports (Engberg et al., 2004; Jones and Taylor, 2001; Svihus et al., 1997, 2001), the addition of whole grains to feed instead of pelleted compound feed has also shown to increase feed conversion and growth of broilers. Furthermore, whole wheat feeding significantly increased gizzard weight, increased retention time and reduced pH in gizzard contents compared to pellet fed birds (Engberg et al., 2004; Bjerrum et al., 2005), and was also instrumental in reducing *Salmonella* population. In addition uniform particle size has been shown to contribute to the development and integrity of the GI tract which subsequently enhanced gut motility and backflow mechanisms in poultry

including reverse peristalsis of compound from nitrogenous-based from the cloaca to the ceca (Karasawa, 1989).

Alfalfa is being used widely as animal feed and as a high fiber feed source, is relatively high in protein, and has one of the slowest rates of passage through the avian system (Matsushima, 1972; Sibbald, 1979; Garcia et al., 2000). Dietary alfalfa is well balanced in amino acids and rich in vitamins and when fed to chickens as dietary supplement gives carcasses and egg yolks their desirable yellow color (Sen et al., 1998; Ponte et al., 2004; Mourão et al., 2006). In addition, alfalfa may have advantages associated with the fermentation properties by cecal microflora capable of limiting *in vitro* growth of *Salmonella* Typhimurium when alfalfa is present and has been shown to limit *in vivo Salmonella* Enteritidis colonization in laying hens (Donalson et al., 2004; Woodward et al., 2005; McReynolds et al., 2005).

Microbial ecology in GI tract of avian species

Historically, the microbial composition of the GI tract of avian species was not extensively defined compared to what is known about microorganisms in ruminants (Ricke and Pillai, 1999). There is the perception that the role of microorganisms in chickens is not as important as is the case in ruminants (Józefiak et al., 2004). Ceca are the major fermentation organs in GI tract of chickens and contain the largest number of bacteria (Annison et al., 1968; Barnes et al., 1972, 1973; Barnes, 1979). A previous report (Zhu et al., 2002) indicated that only 10 to 60% of microorganism in the ceca can be propagated using anaerobic culture techniques. However, over 200 different bacteria

have been isolated and characterized (Barnes et al., 1979). These bacteria are known to be influenced by various factors including diet, health and age.

It has been suggested that the avian digestive microbial ecology is altered during dietary stress such as molting, which leaves the GI tract vulnerable to pathogen infection and colonization (Durant et al., 1999; Holt, 2003; Ricke, 2003b). In the poultry industry feed deprivation is a procedure employed to induce molting to achieve a rapid and economical new egg-laying cycle (Brake, 1993; Holt, 1995). Changes in dietary composition of the GI tract of poultry such as feed deprivation have negative consequences on microbial population. It has been proposed that dietary fiber can be utilized preferentially by *Lactobacillus* and *Bifidiobacteria* species (Kaplan and Hutkins, 2000), which leads to the production of lactic acid and SCFA, which supports the maintenance of normal microbial populations, low pH and preventing the establishment of *Salmonella* (Fuller and Turvey, 1971; Juven et al., 1991). Studies have shown that feed deprivation significantly compromises the immune system (Holt, 1992a, 1992b) making laying hens susceptible to pathogens including *Salmonella* spp. It was demonstrated that molted hens which shed significantly more SE in their feces (Holt, 1993; Holt et al., 1995), normally show much higher levels of SE invasion in their internal organs including liver, spleen and ovaries (Thiagarajan et al., 1994; Holt et al., 1995; Durant et al., 1999). These findings suggest that feed deprivation promotes pathogen invasion in molted hens, which becomes problematic for the poultry industry.

The altering of passage rate (flow rates) represents the amount of digesta that passes a point along the GI tract in a given time (Brant and Thacker, 1958), it is considered

essential in maintaining the overall microbial population and integrity of the GI tract of chickens. Passage rate may vary in different segment of the GI tract and is dependent on the feed composition and texture (Dänicke et al., 1997; Mikkelsen et al., 2004). Adequate feed retention time is essential especially in the ceca in order to encourage microbial degradation for long periods of time (McNab, 1973), which leads to the production of important metabolites which maintain the integrity and retain correct complement of microbial diversity. The lower GI tract of most animal species including poultry is normally populated by large numbers of microorganisms (Barnes et al., 1972), and through various competitive niches and virulence capabilities, some are able to survive. To withstand the flow rates of food material GI tract microorganisms utilize mechanisms such as surface mucus colonization (Rozee et al., 1982) deep mucus and crypt association (Phillips et al., 1978) specific adhesions (Suegara et al., 1975), and development of specialized insertional structures (Chase and Erlandsen, 1976). Changes in the passage rates that are representative of dilution rates can alter the limiting nutrient and therefore could ultimately affect microflora composition in the GI tract ecology.

CHAPTER III

CELL YIELDS AND GENETIC RESPONSES OF A *SALMONELLA* TYPHIMURIUM POULTRY ISOLATE AT DIFFERENT DILUTION RATES IN STEADY STATE CONTINUOUS CULTURE

Introduction

Salmonellosis is among the major gastrointestinal infections in humans. It is responsible for over 600 deaths and 1.4 million illnesses in the USA annually (Mead et al., 1999). The consumption of raw poultry meat and by-product are partially implicated for the cause of foodborne salmonellosis (Bryan and Doyle, 1995; Humphrey, 2000) and costs an estimated US $ 2.3 billion annually for medical care and loss of productivity (Frenzen, et al., 1999). *Salmonella* has the capability to survive various environmental conditions including stress, pH changes, nutrient deprivation, oxygen tension, osmotic shock and heat stress (Foster and Spector, 1995). These conditions may significantly determine survival and virulence potential (Archer, 1996), especially in the gastrointestinal (GI) tract microenvironment of farm animals. The effects of *Salmonella* Typhimurium (ST) pathogenesis is dependent on the host infected, as it causes gastroenteritis in humans while the same organism can cause lethal enteric fever in mice (Carter and Collins, 1974). Likewise age is a factor as young chicks exposed to ST may subsequently develop clinical gastroenteritis while adult chickens typically become asymptomatic (Barrow et al., 1987).

Since passage rate (flow rates) represents the amount of digesta that passes a point along the GI tract in a given time (Brant and Thacker, 1958), it is considered essential in

maintaining the overall microbial population and integrity of the GI tract of chickens. Passage rate varies in different segments of the GI tract and is dependent on the feed composition (Dänicke et al., 1997). By determination of passage rate via markers Tukey et al. (1958) observed an initial appearance of excreta 2 to 2.5 h after intake, however, most of the marker was excreted within 12 h. Adequate feed retention time is essential, especially in the ceca, in order to encourage microbial degradation of feed substrate for long periods of time (McNab, 1973). To withstand the flow rates of food material GI tract microorganisms utilize mechanisms such as surface mucus colonization (Rozee et al., 1982) deep mucus and crypt association (Phillips et al., 1978) specific adhesions (Suegara et al., 1975), and development of specialized insertional structures (Chase and Erlandsen, 1976). Changes in the passage rates that are representative of dilution rates can reduce the microflora population and therefore reduce the digestion of nutrients (Choct et al., 1996). In an *in vitro* study of a mixed ruminal bacterial culture with glucose as the growth-limiting factor (Isaacson et al., 1975), indicated dilution rates corresponded with an increasing microbial glucose yield. This was largely linked to the level of maintenance energy of bacteria. The continuous culture model has been proven to simulate bacterial interaction of microbial ecosystems including the GI tract of chickens (Nisbet et al., 2000). The current study utilized continuous culture to grow ST under various dilution rates. The underlying rationale was to mimic and evaluate the pathogenesis of ST in the GI tract during shifts in passage rate. The objectives of this study were to determine cell yields and genetic response of ST during low and high dilution.

Materials and methods

Bacterial strain and media preparation

The ST chicken isolate selected for resistance to novobiocin and naladixic acid (NO: NA) has been previously described by Ziprin et al., (1990). Maintenance and conformation of bacterial culture integrity was described previously by Ricke et al. (1988). Luria Bertani (LB) medium containing 1% Bacto^{tm} tryptose (Difco Laboratories, Detroit, MI), 0.5% yeast extract (Difco) and 0.1% glucose. The pH (6.5) of medium was adjusted accordingly with NaOH and HCl (Sigma Chemical Co., St Louis, MO).

Preparation of Salmonella Typhimurium inoculum

Samples of ST were taken from a frozen glycerol stock, thawed and streaked onto an LB agar plates. Plates were incubated (Precison Scientific, Winchester, VA) overnight at 39^{o}C. After incubation, colonies were used to inoculate 3 mL of LB broth in a 15 mL borosilicate tube. Tube was incubated at 39^{o}C for 18 h. The inoculum was vortexed and 1 mL used to inoculate each of two continuous culture systems.

Establishment of continuous culture systems

Two continuous culture systems were established in BioFlo 110 chemostat/fermentor (New Brunswick Scientific Company, Edison, NJ) with a total vessel volume of 1.0 liter. Chemostats were prepared and calibrated according to New Brunswick Scientific Company Handbook. LB broth medium (described in the previous section) and chemostats were sterilized (40 min at 21/psi pressure and at 121^{o}C), with a 500 mL medium volume each. Chemostats were maintained anaerobically with a continuous stream of O_2-free CO_2 (Russell and Baldwin, 1979) at 39^{o}C and agitated at 100 rpm with

a 98% turnover rate. The two chemostats (designated Trials 1 and 2) were operated concurrently and used to facilitate changes in dilution rates (D). Chemostats were adjusted to 8 different dilution rates including, 0.0125 h^{-1}, 0.025 h^{-1}, 0.05 h^{-1}, 0.1 h^{-1}, 0.27 h^{-1}, 0.54 h^{-1}, 1.08 h^{-1} and 1.44 h^{-1}. All samples taken from these chemostats were removed from the growth vessel at steady state and not before four volume turnovers. At steady state, the dilution rate was considered to be equal to the growth rate of the microorganism in the chemostat culture (Pirt, 1987). Dilution rate changes were implemented by altering the flow rate of fresh LB medium (diluent) into the chemostats.

Sample collection

For each sample, an 8-mL aliquot of culture was collected from chemostats at designated time points using a sterile pipette (Drummond Scientific Co, Bromall, PA). Chemostats were monitored by determining the pH (Corning 430, Corning, NY) and OD (A_{600}), with a UV-visible light spectrophotometer (Spectronic 20 D, Thermo Spectronic Cooperation, Buffalo, NY). An aliquot (400 µL) of the culture was preserved immediately in 800 µL of RNAlater (Sigma, St. Louis, MO) and stored at –20^{o}C, (Kenmore, Sears Corp., Chicago, IL) these were used for analyses of gene expression (*rpoS* and *hilA*) at a later time. Three milliliters of the sample was centrifuged (Labnet, Hermle Z180M, Burladingen, Germany) at 6,000 x *g* for 6 min. The supernatant was aspirated from the cell pellets and filtered through a 0.45 µm- pore size membrane filter (Pall Cooperation, Ann Arbor, MI). The filtrate was stored at -20^{o}C for glucose and short chain fatty acid (SCFA) analyses. The cells were resuspended into 300 µL of a sterile 0.9% NaCl solution and stored at -20^{o}C to be used for protein analysis.

Protein determination

Protein determination was done using the method described by Lowry et al. (1951). Bovine serum albumin (BSA), (Sigma, St. Louis, MO) was used as protein standard for determining the level of protein in the samples. Protein samples with the enzyme mixture were measured on a spectrophotometer (Gilford Instrument, Model 260, Oberlin, OH) at OD_{600} and recorded. Calculations of protein concentrations were conducted based on linear regression of a standard sample (BSA), intercept and the resulting slope multiplied by the respective results from the enzymatic reaction of samples.

Glucose and yield glucose (Y_{glc}) determination

Glucose determination was done according to Bergmeyer and Klotsch, (1965). The transfer of enzyme mixture was read at OD_{340} from spectrophotometer (Gilford Instrument). Glucose level from the culture medium was subtracted from the original sample and was used to calculate quantity of glucose disappeared. Y_{glc} was based on mmol glucose disappearance. Y_{glc} was calculated based on the mg cell protein per mmol of glucose disappeared.

SCFA concentration and yield ATP determination

The concentration of SCFA from the chemostat samples was determined by gas chromatography as previously described by Corrier et al. (1990). The analyses were conducted with a gas chromatograph equipped with a flame ionization detector and peak profiles integration-quantification integrator (Shimadzu Corp., Columbia, MD). Each sample peak profile was integrated and quantified relative to an internal standard of

methyl-butyric acid placed in the same sample. Analyses were conducted at an oven temperature of 200^{o}C and a flow rate of 85 mL/min. The concentration of each acid was expressed in μmol/mL. Yield ATP (Y_{ATP}) was calculated based on the amount of cell protein concentration per mmol ATP assumed for each fermentation product (acetate propionate, butyrate) (Ricke and Schaefer, 1996; de Vries et al., 1970).

RNA extraction and primer design

Culture samples from chemostats stored in RNAlater (Sigma, St. Louis, MO) were thawed and prepared for RNA extraction. RNA was extracted from ST cultures according to the RNeasy Mini Kit (Qiagen, Valencia, CA). *Salmonella* RNA was subjected to reverse transcriptase-PCR (RT-PCR) to obtained cDNA. Primers were designed for *hilA* and 16S rRNA genes using sequences data obtained from the genBank website and optimized using primer express 1.0 Software (Perkin-Elmer Applied, Biosystems, Foster City, CA). Optimized sequences were processed on the NCBI website in order to determine their cross-reactivity with other species of bacteria. Primers used in this study are listed in Table 1 (McClelland et al., 2001).

Reverse transcriptase reaction

To obtain cDNA for real-time PCR, reverse transcription reactions (RT) were performed using the reagents from the TaqMan® OneStep RT-PCR Kit. Each 20-μL reaction contained 10X RT-PCR Buffer, 500 μM of dNTPs Mix, 20U of RNase Inhibitor, 25 mM of $MgCl_2$, 200 ng of RNA, 2.0 μL of each primer (2.5μM), MultiScribe™ RT 1.25/UμL and RNase-free water, to a final volume of 20 μL. A positive RT control reaction was run to ensure that the procedure was performing

TABLE 1: Primer sequences used in real-time PCR reactions (McClelland et al., 2001)

hilA FWD	5’ TATCGCAGTATGCGCCCTT 3’
hilA REV	3’ TCGTAATGGTCACCGGCAG 5’
16srRNA FWD	5’ TGCGCGACCAGGCTAAA 3’
16srRNA REV	3’ TTCACCACTGGCAGGTATTAAGC 5’
rpoS FWD	5’ GCACGTGAGTTGTCGCATAAA 3’
rpoS REV	3’ TTATCCAGTTGCTCTGCAATTTCT 5’

correctly. The positive (+) control reaction contained all of the same components except that a DNA template was used instead of RNA template. To determine if RNA samples were contaminated with DNA, two negative (-) RT reactions were run on each RNA sample. One (-) RT control reaction contained the same components as the positive (+) RT reactions, except it lacked the RNA sample template and contained additional water to ensure that the final concentration of the components remained the same. The other (-) RT reaction did not contain the RT enzyme to ensure that there was no RNA contamination. All one-step RT reactions were performed on a Gene Amp PCR System from (Perkin Elmer, Wellesley, MA) under the following conditions: Incubation for 10 min at 25˚C, reverse transcription for 30 min at 45˚C, reverse transcriptase inactivation for 5 min at 95 ^{o}C, three-step cycling 1 min at 94˚C 4, 1 min at 53˚C and 1 min at 72˚C for 40 cycles.

Real-time PCR reaction

Real-time PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City CA). Each 20-µL SYBR Green PCR reaction contained 1 µL of cDNA, 0.2 µL (µM) of each primer, 10 µL of 1X SYBR® Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), 8.6 µL PCR-water. Thermal cycling conditions were as follows: 48^{o}C for 30 min, 95^{o}C for 10 min, and 40 repeats of 95^{o}C for 15 second and 60^{o}C for 1 min. A dissociation curve was run following the real-time reaction to determine if the primers generated a specific product. The same (+) and (-) RT reactions were run for the real-time PCR as for RT reactions.

Gene analysis and expression

Real-time PCR reactions (Bustin, 2000; Orlando et al., 1998; Livak and Schmittgen, 2001; Peirson et al., 2003) were run to determine the expression of three genes. Each gene was analyzed as triplicate samples. Data were analyzed using the relative quantification method ($2^{-\Delta\Delta CT}$), (Livak and Schmittgen, 2001), which describes the change in expression of the target gene (*rpoS* and *hilA*) relative to a reference gene (16S rRNA) from an untreated ST control sample. Data were analyzed by averaging the C_T value (cycle at which each sample amplification curve crossed a specific threshold, ΔC_T) for each triplicate sample. The resulting ΔC_T values of the target genes (*rpoS* and *hilA*) were determined by normalizing to the endogenous control gene (16S rRNA) values. These samples were subsequently subtracted from the 16S rRNA gene from the untreated ST control sample (Fey et al., 2004). The resulting $\Delta\Delta C_T$ was then used to calculate relative expression using the formula $2^{-\Delta\Delta CT}$ (Guilietti et al., 2001; Livak and Schmittgen, 2001; Lehman and Kreipe, 2001).

Statistical analysis

Data were analyzed using the one way ANOVA subjected to linear regression using PC-SAS version 9.0 (SAS Institute Inc., Cary, N.C.), where *a* was the slope term, *b* was the intercept and *r* was the correlation coefficient. Differences between means were determined using least square means and Tukey's honest significance test. Statistical variation was also estimated by the standard error of the mean. All statistical analyses were considered significant at ($P < 0.05$).

Results and discussion

Glucose cell yield response

Continuous culture techniques have been used to simulate GI tract microenvironment of humans (Coleman et al., 1996; Ushijima and Seto, 1991) and various animal species including chickens (Nisbet et al., 2000; Hume et al., 1997) and ruminants (Isaacson et al., 1975; Russell, 1986). Studies which model the human colonic ecology (Ushijima and Seto, 1991) demonstrated antagonism of indigenous microflora against enteropathogens from crude human fecal culture in an anaerobic culture system. Parameters such as competition for growth-limiting amino acids and microfloral density contribute to the competitiveness of normal microflora to antagonize pathogens in these conditions. In the current study ST was grown in LB medium with added glucose and subjected to low (0.0125 h^{-1}, 0.025 h^{-1}, 0.05 h^{-1} and 0.1 h^{-1}) and high (0.27 h^{-1}, 0.54 h^{-1}, 1.08 h^{-1} and 1.44 h^{-1}) dilution rates (D). The highest dilution rates represented nutrient-rich conditions while the lowest D represented nutrient-limiting conditions (Crawford et al., 1980; Hoover et al., 1984). Dilution rates were selected to mimic the cecal microenvironment of poultry during dietary shifts as well as changing passage rates in chickens. Various poultry competition exclusion culture chemostats utilized D of 0.0416 h^{-1} for maintenance of poultry cecal bacterial cultures that were effective against *Salmonella* spp *in vivo* (Nisbet et al., 1993; 1994). In addition, the average dilution rates in ruminants (0.04 h^{-1} and 0.2 h^{-1}) (Isaacson et al., 1975) also are in this D range. The LB medium used was considered equivalent in nutritional composition to a typical corn

soybean-based poultry feed and was formulated to simulate the poultry feed (Audisio et al., 1999).

The optimal growth conditions for ST protein formation in LB monoculture were observed between 0.05 h^{-1} and 0.27 h^{-1} D in Trials 1 and 2 (Figure 1). The highest cell protein yield was observed at D 0.05 h^{-1} (411.94 ± 16.97 mg/L) in Trial 2 and the lowest at D 1.08 h^{-1} (171.22 ± 2.41 mg/L) in Trial 1. With the exception of D 0.1 h^{-1}, in Trial 2, cell protein concentration increased proportionally with an increase in dilution rate at each steady state but after D 0.27 h^{-1} there was a reduction in cell protein concentration as the dilution rate increased. This may be the initiation point of washout in the chemostat. Washout occurs as dilution rates approaches critical dilution rate (D_c). When D_c occurs, the cell population cannot grow fast enough to utilize substrate from the chemostat, therefore, cell population is washed out at dilution rate beyond D_c. If cells are not able to utilize the available substrate, this ultimately causes substrate concentration to accumulate in the chemostat and the number of cells would eventually be reduced (Monod, 1950; Novick and Szilard, 1950; Pirt, 1965).

Glucose concentrations in the chemostats were determined and are shown in Figure 2. A significant portion of glucose disappeared from the chemostat between D 0.0125 h^{-1} and 0.27 h^{-1} in both trials, however at rapid D (0.54 h^{-1}, 1.08 h^{-1} and 1.44 h^{-1}) glucose began to accumulate in the chemostat which corresponded to a noticeable decrease in cell protein synthesis. Therefore cell protein synthesis was influenced by D and

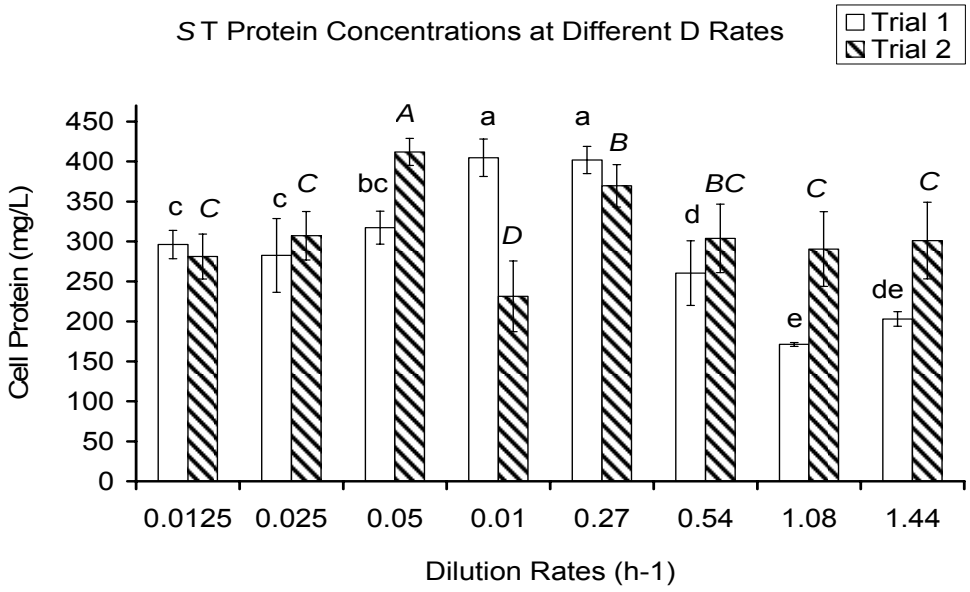
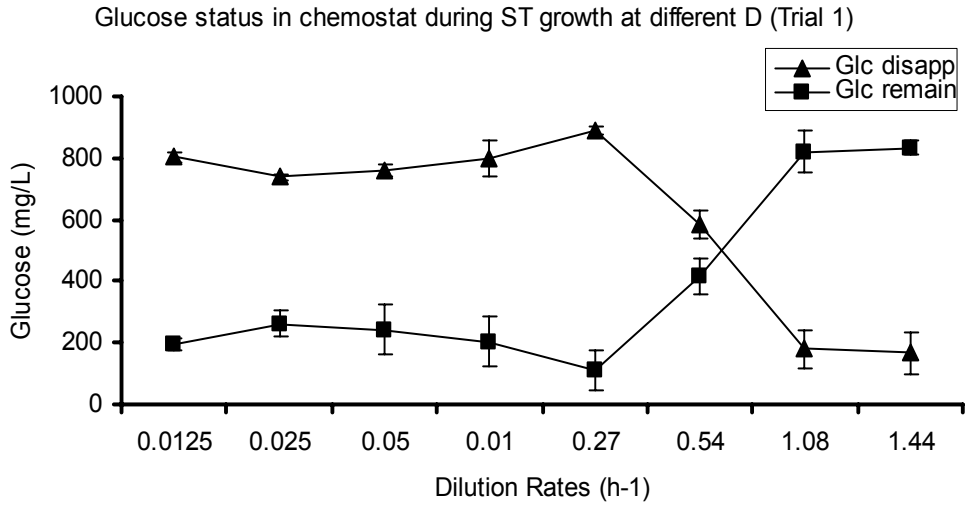


FIGURE 1: Cell protein concentrations of *Salmonella* Typhimurium (ST) at different dilution rates (Trials 1 and 2). Error bars represent std. err (±) of normalized values. Different letters above error bars indicate significant differences *(P* < 0.05); $^{a\text{-}e}$Means within Trial 1. $^{A\text{-}D}$Means within Trial 2. n = 3. D = dilution rates. std err = standard error.

suggested that ST consumption of glucose was feasible at slower D but was less efficient beyond D 0.27 h^{-1} and may be considered as critical D since glucose began to accumulate in the chemostat beyond this point. This data confirm the theory which suggested that as the culture approaches critical D (D_c), cell yield decreased and glucose began to accumulate in the chemostat. Therefore, the presences of ST in a GI tract microenvironment and passage rates of equivalent D are capable of reduced glucose consumption accompanied by lesser cell biomass.

In these experiments, glucose yields were calculated on the basis of mg cell protein per mmol of glucose disappeared from the chemostat (Figure 3). The initial dilution rate (0.0125 h^{-1}) exhibited the lowest glucose yield; 8.45 ± 0.34 and 7.28 ± 0.35 mg cell protein /mmol glucose disappeared) in Trials 1 and 2, respectively, while D between 0.025 h^{-1} and 0.54 h^{-1} gave a consistent glucose yield averaging approximately 10 mg cell protein/mmol glucose. This indicates that ST was able to utilize glucose at a relatively consistent rate between D 0.0125 h^{-1} and 0.54 h^{-1}. Based on the results for both trials, washout of ST cells from the chemostats was initiated at 0.54 h^{-1}, at this point glucose yield was still efficient and probably resulted from metabolic adjustment of cells from maximum growth rate at high D. At high D (1.08 h^{-1} and 1.44 h^{-1}) glucose yield per mg cell protein was exceptionally high, since culture was exposed to critical D (D_c) cells were unable to consume substrate efficiently (Russell, 1986). Driessen et al. (1987) reported that glucose utilization of ST is dependent upon the strain and glucose uptake system. Where the ST strain with phosphotransferase system posted higher cell yield values than on ST strain with only a constitutive galactose permease grown at the same

(a)



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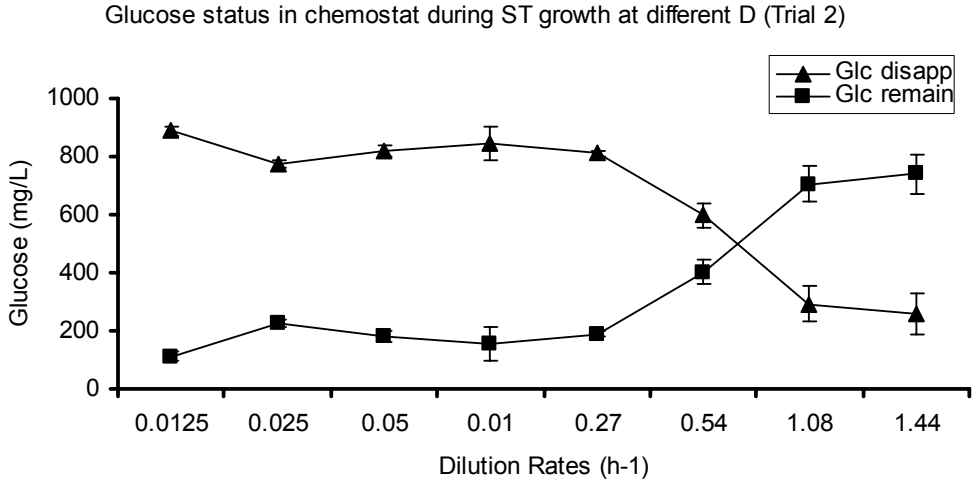


FIGURE 2: Glucose status in chemostat during growth of *Salmonella* Typhimurium at different dilution rates (Trials 1 and 2). (a) Trial 1. (b) Trial 2. Error bars represent std. err (±) of normalized values. Y = yield. D = dilution rates. std err = standard error.

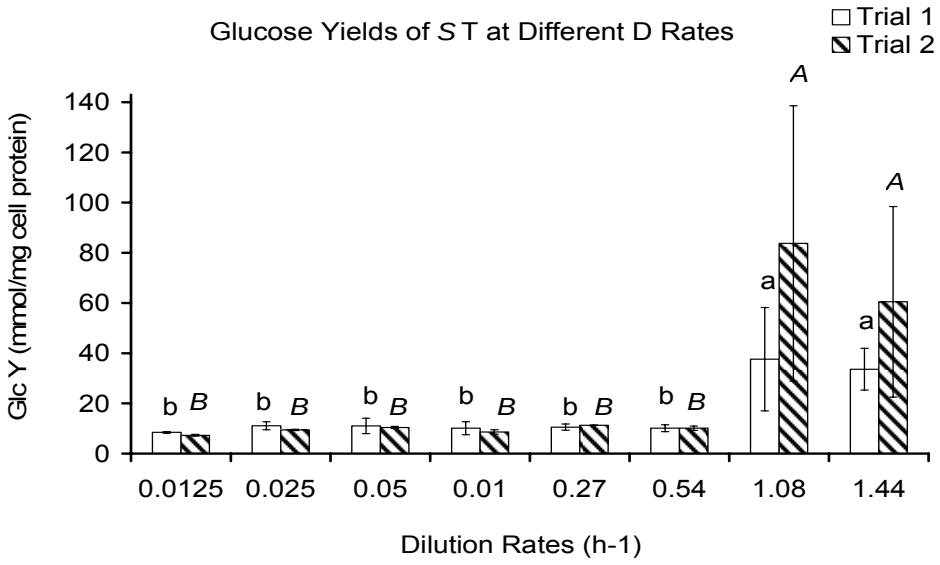


FIGURE 3: Yield glucose of *Salmonella* Typhimurium at different dilution rates (Trials 1 and 2). Error bars represent std. err (±) of normalized values. Similar letters above error bars indicate significant differences *(P* < 0.05); [a-b]Means within Trial 1. [*A-B*]Means within Trial 2. n = 3. Y = yield. D = dilution rates. std err = standard error.

dilution rates (Driessen et al., 1987). In addition, a variation in energy source (glucose, amino acids, lactic acid) can result in discrepancies in anaerobic energy yield, because microorganisms tend to select fermentation pathways based on the compounds available as substrates (Bauchop and Elsden, 1960; DeMoss, et al., 1951; Gunsalus and Gibbs, 1952; Heath, et al., 1958; Hurwitz, 1958).

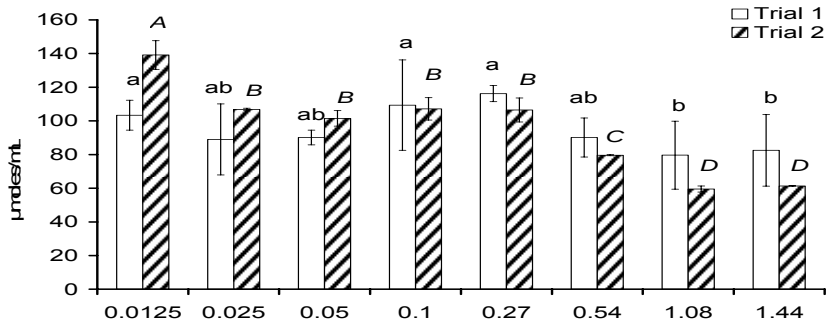
Fermentation and Y_{ATP} cell yield

Short chain fatty acids (SCFA) are end-products of microbial fermentation in GI tract of humans and animals and the primary products include acetate, propionate, butyrate with minor quantities of valerate, isovalerate, isobutyrate (Hungate, 1966; Annison et al., 1968). In these studies ST SCFA production was influenced significantly ($P < 0.05$) by dilution rate in Trials 1 and 2 (Figures 4a, and b). Acetate (Figure 4a) was the most predominant SCFA and was proportionally distributed in the first 5 dilution rates. However, in Trial 2, acetate was significantly lower in D 0.54 h^{-1}, 1.08 h^{-1} and 1.44 h^{-1}, which exhibited similar patterns as cell protein concentration (Figure 1). The highest level of acetate production (139.112 ± 8.51 μmoles/ml) was observed at D 0.0125 h^{-1} in Trial 2 the lowest was (59.56 ± 1.81μmoles/ml) at D 1.08 h^{-1} also in Trial 2. Recently, ST grown in Fed-Batch culture (Van der Wielen et al., 2001) revealed that consumption of glucose was gradually reduced as the concentration of SCFA (acetate, propionate, and butyrate) was increased in the culture vessel. As SCFA was increased in the microenvironment, pH was reduced which provides a higher concentration of undissociated form of SCFA that easily permeate the cell membrane of bacteria (Cherrington et al., 1991a). Our data indicate that propionate production (Figure 4b) was

significantly higher between D rates 0.0125 h^{-1} and 0.27 h^{-1}, however; only a trace amount was detected in D 0.54 h^{-1}, 1.08 h^{-1} and 1.44 h^{-1} which corresponds with much lower glucose consumption (Figure 2) and cell protein concentration.

In the current study the (Ac: Prop) ratio for ST grown in various D is shown in Figure 5. Results showed that Ac: Prop ratio exhibited significant differences ($P < 0.05$) and was influenced by D. In both trials the Ac: Prop ratios were approximately 10**:**1 for the first 5 D (0.0125 h^{-1} to 0.27 h^{-1}). These correspond with the capacity of ST to increase cell protein and an active utilization of glucose. However, as the culture approaches rapid D (0.54 h^{-1}, 1.08 h^{-1} to 1.44 h^{-1}), the Ac:Prop ratio (Trial 1; 65:1, 52:1, 90:1 and Trial 2; 66:1, 78:1, 94:1 respectively) increased with a subsequent decrease in cell protein and glucose utilization from the chemostat. Our experiment support the theory that a decrease Ac:Prop ratio (at lower D) corresponds with an increased metabolic activity which is revealed by increased cell protein and glucose consumption (Russell and Strobel, 1989). An increase Ac: Prop ratio (at higher D) showed a reduced cell protein and glucose consumption. Since glucose was the limiting nutrient in the chemostat, it could be assumed that the formation of acetate and propionate occurred via glucose catabolism Embden-Meyer Parnas (EMP) pathway, including the cleavage of acetyl CoA via acetate kinase to form acetate whereas propionate through the succinate /propionate randomizing pathway, also propionate via the acrylate pathway (Gottschalk, 1986).

(a)



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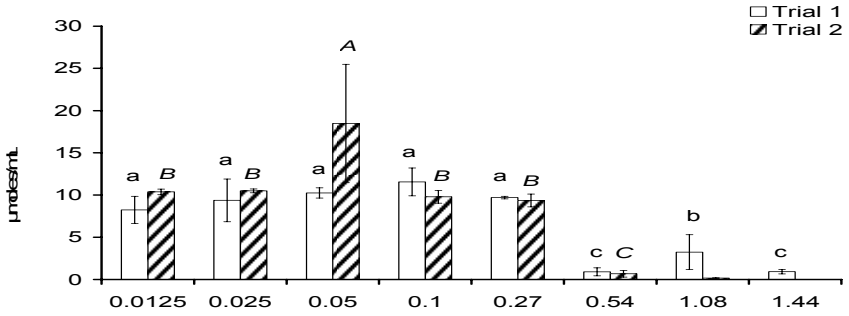

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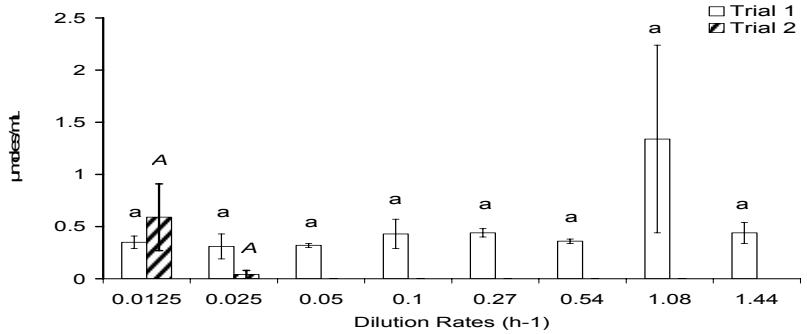


FIGURE 4: Short chain fatty acid production of *Salmonella* Typhimurium at different dilution rates. (a) acetate. (b) propionate. Error bars represent std. err. (± SE) of normalized values. Different letters above error bars indicate significant differences *(P* < 0.05). [a-b]Means within Trial 1. [A-D]Means within Trial 2. SCFA were expressed in µmoles/mL.

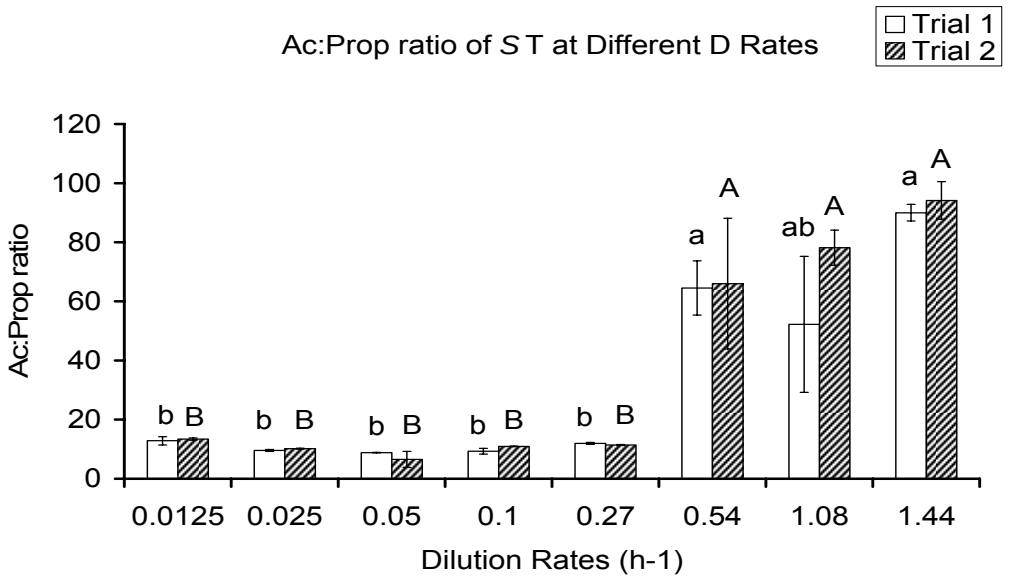


FIGURE 5: Acetate:Propionate ratio of *Salmonella* Typhimurium at different dilution rates. Error bars represent std. err. (± SE) of normalized values. Different letters above error bars indicate significant differences *(P* < 0.05). [a-b]Means within Trial 1. [A-B]Means within Trial 2. Acetate:Propionate ratio was expressed in µmoles/mL. Ac = acetate. prop = propionate.

Yield ATP (Y_{ATP}) is the relationship between the formation of ATP and microbial cell biosynthesis or the amount of dry cell weight an organism produced per g-mole of ATP (Bauchop and Elsden, 1960). In the current study Y_{ATP} was estimated on the basis of cell protein concentration per mmol ATP (Ricke and Schaefer, 1996; de Vries et al., 1970) (Figure 6). Y_{ATP} values varied from 6.4 cell protein/mmol ATP (Trial 2, D0.025 h^{-1}) to 11.7 cell protein/mmol ATP (Trial 1, D0.27 h^{-1}). For the most part ST grown under glucose limiting conditions exhibited a relatively constant Y_{ATP} and was not affected by dilution rates. In both trials, 1 mmol ATP yielded an averaged of approximately 9 mg cell protein. Dilution rates 0.1 h^{-1} and 0.27 h^{-1} yielded (Trial 1: 10.9 and 11.7; Trial 2: 9.27 and 10. 7) the two highest Y_{ATP}, in their respective trials, which corresponded with the highest cell protein concentrations. Historically, it was thought that Y_{ATP} was a biological constant and no more than 10.5 g of cell could be obtained per g-mole ATP (Forrest and Walker, 1971). However, based on a variety of studies Y_{ATP} value relative to g-cell appears to differ by approximately 50%, therefore, it could not be confirmed that Y_{ATP} was generally a biological constant (Stouthamer and Bettenhaussen, 1973). It was later concluded that there was at least a 5-fold range in Y_{ATP} value and that theoretically Y_{ATP} should be three times higher (32 versus 10.5 g of cells per mole of ATP) than the initial value proposed by Bauchop and Elsden, (1960).

rpoS response

In the current study, relative expression of *rpoS* is shown in Figure 7a and 7b. *RpoS* expression showed significant ($P < 0.05$) differences and was observed at all D in both

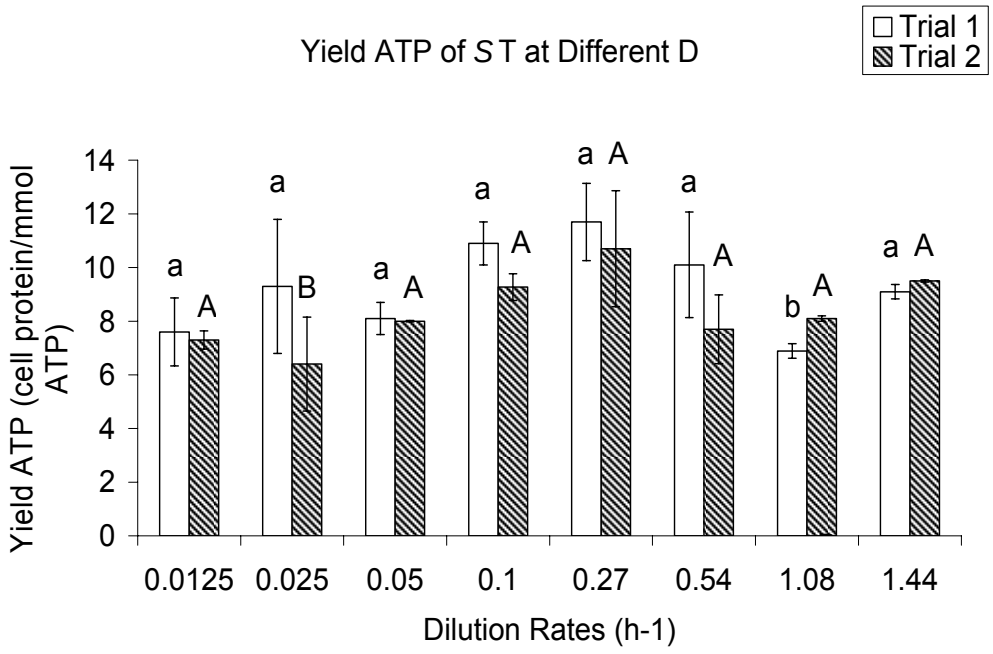
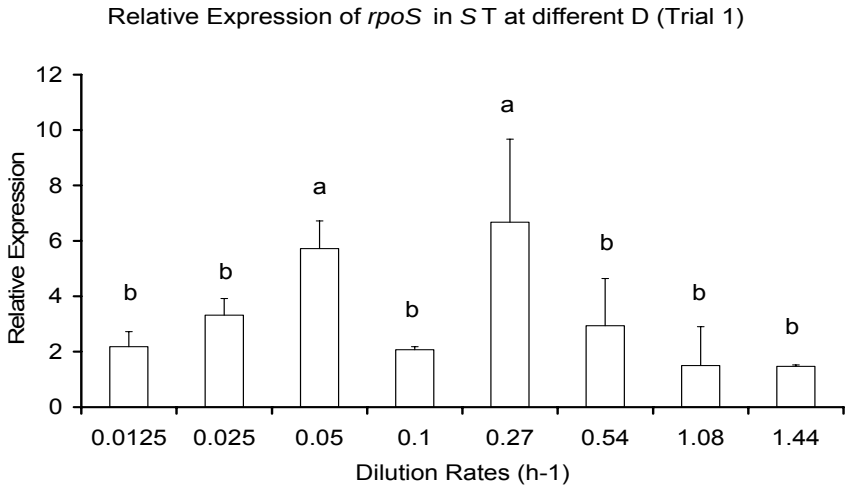


FIGURE 6: Yield ATP (Y_{ATP}) of *Salmonella* Typhimurium grown at different D rates (Trials 1 and 2). Error bars represent std err (± SE) of normalized values. Different letters above error bars indicate significant differences *(P* < 0.05). [a-b]Means within Trial 1. [A-B]Means within Trial 2.

trials. Trial 1 exhibited a similar pattern to cell protein concentration. The two highest *rpoS* expression in Trial 1 (Figure 7a) were observed at D 0.05 h^{-1} and 0.27 h^{-1} which correspond with the two highest cell protein yields in the same trial. In Trial 1, D 0.27 h^{-1} exhibited the highest *rpoS* relative expression (6.67), furthermore, except for D 0.1 h^{-1} there was a proportional increase in *rpoS* expression between initial D and D 0.27 h^{-1} however there was a reciprocal trend from this point onward. The highest *rpoS* expression in Trial 2 (Figure 7b), was observed at D 0.0125 h^{-1} which exhibited a 1.6 fold higher expression than the second highest relative expression (Trial 2) and could be as a result of the initial steady state (at D 0.0125 h^{-1}) that ST cells were exposed to in the chemostat. There is limited information available on the effect of *rpoS* expression of ST in steady state or a continuous culture model. Nevertheless, based on findings from *in vitro* experiments (O'Neal et al., 1994) it is evident that *rpoS* gene is expressed during stationary phase in carbon-poor media, which suggests that dilution rates that facilitated low glucose conditions may play an essential role in triggering *rpoS* induction. In ST, *rpoS* encodes an RNA polymerase sigma factor (δ^S or δ^{38}) known to regulate at least 60 genes in response to environmental signals including various stress conditions, nutrient limitation, osmotic challenge, acid shock, heat shock, oxidative damage, redox potential shifts and stationary phase growth (Jishage et al., 2002; Hengge-Aronis, 1999; Ishihama, 1997; Loewen et al., 1998; Komitopoulou et al., 2004; Hirsch and Elliot, 2005). *RpoS* regulates *Salmonella* virulence and is essential during infection (Hengge-Aronis, 2002). Nutrient deprivation appears to be a critical environmental factor triggering the

(a)



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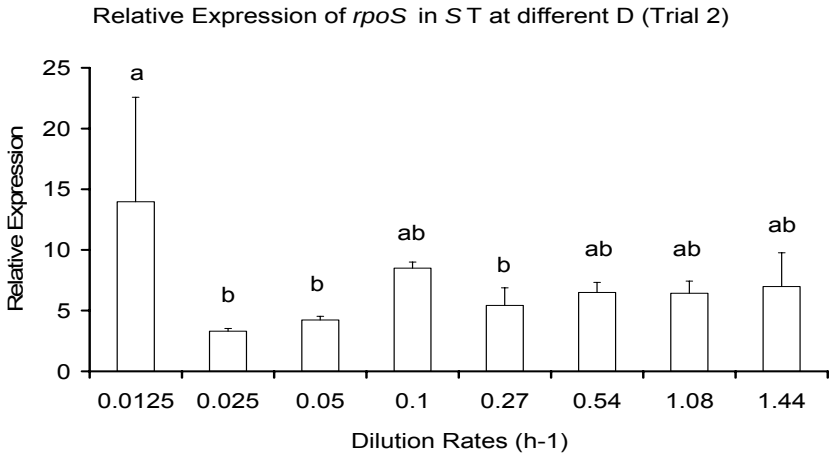


FIGURE 7: Relative expression of *rpoS* gene of *Salmonella* Typhimurium at different dilution rates (a = Trials 1; and b = Trial 2). Error bars represent std. err. (± SE) of normalized values. Different letters above error bars indicate significant differences *(P* < 0.05).

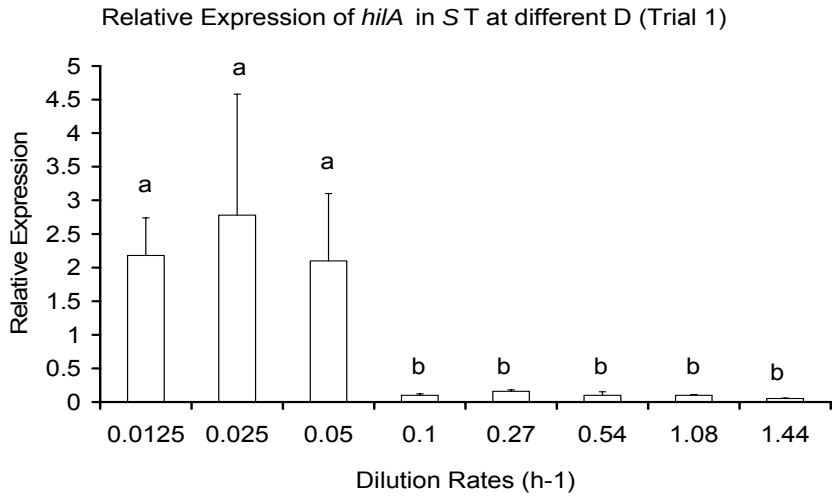
expression of *Salmonella* virulence genes within the phagosomes of host macrophages (Gulig et al., 1993). The survival of *Salmonella* in the intracellular environment may also be a stimulus by *rpoS* induction (Norel et al., 1992).

hilA response

To better understand the impact of changes in D on ST virulence, the level of *hilA* expression was measured when ST was grown in CC and harvested at different D and respective steady state (Figure 8a and b). For the most part results indicated that the three lowest D rates exhibited highest *hilA* relative expression in Trial 1 (Figure 8a). Relatively higher expression of *hilA* was largely observed in Trial 1 at low D (0.0125 h^{-1}, 0.025 h^{-1}, 0.05 h^{-1}), which suggests that low D and less optimal growth conditions could contribute to potential ST virulence in a natural microenvironment including the GI tract of poultry. Relative expression of *hilA* was 21.8- , 27.8- and 21-fold higher in D 0.0125 h^{-1}, 0.025 h^{-1}, 0.05 h^{-1}, respectively, compared to D 0.1 h^{-1}, 0.54 h^{-1} and 1.08 h^{-1}. In Trial 2, D 0.025 h^{-1} and 0.27 h^{-1} showed no difference in *hilA* expression but were significantly higher compared to other D.

Stress conditions for ST under *in vivo* conditions typically have been associated with dietary shifts, such as molting, elevates the level of *Salmonella* invasion of internal organs of poultry, including the GI tract, liver, spleen and ovaries (Holt, 1993; Holt, et al., 1995; Thiagarajan et al., 1994; Durant et al., 1999). The penetration of *Salmonella* pathogens in major tissues is essential to the development of disease (Jones et al., 1994). Invasion is genetically mediated (Collazo and Galán, 1997) by several invasion genes

(a)



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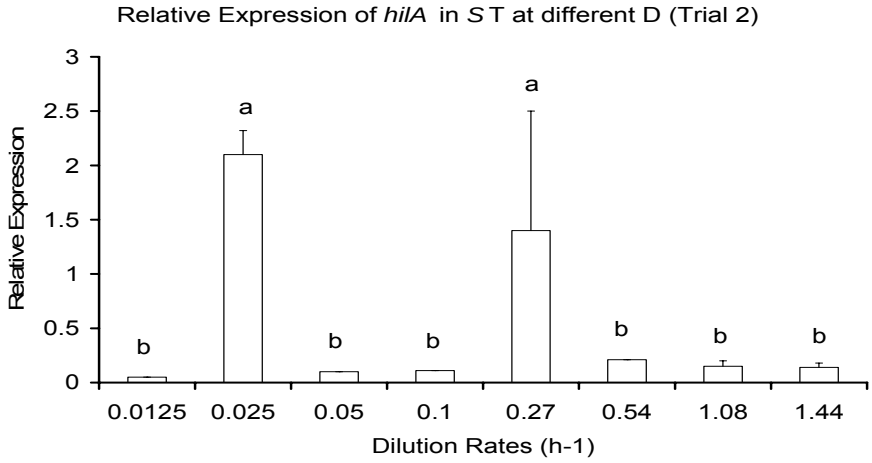


FIGURE 8: Relative expression of *hilA* gene in *Salmonella* Typhimurium at different dilution rates (a = Trials 1; and b = Trial 2). Error bars represent std err (± SE) of normalized values. Different letters above error bars indicate significant differences *(P* < 0.05).

found on a 40 kb *Salmonella* Pathogenicity Island 1 (SPI1) inserted between *flhA* and *muts* chromosomal genes at centisome 63 on the ST chromosomes (Ochman and Groisman, 1996). In ST, *hilA* a transcriptional activator is encoded in SPI1 and is known to regulate invasion in epithelial cells and expression of invasion genes in response to environmental signals (Bajaj et al., 1995). Results suggest that *hilA* expression is up-regulated in microenvironmental conditions that are low in nutrient availability, (Durant et al., 1999) which is representative of the GI tract microenvironment of poultry that are molted via feed removal methods. Conversely, greater nutrient availability causes a down-regulation of *hilA*. In an *in vitro* study (Durant et al., 1999) when sterile crop contents were used to incubate *Salmonella*, *hilA* expression was 1.6 to 2.1-fold higher in crop content from feed withdrawal hens compared to crops from full fed hens, these data corresponded with a 5- to 6-fold increase in SE colonization in the spleen-liver on molted hens to those of unmolted chicken. Durant et al. (2000) also observed that *hilA* expression was nearly 3 fold higher in DLB (lower nutrient concentration) compared to LB (higher in nutrients concentration) broth. Rodriquez et al., (2002) reported that ST EE658 (*hilA*080::Tn*lac*ZY fusion strain) cultured in a high osmolarity and low oxygen condition for 8 h, exhibited a reduced *hilA* expression during exponential phase, however, at stationary phase (3 h post-inoculation) in *hilA* expression increased apparently due to less optimal growth conditions created after the first 2 h in the medium.

Our study examined the physiological and genetic parameters of ST grown at various nutritional levels. The results in our study fully elucidate the concept that ST is

capable of colonizing a microenvironment consisting of limiting nutrient condition at various passage rates including GI tract of chickens. At low D, excessive glucose disappeared from the chemostat between D 0.0125 h^{-1} and 0.27 h^{-1} with increase cell protein concentrations. This demonstrated that ST has the potential to consume glucose efficiently in less than optimal conditions to enhance their survival ability. Therefore, ST is capable of surviving when glucose levels are exceptionally low. In addition, Ac: Prop ratio was approximately 10**:**1 at low and medium range D (0.0125 h^{-1} to 0.27 h^{-1}). This corresponded with the capacity of ST to increase cell protein from glucose utilization. It has been postulated that changes in the bacterial population resulted in a decrease in Ac:Prop ratio which corresponded with increased metabolism to generate favorable energy availability in the form of ATP. Furthermore, steady state and slow growth have been shown to influence genetic responses and could play a significant role in ST virulence and colonization. These results are important not only for understanding these mechanisms but also to emphasize the extensive use of the continuous culture model to simulate ST growing conditions that might help to define specific parameters linked to ST virulence, pathogenesis, and colonization, and could therefore clarify some of the inconsistencies in dealing with *Salmonella* colonization in the GI tract ecosystems.

CHAPTER IV

GLUCOSE YIELD AND GENETIC RESPONSES IN A POULTRY ISOLATE OF *SALMONELLA* TYPHIMURIUM IN A CONTINUOUS CULTURE DURING SHIFTS IN pH

Introduction

Foodborne pathogens such as *Salmonella* have the capability to survive various environmental stress situations including extreme pH, nutrient deprivation, oxygen stress, osmotic shock and heat stress (Foster and Spector, 1995), which may significantly determine the survival and virulence potential (Archer, 1996). The *in vitro* invasion of ST into cultured mammalian epithelial cells is complex and involves genetic loci and host factors (Finlay et al., 1988; Betts and Finlay, 1992). Genes are required for the intestinal phase of ST invasion and are located on *Salmonella* pathogenicity island 1 (SP11) located at centisome 63 on the chromosome (Galán and Sansonetti, 1996). HilA (Hyper Invasive Locus) a member of the OmpR/ToxR family of transcriptional activator coordinately regulates the expression of invasion genes in response to environmental conditions including pH, osmolarity, oxygen tension and low nutrients (Bajaj et al., 1996) and is encoded on SPI1 and directly activates the type three secretion systems (TTSS) genes (Lee et al., 1992; Bajaj, et al., 1995; Ahmer et al., 1999; Lostroh and Lee, 2001). The *hilA* encoding protein and is thought to be a requirement for *Salmonella* invasion due to its transcriptional properties (Bajaj, et al., 1995; Bajaj et al., 1996). HilA directly activate the expression of the *invF* and *prgH* operons that encode the inv, *spa*,

prg and *Org* protein components of the type III secretion system (Bajaj, et al., 1995; Lostroh et al., 2000).

RpoS encodes an RNA polymerase sigma factor (δ^S or δ^{38}) known to regulate at least 60 genes in response to environmental signals including various stress conditions, nutrient limitation, osmotic challenge, acid shock, heat shock, oxidative damage, redox potential and steady and stationary growth phases (Hengge-Aronis, 1999; Ishihama, 1997; Loewen et al., 1998; Jishage et al., 2002; Komitopoulou et al., 2004; Hirsch and Elliot, 2005). *RpoS* exhibits several low pH inducible acid defense systems that expand the limit of pH tolerance of microorganisms including ST and are referred to as Acid Tolerance Response (ATR) (Foster, 1995; Lee et al., 1995, 1994). This mechanism also involves the induction of a set of approximately 51 acid shock proteins (ASPs) for exponential phase ATR and 15 for stationary phase ATR, at least some of these are needed to protect ST against extreme acid conditions (pH 3) (Foster, 1991, 1993; Lee et al., 1995), in minimal and complex medium.

Changes in gastrointestinal pH encountered by *Salmonella* spp. can occur for a variety of reasons. Upon ingestion ST routinely encounters acidic conditions of the stomach (Snepar, 1982), a microenvironment that potentially destroys most microorganisms. In addition, the poultry industry management practices such as removal of feed to induce molt may be responsible for sudden changes in the gastrointestinal pH (Durant et al., 1999; Ricke, 2003a), which could influence *Salmonella* virulence, growth and nutrient availability. Intestinal microorganisms such as *Lactobacilli* are known to provide protective mechanisms against *Salmonella* in the ceca (Barnes et al., 1980;

Corrier et al., 1995; Nisbet et al., 1994; Nurmi and Rantala, 1973; Van der Wielen et al., 2001, 2002) and the crop (Barnes et al., 1980; Durant et al., 1999, 2000). It is well documented that decreased *Salmonella* colonization in the ceca has been linked to an increase in volatile fatty acids (VFA) and a decreased in pH (Barnes et al., 1979; Nisbet et al., 1994; Corrier et al., 1995; Van der Wielen et al., 2001, 2002). Van der Wielen et al., (2001) demonstrated in a batch-fed competitive exclusion co-culture that acetate, propionate and lactate inhibited *Salmonella* growth at pH 5.8, but did not at neutral pH. At pH 5.8 the total undissociated VFA were significantly higher compared to the dissociated form at neutral pH (Van der Wielen et al., 2002). At low pH (5.8), it is thought that SCFA promote bacteriostatic action (Cherrington et al., 1991b) by increasing the concentration of undissociated acid, which can permeate the cell membrane of bacteria which ultimately caused depletion in bacterial ATP, hence, no replication. While a bacteriostatic activity was observed on *Enterobacteriaceae* the organic acid did not inhibit beneficial GI tract bacteria such as *Lactobacilli* (Van der Wielen et al., 2000).

Continuous culture techniques provide an *in vitro* model to study GI tract metabolism (Isaacson et al. 1975; Russell, 1986; Coleman et al., 1996; Ushijima and Seto, 1991; Hume et al., 1997; Nisbet et al., 2000). The growth of bacteria in CC in chemostat provides a more accurate reflection and control conditions that are closely related to the *in vivo* ecosystem (Isaacson et al., 1975). CC techniques have been used to simulate GI tract microenvironment of human (Coleman et al., 1996; Ushijima and Seto, 1991) and various animal species including chickens (Nisbet et al., 2000; Hume et al.,

1997) ruminant (Isaacson et al. 1975; Russell, 1986). This study utilized the continuous culture to mimic and evaluate the pathogenesis of ST in the GI tract of chickens during shifts in pH. The objectives of this study are to evaluate cell yields and virulence genes response (*hilA* and *rpoS*) of ST during various pH shifts which are representative of variable pH ranges in the chicken GI tract that might be expected to be encountered during changes in the microenvironment.

Materials and methods

Bacterial strain and media preparation

Salmonella Typhimurium strain, was selected for resistance to novobiocin and nalidixic acid (NO: NA) has been previously described by Ziprin et al. (1990). Maintenance and confirmation of bacterial culture integrity was described previously by Ricke et al. (1988). Luria Bertani (LB) medium containing 1% Bacto^{tm} tryptose (Difco Laboratories, Detroit, MI), 0.5% yeast extract (Difco) and 0.1% glucose. The pH (6.5 to 8.0) of medium was adjusted accordingly with NaOH and HCl (Sigma Chemical Co., St Louis, MO).

Preparation of Salmonella Typhimurium *inoculum*

Salmonella Typhimurium was streaked on an LB agar plate and incubated (Precison Scientific, Winchester, VA) overnight at 39 ^{o}C. After incubation, colonies were used to inoculate 3 mL of LB broth in a 15-mL borosilicate tube. The tube was incubated at 39^{o}C for 18 h. The inoculum was vortexed and used to inoculate the continuous culture systems.

Establishment of continuous culture systems

The CC systems were established in 1.0-liter volume Bio-Flo 110 chemostat/fermentors (New Brunswick Scientific Company, Edison, NJ). The chemostats were prepared and calibrated according to New Brunswick Scientific Company Handbook (New Brunswick Scientific Company, Edison, NJ). LB broth medium (described in the previous section) and the chemostats were sterilized (40 min at 21psi pressure and at 121^{o}C), with 500 mL media volumes. The chemostats were maintained anaerobically with a steady stream of O_2-free CO_2 (Russell and Baldwin, 1979) at 39^{o}C and agitated at 100 rpm. LB medium in chemostat vessels and diluent bottles were initiated at pH 6.5 and a constant dilution rate of 0.05 h^{-1}. Samples were taken (0, 1, 3, 6, and 96 h) after which diluent bottles were subsequently changed from pH 6.5 to 7.25 and 8.0; each cycle initiated at steady states (obtained after four volume turnovers or 96 h). The chemostats were monitored by determining the pH (Corning 430, Corning, NY) and OD (A_{600}) with a UV-visible light spectrophotometer (Spectronic 20 D, Thermo Spectronic Cooperation, Buffalo, NY).

Sample collection

For each sample an 8-mL aliquot of culture was collected from chemostats at designated periods using a pipette (Drummond Scientific Co, Bromall, PA). The pH and OD readings (OD_{600}) were determined. An aliquot (400 µL) of the culture was preserved immediately in 800 µL of RNAlater (Sigma, St. Louis, MO) and stored at –20^{o}C, these were used for analyses of gene expression (*hilA*, *rpoS* and 16S rRNA). Three milliliters (3 mL) of sample was centrifuged (Labnet, Hermle Z180M, Burladingen, Germany) at

6,000 x g for 6 min. The supernatant was aspirated from the cell pellets with a syringe and was filter sterilized through a 0.45 μm- pore size membrane filter (Pall Corperation, Ann Arbor, MI). Supernatant was stored at -20^{o}C for glucose and SCFA analyses while cells were resuspended in 150 μL of sterile 0.9 % NaCl solution for protein analysis.

Protein determination

Protein determination was done using the method described by Lowry et al. (1951). Bovine serum albumin (BSA), (Sigma, St. Louis, MO) was used as protein standard for determining the level of protein in the samples. Protein samples with the enzyme mixture were measured on a spectrophotometer (Gilford Instrument, Model 260, Oberlin, OH) at OD_{600} and recorded. Protein concentrations were calculated based on linear regression of a standard sample (BSA), intercept and the resulting slope multiplied by the respective results from the enzymatic reaction of samples on a spectrophotometer (Gilford Instrument).

Glucose and Y_{glc} determination

Glucose determination was done according to Bergmeyer and Klotsch, (1965). The transfer of enzyme mixture was read at OD_{340} from spectrophotometer (Gilford Instrument). Glucose level from the culture medium was subtracted from the original sample and was used to calculate quantity of glucose disappeared. Y_{glc} was based on mmol glucose disappearance. Y_{glc} was calculated based on the mg cell protein per mmol of glucose disappeared.

SCFA concentration and Y_{ATP} determination

Gas chromatography was used to determine the concentration of SCFA from the chemostat samples, as previously described by Corrier et al. (1990). The analyses were conducted with a gas chromatograph equipped with a flame ionization detector and peak profiles integration-quantification integrator (Shimadzu Corp., Columbia, MD.). Each sample peak profile was integrated and quantified relative to an internal standard of methyl-butyric acid placed in the same sample. Analyses were conducted at an oven temperature of 200^{o}C and a flow rate of 85 mL/min. The concentration of each acid was expressed in μmol/mL. Y_{ATP} was calculated based on the amount of cell protein produced per mmol ATP generated, in the form of fermentation end-products (acetate propionate, butyrate) (Ricke and Schaefer, 1996; de Vries et al., 1970).

RNA extraction and primer design

Culture samples from pH shift chemostats were stored in RNAlater (Sigma, St. Louis, MO) removed from the freezer thawed and prepared for RNA extraction. RNA was extracted from ST cultures according to the RNeasy Mini Kit (Qiagen, Valencia, CA). Primers were designed using sequences obtained from the genBank website for *hilA, rpoS* and 16S rRNA gene sequences primer express 1.0 Software (Perkin-Elmer Applied, Biosystems, Foster City, CA). Sequences were subsequently blasted on the NCBI website in order to determine if they were compatible with any other species of bacteria. The primers used in this study (McClelland et al., 2001) are listed in Table 2.

TABLE 2: Primer sequences used in real-time PCR reactions (McClelland et al., 2001).

hilA FWD	5’ TATCGCAGTATGCGCCCTT 3’
hilA REV	3’ TCGTAATGGTCACCGGCAG 5’
16srRNA FWD	5’ TGCGCGACCAGGCTAAA 3’
16srRNA REV	3’ TTCACCACTGGCAGGTATTAAGC 5’
rpoS FWD	5’ GCACGTGAGTTGTCGCATAAA 3’
rpoS REV	3’ TTATCCAGTTGCTCTGCAATTTCT 5’

Reverse transcriptase reaction

To obtain cDNA for real-time, reverse transcription reactions were performed using the reagents from the TaqMan® OneStep RT-PCR Kit. Each 20-µL reaction contained 10X RT-PCR Buffer, 500 µM of dNTPs Mix, 20U of RNase Inhibitor, 25 mM of $MgCl_2$, 200 ng of RNA, 2.0 µL of each primer (2.5µM), MultiScribe™ RT 1.25/UµL and RNase-free water to a final volume of 20 µL. A positive RT control reaction was conducted to ensure the procedure was working correctly. The positive (+) control reaction contained all of the same components except that a DNA template was used instead of RNA template. To determine if RNA samples were contaminated with DNA, two negative (-) control RT reactions were run on each RNA sample. One (-) RT reaction contained the same components as the positive (+) RT reactions, except it lacked the RNA sample template and contained more water to ensure that the final concentration of the components remained the same. The other (-) RT reaction did not contain the RT enzyme to ensure that there was no RNA contamination. All one-step RT reactions were performed on a Gene Amp PCR System from Perkin Elmer (Wellesley, MA) under the following conditions: Incubation for10 min at 25˚C, Reverse Transcriptase for 30 min at 45˚C, Reverse Transcriptase inactivation for 5 min at 95 ^{o}C, three step cycling 1 min at 94˚C 4, 1 min at 53˚C and 1 min at 72˚C for 40 cycles. The samples were then held at 4˚C until the samples could be removed.

Real-time PCR reaction

Real-time PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City CA). Each 20-µL SYBR Green

PCR reaction contained 2 µL RNA, 0.2 µL (µM) of each primer, 10 of µL 1X SYBR® Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), 0.1 µL MultiScribed Reverse Transcriptase (Perkin-Elmer, Applied Biosystem, Foster City, CA), 0.4 µL RNase Inhibitor (Perkin-Elmer, Applied Biosystem, Foster City, CA), and water. Thermal cycling conditions were as follows: 48^{o}C for 30 min, 95^{o}C for 10 minutes, and 40 repeats of 95^{o}C for 15 second and 60^{o}C for 1 min.

Gene analysis and expression

Real-time PCR reactions (Bustin, 2000; Orlando et al., 1998; Livak and Schmittgen, 2001; Pierson et al., 2003) were run to determine the expression of three genes. Each gene was analyzed in triplicates. Data was analyzed using the relative quantification method ($2^{-\Delta\Delta CT}$), (Livak and Schmittgen, 2001), which describes the change in expression of the target gene (*rpoS* and *hilA*) relative to a reference gene (16S rRNA) from an untreated ST control sample (steady state). Data was analyzed by averaging the C_T value (cycle at which each sample amplification curve crosses a specific threshold, ΔC_T) for each triplicate sample. The resulting ΔC_T values of the target genes were (*rpoS* and *hilA*) normalized by subtracting the ΔC_T values of 16S rRNA control gene from the target genes (*rpoS* and *hilA*) values. These samples were then subtracted from the 16S rRNA gene from the untreated ST control sample, prepared according to Fey et al. (2004). The resulting $\Delta\Delta C_T$ was used to calculate relative expression using the formula $2^{-\Delta\Delta CT}$ (Guilietti et al., 2001; Livak and Schmittgen, 2001; Lehman and Kreipe, 2001).

Statistical analysis

Data were analyzed using the one-way ANOVA subjected to linear regression using PC-SAS version 9.0 (SAS Institute Inc., Cary, N.C). Differences between means were determined using least square means and Tukey's honest significance test. Statistical variation was also estimated by the standard error of the mean. All statistical analyses were considered significant at ($P < 0.05$).

Results and discussion

Cell protein concentration

In our studies ST grown in continuous culture was subjected to three different pH shifts from diluents entering the chemostat, while growing in LB medium with added glucose from a diluent bottle set at pH 6.5 and a constant dilution rate of 0.05 h^{-1}. LB minimal medium used was considered equivalent in nutritional composition to a typical corn soybean-based poultry feed and was formulated to simulate the poultry feed (Audisio et al., 1999) and the cecal microenvironment of poultry when nutrients are limiting. Samples were taken (0, 1, 3, 6, and 96 h) after which diluent bottles with fresh medium were subsequently changed from pH 8.0 to 6.5, 6.5 to 7.25, and 7.25 to 8.0. These pH of the media were selected to generate internal chemostat media that would correspond to pH shifts that occurred in GI tract of the chicken under normal and less optimal conditions (cecal pH, 5.6 to 6.7; crop pH, 5.7 to 6.3 from Hume et al., 1996),

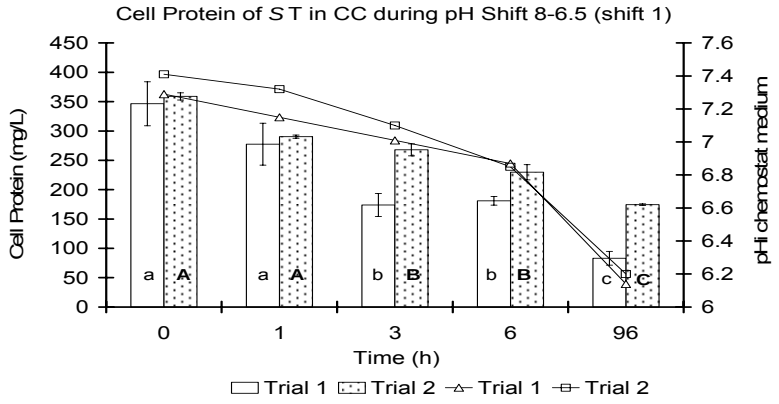
Cell protein concentrations in both Trials exhibited similar trends (Figure 9), however, in Trial 2, ST exhibited a slightly higher cell protein concentration. Cell protein concentrations were influenced by pH shifts and gave a similar pattern when

compared to glucose yields in pH shifts 1 (7.41 to 6.14) and 2 (6.84 to 7.23) in both Trials. The highest cell protein concentrations (359.0 ± 6.14 mg cell protein) was observed at 0 h in pH shift 1 (pHi 7.41 in Trial 2) in Trial 2 while the lowest was (27.71 ± 13.10 mg cell protein) at 0 h in shift 2 (pH 6.99 in Trial 1). For the most part as pH increased so did the cell protein concentration except in pH shift 2 (pHi 6.15 to 6.98) where cell protein concentration remained constant with an increased pH. Cell protein concentrations are usually determined on the basis of the depletion of energy source, however the estimation of energy source utilization do not take into consideration the energy source that is incorporated into cell materials (Pirt, 1965, 1982).

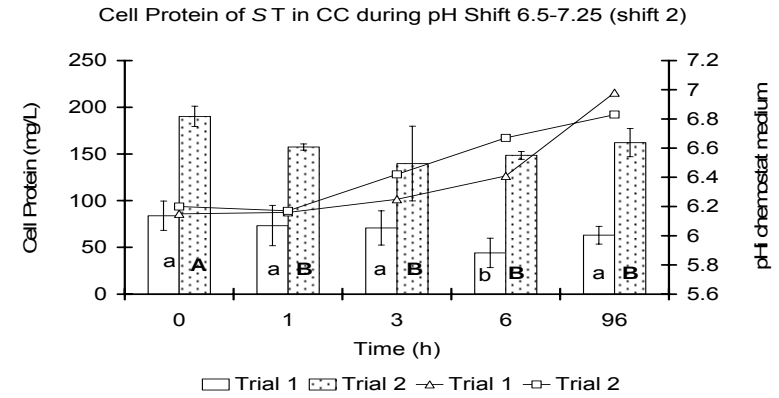
Glucose response

Total glucose concentration in the chemostat was estimated at 1000 mg/L. Our data clearly suggested that glucose utilization by ST was of significant proportion (Figure 10). pH shift 1 (Trial 1) demonstrated the highest glucose disappearance from the chemostat ranging from 829.87 (0 h) to 603.9 mg/L (96 h) at pH 7.29 and 6.2, respectively. In shift 3, (Trial 2) glucose disappearance from chemostat ranged from 528.59 (0 h) to 743.27 mg/L (96 h) at pH 6.68 to 7.23, respectively. In shift 1(7.41 to 6.14), in both trials, highest glucose utilization corresponded to highest actual pH, which also exhibited similar trend in cell protein concentration. Therefore, glucose disappearance appears to be influence by pH in which higher glucose disappeared corresponded to with high pH.

(a)



(b)



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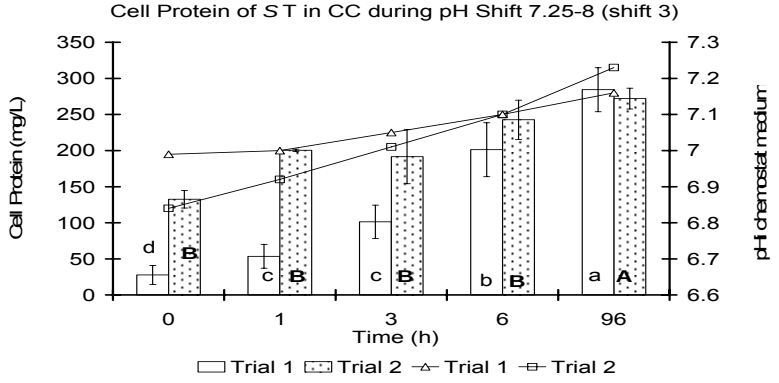
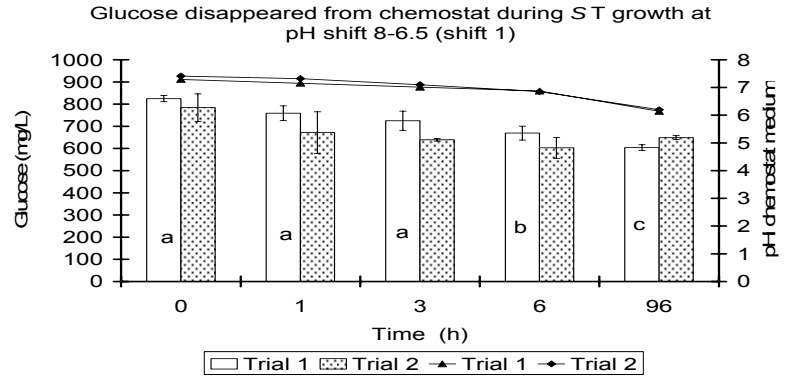


FIGURE 9: Cell protein concentration of *Salmonella* Typhimurium grown in continuous culture during three different pH shifts (Trials 1 & 2). (a) pH shift 1 (pHi 7.41 to 6.14) =8-6.5. (b) pH shift 2 (pHi 6.15 to 6.98) = 6.5-7.25. (c) pH shift 3 (pHi 6.84 to 7.23) = 7.25-8. Primary Y axis (bar graph) indicates cell protein concentration (mg/L) whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error of mean. Different letters indicate significant differences *(P* < 0.05). [a-c]Means within Trial 1. [A-C]Means within Trial 2.Y= yields. CC = continuous culture. pHi = internal pH.

(a)



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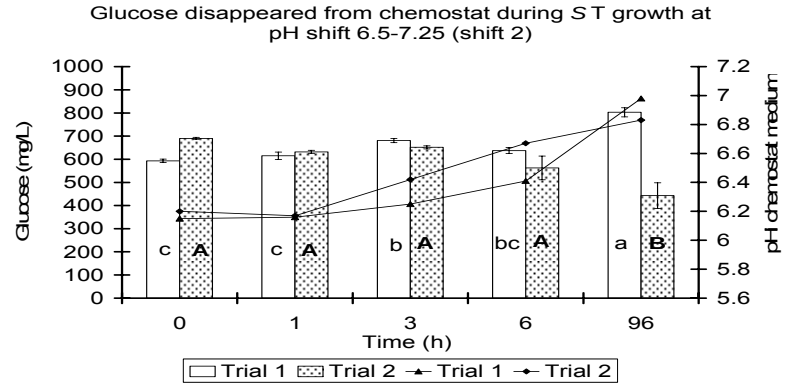


(a)

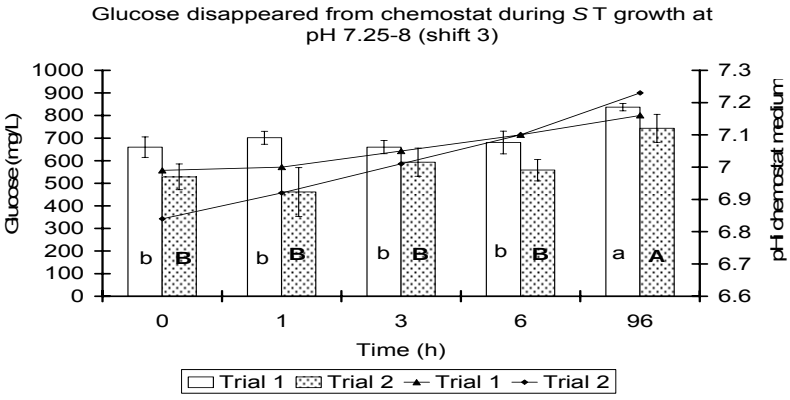
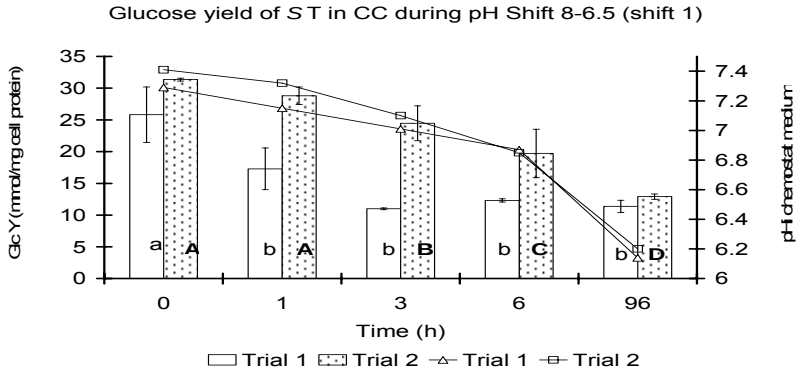


FIGURE 10: Glucose disappearance from chemostat during *Salmonella* Typhimurium grown in continuous culture during three different pH shifts (Trials 1 & 2). (a) pH shift 1 (pHi 7.41 to 6.14) =8-6.5. (b) pH shift 2 (pHi 6.15 to 6.98) = 6.5-7.25. (c) pH shift 3 (pHi 6.84 to 7.23) = 7.25-8. Primary Y axis (bar graph) indicates glucose disappeared (mg/L) whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error of mean. Different letters indicate significant differences *(P* < 0.05). $^{a\text{-}c}$Means within Trial 1. $^{\mathbf{A\text{-}B}}$Means within Trial 2. CC = continuous culture. pHi = internal pH.

(a)



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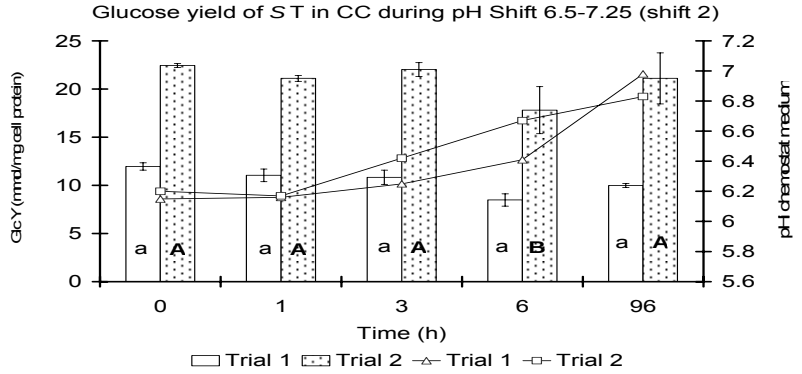


(c)

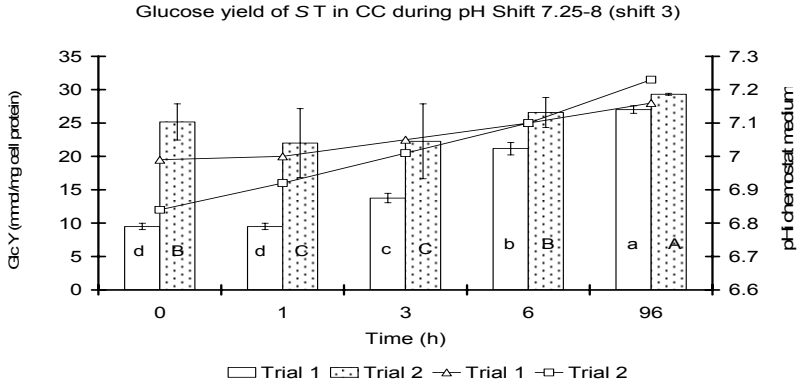


FIGURE 11: Glucose yield response of *Salmonella* Typhimurium grown in continuous culture during three different pH shifts (Trials 1 & 2). (a) pH shift 1 (pHi 7.41 to 6.14) =8-6.5. (b) pH shift 2 (pHi 6.15 to 6.98) = 6.5-7.25. (c) pH shift 3 (pHi 6.84 to 7.23) = 7.25-8. Primary Y axis (bar graph) indicates glucose Y (mmols of glucose disappeared) whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error of mean. Different letters indicate significant differences *(P* < 0.05). $^{a\text{-}d}$Means within Trial 1. $^{A\text{-}D}$Means within Trial 2.Y= yields. glc = glucose. CC = continuous culture. pHi = internal pH.

Glucose yield

Glucose yields are shown in Figure 11. Data indicated significant ($P < 0.05$) differences within trials with pH shifts 1 and 3 exhibiting similar patterns for both trials. Highest (pHi 7.41 in Trial 2) and the lowest (pHi 6.14 in Trial 1) pH were observed at 0 and 96 h respectively, the highest glucose yield (31.35 ± 0.23 g cell/mol glc disappeared) was observed at 0 h in pH (pHi 7.41 in Trial 2) while the lowest glc yield (8.48 ± 0.64 g cell/mol glc disappeared) occurred at 6 h in pH (pHi 6.41 in Trial 1). For the most part as pH increased so did the glucose yield except in pH (pHi 6.15 to 6.98) where glucose yield remained constant while pH increase noticeably at 96 h. pH 7.41 to 6.14 demonstrated a reciprocal trend in glucose utilization when compared to pH 6.84 to 7.23, glucose yield in pH 6.15 to 6.98 (intermediate shift), indicated that glucose was utilized at a consistent level. Changes in the internal pH (pHi) of the chemostat, also indicated linearity and glucose utilization by ST in chemostats were influenced significantly ($P < 0.05$) by changes in pH levels. Results from pH 7.41 to 6.14 and 6.84 to 7.23 suggested that as pH decreased from 7.41 to 6.14 in both Trials, ST consumption of glucose was also reduced along with lesser disappearance of glucose in the chemostat. Glucose utilization patterns appeared to exhibit consistent trends in pH 6.84 to 7.23, however, as pH increased ST utilization of glucose increased. The reduction in pH generated from infused media and SCFA production could combine to create a bacteriostatic effect, which minimizes ST glucose consumption in the presence of undissociated weak acids (Wilson et al., 2003). It has also been reported that ST glucose utilization could be influenced by the strain and glucose uptake system (Driessen et al., 1987).

SCFA production

ST production of SCFA in CC at various pH shifts is shown in Figure 12. There were significant differences ($P < 0.05$) between SCFA production in Trial 1 and 2. In Trial 1 SCFA production was significantly higher compared to Trial 2. Acetate (Figure 12a) was the predominant SCFA with an average production of 150 μmoles/mL in pH (pHi 7.29 to 6.14) and 3 (6.99 to 7.16) in Trial 1 compared to 85 μmoles/mL in pH shift 1 (7.41 to 6.2) and 3 (6.84 to 7.23) in Trial 2. The average acetate production in pH (6.15 to 6.98) was approximately 100 μmoles/mL and 75 μmoles/mL in Trials 1 and 2 respectively. The highest acetate production (170.3 ± 73.12 μmoles/mL) was observed at 6 h pH (6.68) shift 3 in Trial 1 (Figure 12a) and the lowest (65.78 ± 1.87 μmoles/mL) seen at 1 h (pH7.32) in pH shift 1 in Trial 2 (Figure 12b). Shifts in other SCFA production were also detected, including propionate (Fig. 12c and 12d) and butyrate (Figure 12e and 12f). The SCFA production demonstrated a higher numerical productivity in pH shift 1 (7.41 to 6.16) and 3 (6.99 to 7.23) than in pH shift 2 (6.15 to 6.98) in both Trials. Results in pH shifts 2 (lowest pH in chemostat yielded the lowest level of glucose utilization and SCFA production), support the concept of SCFA antimicrobial properties (Cherrington et al., 1991b; Dorsa, 1997; El-Gedaily et al., 1997). In addition, low pH has been proven to inhibit the colonization of ST by competitive exclusion of native microflora in the GI tract (Hoiseth and Stocker, 1981; Hentges, 1983; Van der Wielen et al., 2001; 2002). A correlation between VFA on development of cecal microflora has shown that isolate of *Enterobacteriaceae* strains from chickens' ceca were more vulnerable to VFA than *Enterococci* and *Lactobacilli* (Van der Wielen et al., 2000). A gradual decrease in

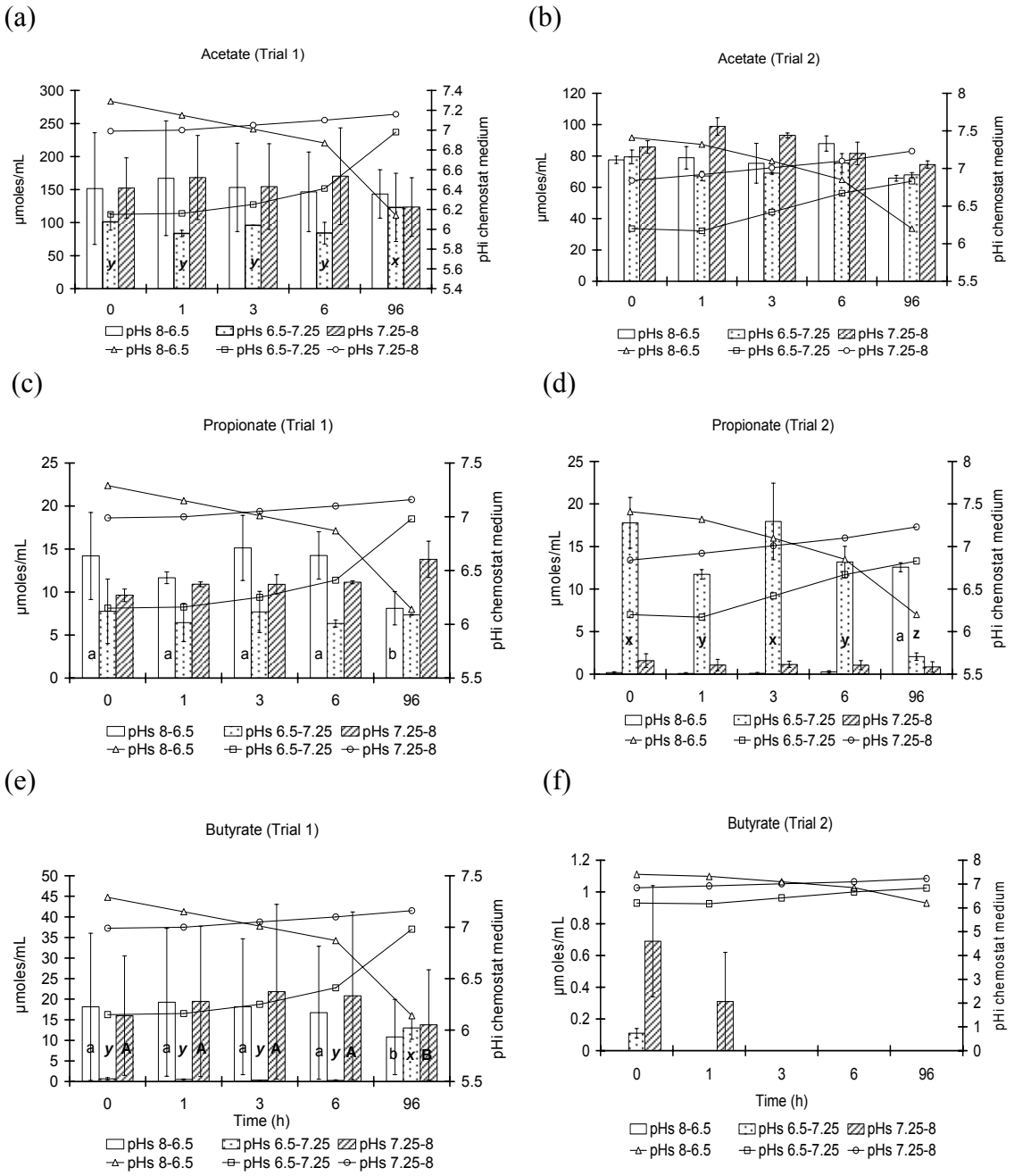
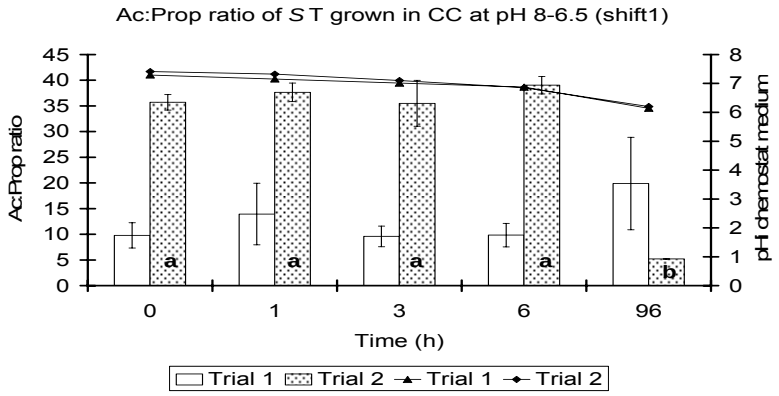
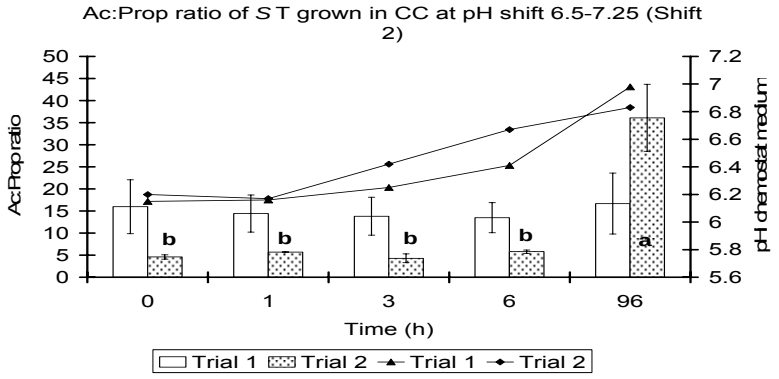


FIGURE 12: Short chain fatty acids (SCFA) production of *Salmonella* Typhimurium grown in continuous culture during three different pH shifts (Trials 1 & 2). a & b = pH shift 1 (pHi 7.41 to 6.14), 8-6.5. c & d = pH shift 2 (pHi 6.15 to 6.98), 6.5-7.25. e & f = pH shift 3 (pHi 6.84 to 7.23), 7.25-8. Primary Y axis (bar graph) indicates µmoles/mL of SCFA whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error (± SE) of mean. $^{a-b}$Means within pHi 7.41 to 6.14 (8 -6.5) without common letter differ significantly ($P < 0.05$). $^{x-z}$Means within pHi 6.15 to 6.98 (6.5-7.25) without common letter differ significantly ($P < 0.05$). $^{A-B}$Means within pHi 6.99 to 7.23 (7.25-8) without common letter differ significantly ($P < 0.05$). pHs = pH shift.

(a)



(b)



(c)

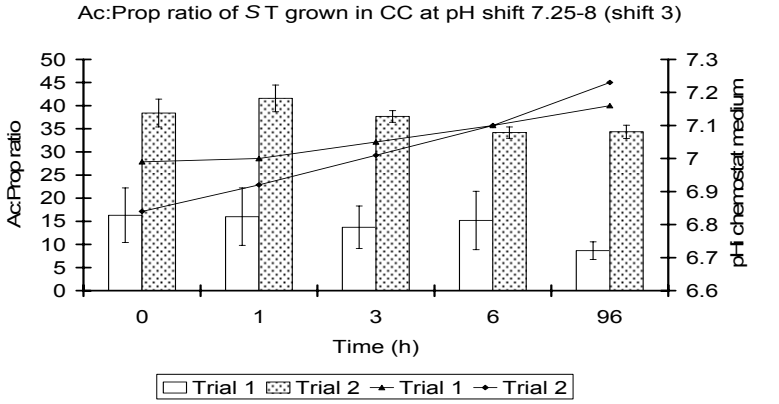


FIGURE 13: Acetate:Propionate ratio of *Salmonella* Typhimurium grown in continuous culture during three different pH shifts (Trials 1 & 2). (a) pH shift 1 (pHi 7.41 to 6.14) =8-6.5. (b) pH shift 2 (pHi 6.15 to 6.98) = 6.5-7.25. (c) pH shift 3 (pHi 6.84 to 7.23) = 7.25-8. Primary Y axis (bar graph) indicates Acetate:Propionate ratio whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error of mean. Different letters indicate significant differences *(P* < 0.05). [a-b]Means within Trial 2. CC = continuous culture. pHi = internal pH.

maximal specific growth rate of *Enterobacteriaceae* was observed with a corresponding increase VFA to the culture medium. However, the growth of *Lactobacilli* was not affected by an increase of VFA (Van der Wielen et al., 2000).

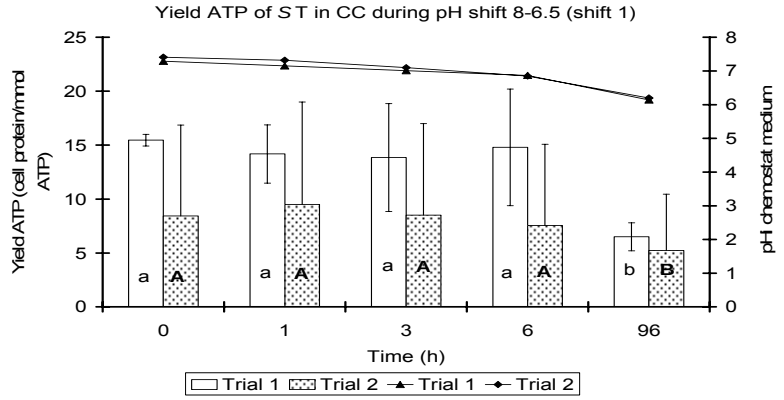
Acetate: propionate ratio

The acetate: propionate ratio of ST has shown inconsistency between both a trials in different pH shifts (Figure 13). Trial 1 showed significantly ($P < 0.05$) lower acetate: propionate ratio in shifts 7.29 to 6.14 and 6.99 to 7.16, ranging from 10 (shift 1, pH 7.01) to 20:1 (shift 1, ph 6.2). However, in shift 6.2 to 6.83 (Figure 5b), Trial 2 was significantly lower than Trial 1, ranging from 4 (pH 6.42) to 36: 1 (pH 6.8). There is the conception that low acetate: propionate ratio interplays with an efficient cell turnover ratio of bacterial cell and increased acetate: propionate ratio showed a reduction (Russell and Strobel, 1989). Based on these results, Trial 1 exhibited a relatively low acetate: propionate ratio that could explain the high levels of cell protein concentration, however except for shift 2 (6.2 to 6.83) other shift in Trial 2 produce high levels of acetate: propionate ratio.

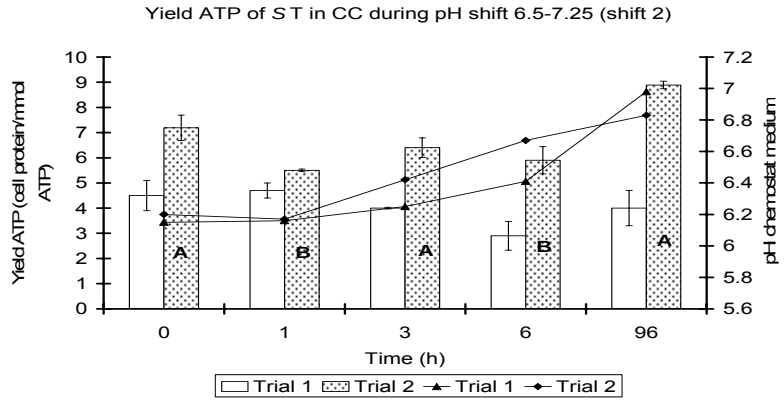
Yield ATP response

In the current study yield ATP (Y_{ATP}) was estimated on the basis of cell protein concentration per mmol ATP (Ricke et al., 1996; de Vries et al., 1970) (Figure 14). Y_{ATP} of ST grown in different pH exhibited similar trend to cell protein concentration. In both trials, Y_{ATP} ranges from 2.9 (pHi 6.41) to 16.95 (cell protein/mmol ATP, pHi 7.16). In pH shift 1 and 2, (Trial 1) higher Y_{ATP} shift corresponded with higher levels of pH (15.46 cell protein/mmol ATP at pHi 7.27, Figure 14a and 16.95 cell protein/mmol ATP

(a)



(b)



(c)

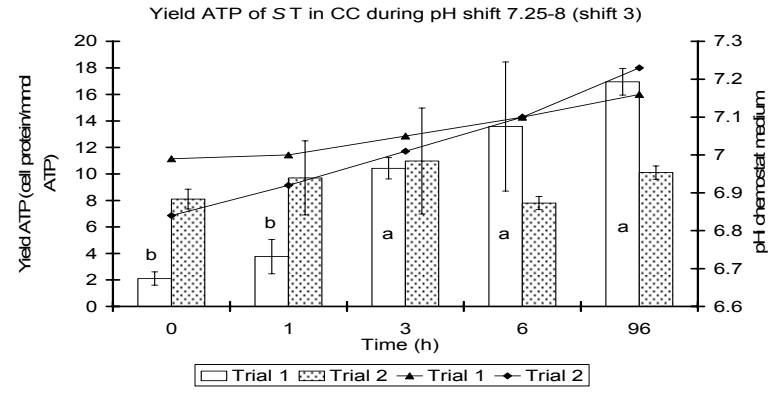


FIGURE 14: Yield ATP of *Salmonella* Typhimurium grown in continuous culture during three different pH shifts (Trials 1 & 2). (a) pH shift 1 (pHi 7.41 to 6.14) =8-6.5. (b) pH shift 2 (pHi 6.15 to 6.98) = 6.5-7.25. (c) pH shift 3 (pHi 6.84 to 7.23) = 7.25-8. Primary Y axis (bar graph) indicates Yield ATP (mg cell protein/mmol ATP) whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error of mean. Different letters indicate significant differences *(P* < 0.05). [a-b]Means within Trial 2. [A-B]Means within Trial 2. Y= yields. CC = continuous culture. pHi = internal pH.

at pHi 7.16, Figure 6b). Based on our results Y_{ATP} fermentation products appeared to be influenced by pH and could also determined metabolism and growth physiology of ST. The estimation of energy source utilization for cell biosynthesis does not take into consideration the energy source that is incorporated into cell materials (Pirt, 1965; 1982). However, if the ATP production from catabolism is minimal then most of the energy source will be used for energy, conversely if the ATP production is high, the fraction of energy source used for carbon can be of significant proportion (Russell and Cook, 1995). Cell growth in a minimal medium where Y_{ATP} value of 32 g of cell per mol of ATP, it is possible that 90% of the energy source could be used as carbon (Russell and Cook, 1995). Contribution of energy source to carbon can be relatively low, Bauchop and Elsden, (1960) suggested that *Enterobacter faecalis* contributed as little as 4% of its energy source to cell carbon.

Responses of rpoS and hilA genes in three different pH shifts.

In our studies relative expression of *rpoS* genes was also determined under similar conditions as *hilA* and is shown in Figure 15. There were significant differences ($P < 0.05$) within both Trials. In pH shift 7.29 to 6.14 (Trial 1, Figure 15a), the relative expression of *rpoS* at 0 and 1 h was 4 fold higher than 3 and 6 h. In Trial 2 (Figure 15b), there was a similar trend compared to Trial 1, *rpoS* expression at 0 h was 1.8- and 2-fold higher than 1 and 3 h respectively. In pH shift 6.15 to 6.98 (Figure 15c, Trial 1), there was a proportional increase in *rpoS* expression consistent with a steady increase in pH from 0 to 6 h. At 6 h (pHi 6.98) *rpoS* expression was 2-fold higher than time 3 and 1 h

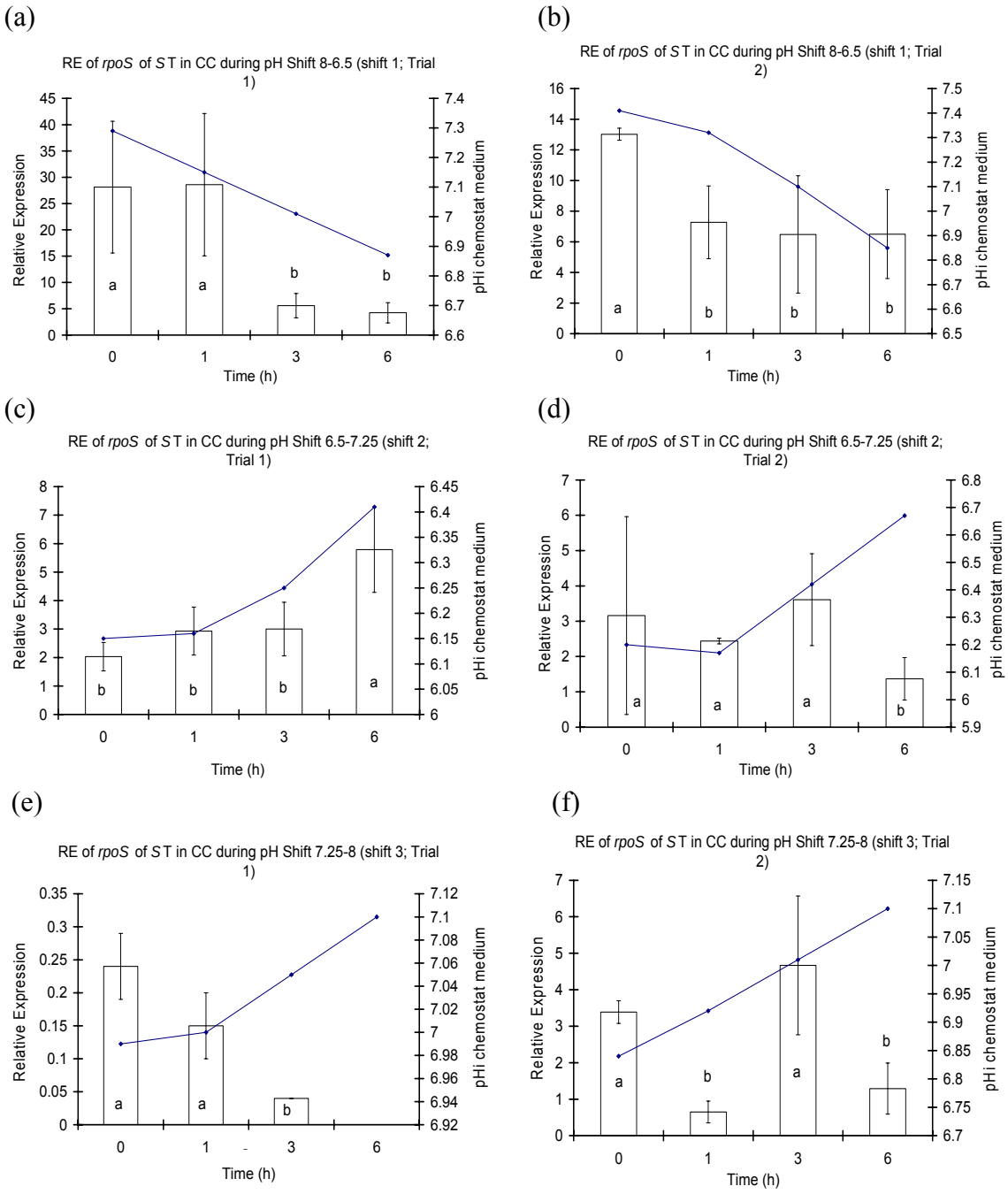


FIGURE 15: Relative Expression of *rpoS* gene in *Salmonella* Typhimurium grown in continuous culture (CC) during three different pH shifts (Trials 1 & 2). a & b = pH shift 1 (pHi 7.41 to 6.14), 8-6.5. c & d = pH shift 2 (pHi 6.15 to 6.98), 6.5-7.25. e & f = pH shift 3 (pHi 6.84 to 7.23), 7.25-8. Primary Y axis (bar graph) indicates Relative Expression whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error (± SE) of mean. Means within Trials 1 and 2 without common letter differ significantly ($P < 0.05$). RE = relative expression. pHi = internal pH.

and 3-fold higher than 0 h (steady state). The relative expression of *rpoS* in pH shift 6.99 to 7.16 (Figure 15e, Trial 1) at the 4 time points were below 1, it was also obvious that the pH shift 6.99 to 7.16 (Figures 15e and f) was much lower than pH shift 1 (7.29 to 6.14) and 2 (6.15 to 6.98). The overwhelming relative expression at 0 h in pH shift 7.29 to 6.14 (Figure 15a) demonstrated that *rpoS* relative expression is influenced by steady state, and glucose utilization, however, pH of the chemostat medium did not influence *rpoS* expression.

Although this experiment involved pH shifts (pHi 7.41 to 6.14) in chemostats, the pH range presumably was not extreme (low) enough to stimulate *rpoS* expression during growth of ST, instead conditions were more suitable for growth. Bearson et al., (1997) confirmed that ST consists of both exponential and stationary phase Acid Tolerance Responses (ATR) that protect cells at pH 3 for several h. Acid Tolerance Response (Bearson et al., 1997) is a mechanism that prepares cells to withstand extremely low pH, cells that are exposed to mild acid conditions are better able to induce ATR, hence, they become more tolerant to extreme acid condition. Induction of ATR genes provide cross protection for cells against high temperature, oxidation, high osmolarity and nutrient deprivation. ATR can be induced in microenvironment including intestine of chickens (ceca), where the pH can be shifted below neutral (pH 7) by high SCFA concentration (Kwon and Ricke, 1998). In addition, ST grown in acetate medium showed that *rpoS* expression elevated 5 fold during exponential growth in medium from cells restricted by slow transport of glucose (Cunning and Elliot, 1999).

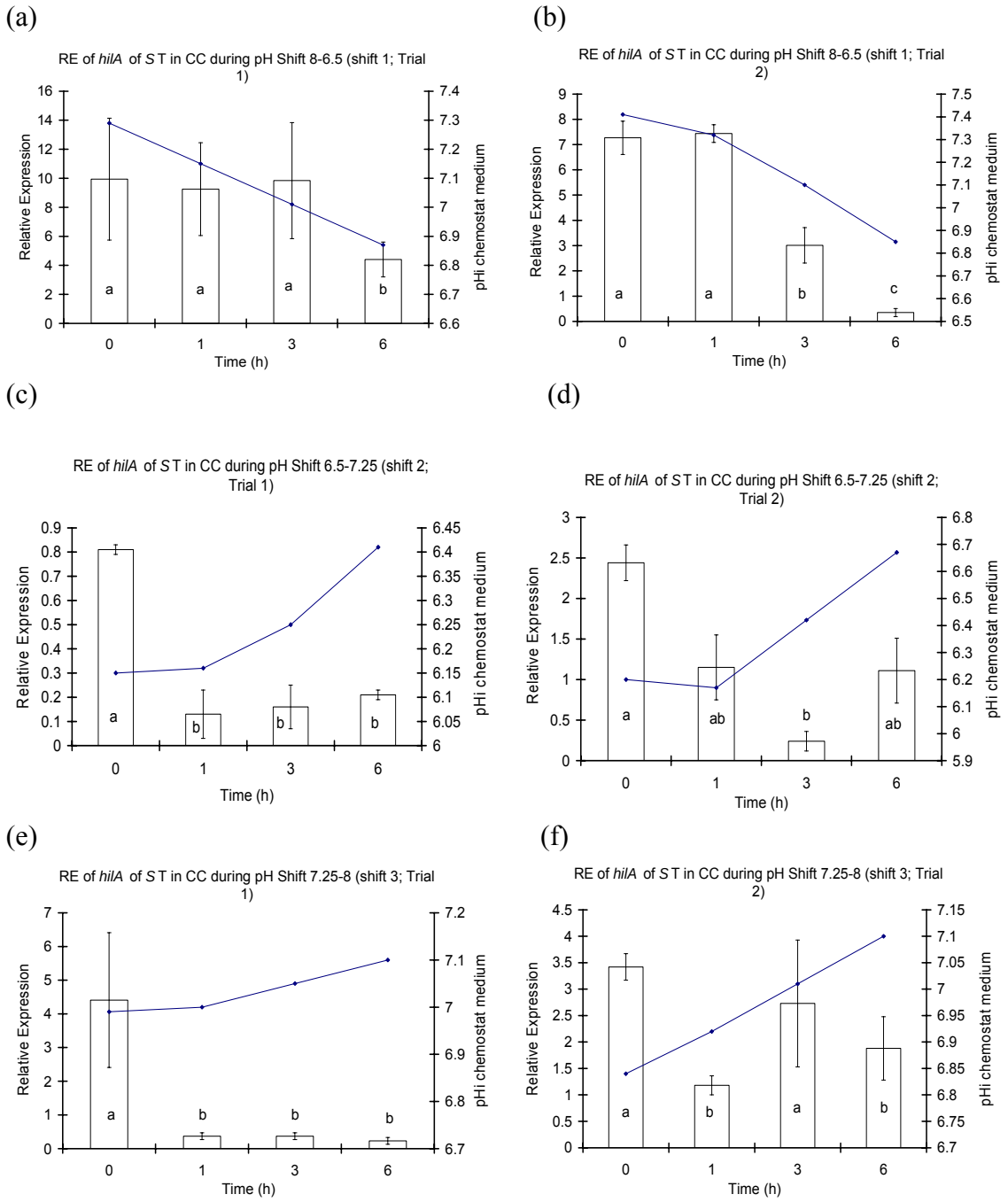


FIGURE 16: Relative expression of *hilA* gene in *Salmonella* Typhimurium grown in continuous culture (CC) during three different pH shifts (Trials 1 & 2). a & b = pH shift 1 (pHi 7.41 to 6.14), 8-6.5. c & d = pH shift 2 (pHi 6.15 to 6.98), 6.5-7.25. e & f = pH shift 3 (pHi 6.84 to 7.23), 7.25-8. Primary Y axis (bar graph) indicates Relative Expression whereas secondary axis indicates (line graph) internal pH of chemostat medium during pH shifts. Error bars represent standard error (± SE) of mean. Means within Trials 1 and 2 without common letter differ significantly ($P < 0.05$). RE = relative expression. pHi = internal pH.

Conditions related to stress and dietary shifts caused from changes in nutrient supply have altered the functions of bacteria that could influence tendencies of virulence capabilities among resident bacteria the GI tract microenvironment (Alverdy and Stern, 1998). Shift of pHi 7.41 to 6.14 (Figure 16a, Trial 1) revealed that at time 0, 1 and 3 h yielded similar *hilA* expression and were 2-fold higher compared to time 6 h, while in Trial 2 (Figure 16b) there was a 2-fold expression on *hilA* at 0 and 1 h higher than 3 h from the same shift. In pH shift 6.15 to 6.98 (Figure 16c, Trial 1), 0 h was 4-fold higher than 1, 3 and 6 h and at pH shift 3, 0 h was 12-fold higher than the other time points. In trial 2 (Figure 16d), at 0 h *hilA* expression was over 2-fold higher than 1 and 6 h in pH shift (6.2 to 6.83). In pH shift (6.99 to 7.16), (Figure 16e, Trial 1) 0 h which is at steady state exhibited an 11.9 fold higher than 1 and 3 h; whereas at 0 h (Figure 16f, Trial 2), *hilA* expression was 2.8- and 1.8-fold higher than 1 and 6 h respectively (Figure 16f, Trial 2). These results indicate that expression of *hilA* gene is elevated at steady state (0 h), ST is experiencing adverse environmental conditions such as lower levels of glucose in chemostat which trigger *hilA* expression in ST. The overall pHi in the chemostat ranged from 7.41 to 6.14 was not consistent with *hilA* expression; this suggested that lowering pH had limited influence on *hilA* expression (Durant et al., 1999). Our studies indicate that at steady state there is an obvious increase in *hilA* expression, at this point glucose remaining in the chemostat is quite low. This supports Durant et al., (2000) who observed in batch culture that a dilution of carbon source in spent LB medium elevates *hilA* expression nearly 3-fold.

Our studies revealed that cell protein concentration, glucose yield, and Y_{ATP} of ST in CC were influenced by an increase in pH (6.14 to 7.41). Cell protein concentration, of ST increased and decreased consistently with a corresponding increase and decrease in pH. Data suggested that as pH decreased from 7.41 to 6.14 in both Trials, ST consumption of glucose was also reduced along with lesser disappearance of glucose in the chemostat, suggesting that the physiological activities of ST were drastically reduced at reduced pH. Y_{ATP} of ST exhibited similar trend to cell protein concentrations. In a previous report (Van der Wielen et al., 2001), ST growth in a fed-batch culture exhibited low growth rates when an increase of SCFA concentrations and lactate were infused into the culture. The administration of define bacterial mixture exhibit similar mechanisms and correlated with a reduction in *Salmonella* in ceca of birds (Nisbet et al., 1996). SCFA are known to decrease growth rates of *Salmonella,* and the sensetivity of *Salmonella* to SCFA is increased with decreasing pH (Cherrington et al., 1991a). As SCFA and lactate are increased in the microenvironment, pH is reduced which provides a higher concentration of undissociated form of SCFA and lactate that easily permeate the cell membrane of bacteria (Cherrington et al., 1991a) and caused bacteriostatic action. These findings may explain the effects of various pH shifts which corresponded with our study and indicated that ST cell protein concentration increased and decreased consistently with a corresponding increase and decrease in pH. Our data for both trials demonstrated that *hilA* and *rpoS* were consistently elevated at 0 h which represented steady states; this may suggest that depletion in nutrient components in a microenvironment may influence genes necessary for *Salmonella* virulence. This

supports Durant et al. (2000) who observed that *hilA* expression was 2.9 fold higher in lower nutrients concentration LB compared to LB with higher in nutrients concentration broth which indicated that *hilA* expression could be influenced by nutritive components. Furthermore an increase lactate concentration reduced *hilA* expression at pH 6.5 and 4 with pH 4 indicating the least *hilA* expression. The observations in our study suggest that pH could be an additional component of microenvironmental stimuli that that regulate *Salmonella* growth physiology and virulence. These *in vitro* results may further explain mechanisms behind *Salmonella* pathogenesis and virulence which could conclude changing pH even within a fairly narrow range may promote its survival.

CHAPTER V

COMPARISON OF *IN VITRO* FERMENTATION AND MOLECULAR MICROBIAL PROFILES OF HIGH FIBER FEED SUBSTRATES INCUBATED WITH CHICKEN CECAL INOCULA

Introduction

It is apparent that the cecal microbial ecology is an important factor in limiting pathogen colonization during dietary stress such as molting. In particular, feed withdrawal as a method for molting hens has been implicated and fermentable dietary fibers have been examined as potential alternative molting approaches (Seo et al., 2001; Ricke, 2003a; Woodward et al., 2005). Dietary fibers are predominantly a constituent of plant cell wall and also consist of non-starch polysaccharides (NSPs) along with non-carbohydrates compound including lignin, protein, fatty acid, and wax (Bach Knudsen, 2001). Upon ingestion, dietary fiber may affect the GI tract by altering its microbial activities, rate of passage, metabolites and digestive efficacy (Bach Knudsen, 2001; Wenk, 2001). Recently, certain dietary fractions including polysaccharides are identified as having the potential to be utilized as prebiotics (Verstegen and Schaafsma, 1999; Cummings and MacFarlane, 2002), possibly by reducing pH and the increase of volatile fatty acids (VFA) (Bedford, 2000; Corrier et al., 1990; Hinton et al., 1990). Prebiotics can be defined as non-digestible carbohydrate fractions fed in diets that are beneficial to host by stimulating the growth of one or more bacteria in the GI tract (Gibson and Roberfroid, 1995). Bacteria recognized as beneficial include species of *Lactobacilli* and

Bifidobacteria, which are suggested to be inhibitory towards pathogens (Gibson and Roberfroid, 1995).

In poultry, extensive strict anaerobic metabolism including methanogenesis fermentation occurs in the ceca in birds fed a variety of diets (Ricke et al., 2004a). In addition, it has been demonstrated that certain microorganisms that are indigenous to the GI tract of poultry have the potential to hydrolyze dietary fiber into oligosaccharides and other low molecular weight carbohydrates (Sunvold et al., 1995) which leads to production of SCFA that serve as energy sources (Hungate, 1966; Kass et al., 1980; Ricke et al., 2004a). Results from studies with four species of birds indicated that metabolizable energy obtained from total VFA production was equivalent to 5-15% of daily requirement for maintenance (Annison et al., 1968; Gassaway, 1976a, b).

Microbial fermentation in the GI tract can also contribute to limiting foodborne pathogen colonization. McHan and Shotts (1993) reported in an *in vitro* study, that toxic effect of SCFA to some *Enterobacteriacae* showed a 50-80% reduction in *Salmonella* Typhimurium (ST) population in the presence of SCFA. SCFA also have a bacteriostatic effect on some enteric bacteria including ST, these organic acids do not inhibit beneficial GI tract bacteria such as *Lactobacillus* (Van der Wielen et al., 2000). It has been suggested that propionic acid was more effective in inhibiting pathogenic bacteria (Marounek et al., 1999), while others observed that acetate is more effective (Van der Wielen et al., 2000).

It has been shown that anaerobic cecal bacterial culture from mature chickens or other competitive exclusion (CE) cultures are widely used for poultry and demonstrated

its effect in limiting *Salmonella* invasion significantly (Nurmi and Rantala, 1973; Ziprin et al., 1993; Nisbet, 2002). The particle size of feed structure has been identified as an influential factor in reducing *Salmonella* numbers in the GI tract (Mikkelsen et al., 2004). The benefits of uniform particle size has also been shown to be involved in the development of the GI tract and associated with enhance gut motility and backflow. Compound from nitrogenous-based are transported from the cloaca to the ceca as a result of reverse peristalsis (Karasawa, 1989), it was postulated that a course diet could enhance this mechanism. In addition, several studies have been carried out to evaluate the effects of feed structure on performance of poultry (Nir et al., 1995, 1994a; Svihus et al., 1997, 2001; Jones and Taylor, 2001; Engberg et al., 2002, 2004).

It has been suggested that the ceca contain the largest number of microorganism in the GI tract of poultry (Barnes et al., 1972; 1973; Barnes, 1979) more than 200 different bacteria have been isolated and most of these are strict anaerobes (Barnes, 1979). Molecular techniques have been used to compare bacterial diversity in chicken cecal microenvironment and for potential probiotics sources (Ricke and Pillai, 1999; Zhu et al., 2002; Hume et al., 2003; Ricke et al., 2004a), however, some DNA isolation techniques have their limitation regarding amplification and cloning, and tend to favor the detection of certain bacteria and nucleic acid sequences. The PCR-based Denaturing Gradient Gel Electrophoresis (DGGE) has been widely used in the last two decades and facilitates unique visualization of PCR products (amplicons) representing predominant (composing 1% or more of the total community) diversity of digestive bacteria (Muyzer et al., 1993; Lee et al., 1996; Murray et al., 1996). The objectives of this study were to

investigate and compare the *in vitro* potential fermentability of high fiber feed sources that could be used to formulate a more refined alternative induced molting diet that retains and potentially promotes the protective microflora in GI tract of poultry, also the use of a Polymerase Chain Reaction-based Denaturing Gradient Gel Electrophoresis (DGGE) method to determine the bacterial profile of each sample after fermentation.

Materials and methods

Experimental protocol

Cecal contents were obtained from single comb Leghorn hens (>50 wks old) fed on a commercial layer ration. Cecal contents were diluted at 1:3000 (Donalson et al., 2004) with anaerobic dilution solution (Bryant, 1973) to formulate inoculum. Ten high fiber feed substrates (HFFS) including soybean meal, soybean hull, beet pulp, wheat middling, ground sorghum, cotton seed meal, 100% alfalfa meal, 90% alfalfa + 10% commercial layer ration (A90), 80% alfalfa + 20% commercial layer ration (A80), 70% alfalfa + 30% commercial layer ration (A70), were ground through a 1-mm screen in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA). A quantity (0.5 g) of each feed substrate was places in serum tubes, with 10 ml inoculum (feed + inoculum) and/or 10 ml dilution solution (feed + no inoculum) added anaerobically (Russell and Baldwin, 1979). There were two trials and samples for analysis were done at two time periods (0 and 24 h). All 24 h serum tubes were capped with butyl rubber stopper and crimp metal seals then pressurized using a CO_2: H_2 manifold (Balch and Wolfe, 1976) and incubated at 39^{o}C. For each time point (0 h and 24 h) samples were centrifuged (10,000 x g) for 10 min and supernatant were aspirated, and stored at -20^{o}C for determining SCFA

concentration, substrate pellets were used to determined bacterial profile using PCR-based DGGE.

SCFA concentration

The concentration of SCFA from samples was determined by gas-liquid chromatography as previously described by Corrier et al. (1990). The analyses were conducted with a gas chomatograph equipped with a flame ionization detector and peak profiles integration-quantification integrator (Shimadzu Corp., Columbia, MD). Each sample peak profile was integrated and quantified relative to an internal standard of methyl-butyric acid placed in the same sample. Analyses were conducted at an oven temperature of 200^{o}C and a flow rate of 85 ml/min. the concentration of each acid was expressed in micromoles per milliliter.

PCR-based DGGE procedure

Genomic DNA was isolated from 1.0 mL of each sample using a QIAamp DNA mini kit according to instructions described in the manual (Qiagen, Valencia, California). Denaturing gradient gel electrophoresis was run according to the method of Muyzer et al., (1993) with modification, using bacterial specific PCR primers to conserve region flanking the variable V3 region of 16S rDNA genes. PCR was run with a 50-µL total reaction volume. Primers (50 pmol of each per reaction mixture; primer 2, 5'-ATTACCGCGGCTGCTGG-3', and primer 3 with a 40-base G-C clamp (Sheffield et al., 1989; Muyzer et al., 1993), *5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGGCCTACGGGAGGCAGCAG-3'*), were mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO) according to kit instructions,

with 250 ng (50 ng of DNA pooled from 3 samples each) template DNA. Acetamide (5%, wt/vol) was added to eliminate preferential annealing (Reysenbach et al., 1992), also 10 mg/ml bovine saline albumin (BSA) and deionized water to make up a final 50-uL volume reaction. Amplification was done on a PTC-200 Peltier Thermal Cycler with the following program: 1) Denaturation at 94.9 ^{o}C for 2 min; 2) Subsequent denaturation at 94.0^{o}C for 1 min; 3) annealing at 67.0 ^{o}C for 45 s; -0.5^{o}C per cycle [touchdown to minimize spurious by-product (Don et al., 1991; Wawer and Muyzer, 1995)]; 4) extension at 72.0^{o}C for 2 min; 5) repeat step 2 to 4 for 17 cycles; 6) Denaturation at 94^{o}C for 1 min; 7) annealing at 58.0^{o}C for 45 s; 8) repeat step 6 to 7 for 12 cycles; 9) extension at 72.0^{o}C for 7 min; 10) 4.0^{o}C final.

Gel electrophoresis

Polyacrylamide gels (8% vol/vol) acrylamide-bisacrylamide ratio 37.5:1) were cast with a 35 to 60% urea-deionized formamide gradient; 100% denaturing acrylamide was 7 M urea and 40% deionized formamide. Amplified samples (4 µL of pooled template) were mixed with an equal volume of 2x loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and 7 µL were placed in each sample well (16-well comb). Gels were placed in a DCode Universal Mutation Detection System for electophoresis in 1x TAE (20 mM Tris (pH 7.4), 10 mM sodium acetate, 0.5 M ETDA) buffer at 59^{o} C for 17 h at 60 V. Gels were stained with SYBR Green 1 (1:10,000 dilution) for 40 min. Amplified fragment pattern relatedness of samples were determined with Molecular Analysis Fingerprinting Software, version 1.610 based on the Dice similarity coefficient and the unweighted pair group method

using arithmetic averages (UPGMA) for clustering. Dice coefficient (values between 0 and 1) is an arithmetic method, which determines the degree to which banding patterns are alike. Clusters (groups) were determined by sequentially comparing the patterns and the construction of a dendrogram reflecting the related similarities. The amount of similarity was reflected by the relatively closeness or grouping and is indicated by the percentage similarity coefficient bar located above each dendrogram.

Statistical analysis

Data from concentration of SCFA were analyzed using the one way ANOVA subjected to linear regression using PC-SAS software version 9.0 (2001), (SAS Institute Inc., Cary, NC.). Differences between means were determined using least square means and Tukey's honest significance test. Statistical variation was also estimated by the standard error of the mean. All statistical analyses were considered significant at ($P < 0.05$).

Results and discussion

Short chain fatty acid production

Results for HFFS with cecal inocula are shown in Figure 17. The two Trials were statistically different ($P < 0.05$), therefore were examined separately. Except for GI where the acetate production was similar, all HFFS yielded numerically higher levels in Trials 2 than in Trial 1, with H1, B1, A1, A91 and A71 at significantly higher levels ($P < 0.05$). Results demonstrated that in all HFFS fermentation, acetate production (Figure 17a) was present in the greatest proportion. Propionate (Figure 17c) and butyrate (Figure 17e) were next in order of magnitude whereas only trace amounts of isovalerate (Figure

17b), valerate (Figure 16d) and isobutyrate (Figure 17f) were present. Alfalfa, soybean and soybean-based HFFS exhibited consistently high SCFA production.

In Trial 1, acetate production (Figure17a) for all HFFS was less than those for Trial 2. For propionate, GI was significantly ($P < 0.05$) higher in Trial 1 than Trial 2, in addition WI, CI, A8I and A7I all revealed higher propionate production but at relatively marginal levels. For butyrate production (Figure17e) all HFFS except GI demonstrated higher levels. It was clear that SCFA production was largely dependent upon HFFS, since treatment with no HFFS + cecal inoculum (NFI) yielded little or no SCFA production as shown in all figures. Isobutyrate, valerate and isovalerate production occurred in trace amounts, however, valerate production seemed to favor alfalfa-based HFFS (Figure 17). In Trials 1 and 2, all HFFS for isobutyrate and isovalerate production were detected at less than 1 μmoles levels.

Similar quantities of HFFS were incubated in anaerobic dilution solution without cecal content (Figure 18) as the controls at the same time points. Samples were processed similar to those with inocula. The results indicate that a treatment of HFFS with cecal inoculum were significantly higher in acetate (Figure 18a), propionate (Figure 18c), and butyrate (Figure 18e) when compared with HFFS without cecal inoculum. However, there were minimal differences in isobutyrate (Figure 18b), valerate (Figure 18d), and isovalerate (Figure 18f) production from HFFS with cecal inoculum compared with HFSS without cecal inoculum. The overall propionate and butyrate fermentation appeared to be influenced by cecal inocula, since their production levels were relatively

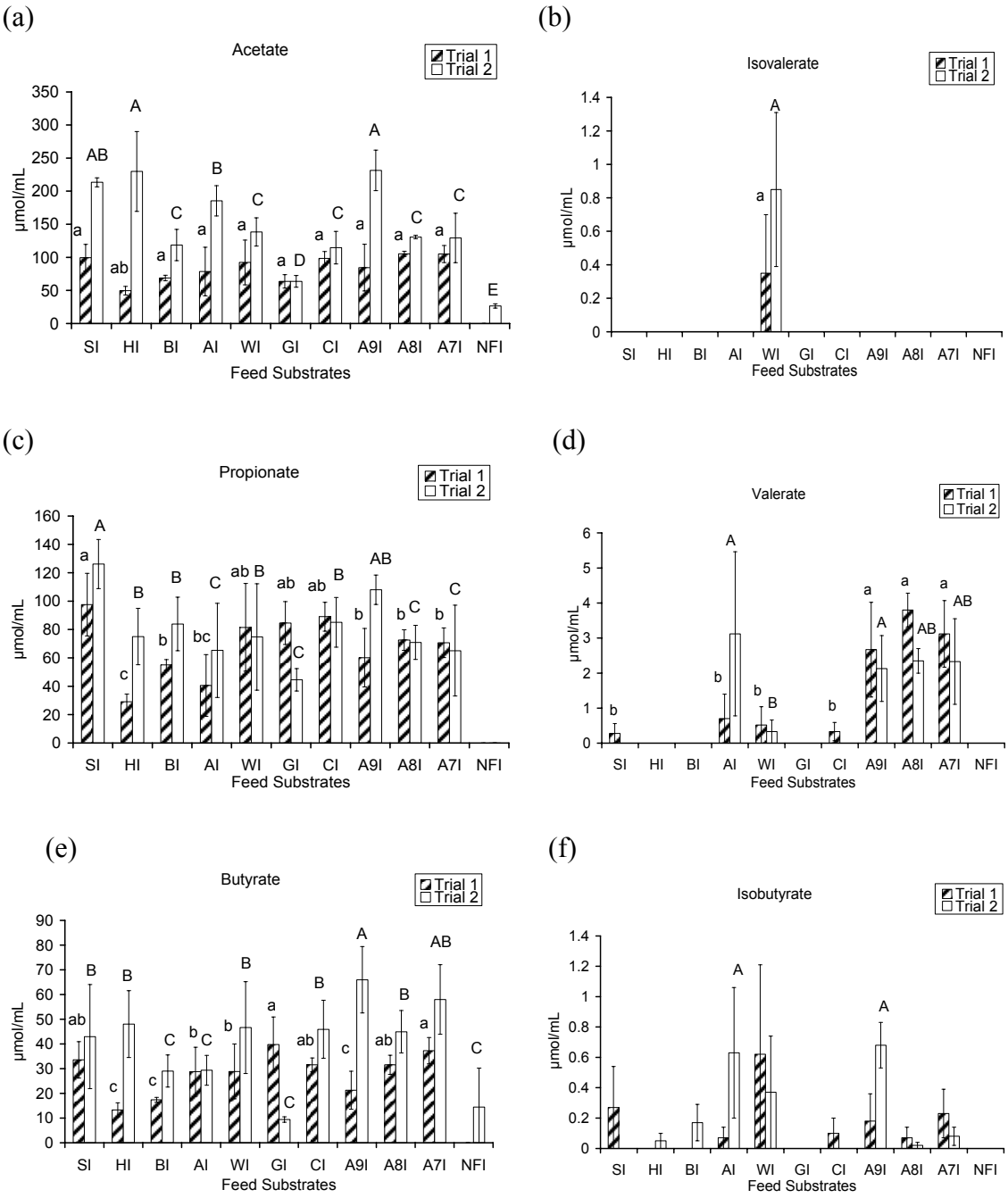


FIGURE 17: Production of short chain fatty acids (SCFA) during *in vitro* fermentation with cecal inoculum on different feed substrates (Trial 1 and 2). 1a = acetate. 1b = isovalerate. 1c = propionate. 1d = valerate. = 1e = butyrate. 1f = isobutyrate. Feed substrates used are: soybean meal (S1), soybean hull (H1), beet pulp (B1), wheat middling (W1), ground sorghum (G1), cotton seed meal (C1), 100% alfalfa meal (A1), 90% alfalfa + 10% commercial layer ration (A91), 80% alfalfa + 20% commercial layer ration (A81), 70% alfalfa + 30% commercial layer ration (A71), and no feed + inoculum (NF1). SCFA measured in μmoles/mL and error bars represent standard error of mean. Letters above error bars indicate significant differences *(P* < 0.05). [a-c]Means within Trial 1. [A-E]Means within Trial 2. n = 3.

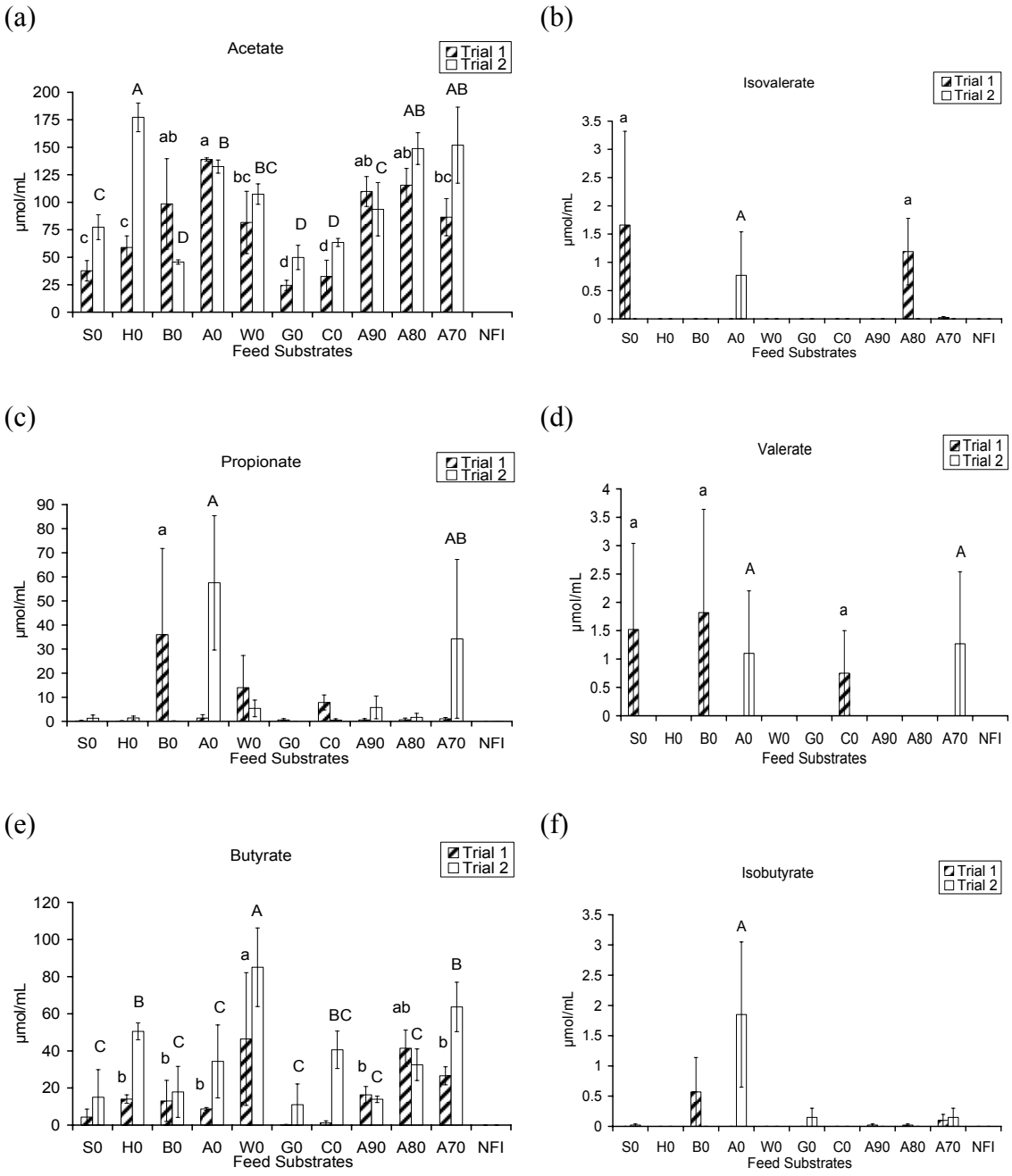


FIGURE 18: Production of short chain fatty acids during *in vitro* fermentation of different feed substrates with anaerobic dilution solution (no cecal inoculum), (Trial 1 and 2). 2a = acetate. 2b = isovalerate. 2c = propionate. 2d = valerate. = 2e = butyrate. 2f = isobutyrate. Feed substrates used are: soybean meal (S0), soybean hull (H0), beet pulp (B0), wheat middling (W0), ground sorghum (G0), cotton seed meal (C0), 100% alfalfa meal (A0), 90% alfalfa + 10% commercial layer ration (A90), 80% alfalfa + 20% commercial layer ration (A80), 70% alfalfa + 30% commercial layer ration (A70), and no feed + inoculum (NF1). SCFA measured in μmoles/mL and error bars represent standard error of mean. Different letters above error bars indicate significant differences *(P* < 0.05). [a-d]Means within Trial 1. [A-D]Means within Trial 2. n = 3.

higher when compared to fermentation without cecal inoculum. The highest acetate production was observed in Trial 2 with H0 (177.25 ± 13.1 μmoles/mL), A70 (151.93 ± 4.55 μmoles/mL), A80 (148.85 ± 14.46 μmoles/mL), in descending order. Butyrate was higher than propionate. For propionate, A0 (57.5 ± 27.86 μmoles/mL) in Trial 2 was the highest yield while W0 in Trial 2 was for butyrate (63.67 ± 13.32 μmoles/mL). For A70, acetate, propionate, butyrate production was consistently high in Trial 2.

The microbial composition of the GI tract of avian species is not extensively defined compared to what is known about microorganisms in ruminants (Ricke and Pillai, 1999). There is the perception that the role of microorganism in chickens is not as important as in the case ruminants (Józefiak et al., 2004). The ceca are the major fermentation organs in GI tract of chickens and contain the largest number of bacteria (Annison et al., 1968; Barnes et al., 1972, 1973; Barnes, 1979). In a previous report (Zhu et al., 2002) indicated that only 10-60% of microorganism in the ceca could be propagated using anaerobic culture techniques. However, over 200 different bacteria have been isolated and characterized (Barnes et al., 1979), these bacteria are influenced by various factors including diet, health and age. In the poultry industry feed deprivation is a procedure employed to induce molting to achieve a rapid and economical new egg-laying cycle (Brake, 1993; Holt, 1995). Changes in dietary composition of the GI tract of poultry such as feed deprivation have negative consequences on microbial population. High fiber containing diets including alfalfa and wheat middling have been examined as alternative diets that would support microbial fermentation and retain the ability to

induce molt (Seo et al., 2001; Ricke, 2003a; Holt, 2003; Donalson et al., 2005; Landers et al., 2005a, b; Woodward et al., 2005). It has been proposed that dietary fiber can be utilized preferentially by *Lactobacillus* and *Bifidiobacteria* species (Kaplan and Hutkins, 2000), which leads to the production of lactic acid and SCFA, this resulted in the maintenance of normal microbial population, low pH and also prevent the establishment of *Salmonella* in the GI tract (Fuller and Turvey, 1971; Juven et al.,1991).

In this *in vitro* study chicken cecal contents and anaerobic dilution solution (Bryant, 1973) were employed to establish cecal inoculum to ferment ten high fiber feed substrates (HFFS) at 0 and 24 h. Similar quantities of HFFS were also exposed to anaerobic dilution solution (without cecal content) as control at the same time points. Six short chain fatty acids (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) were measured. Dietary fiber fractions are fermented by cecal microorganism and have been shown to form end products such as SCFA, ammonia, CO_2 and methane (Pinchasov and Elmaliah, 1994; Marounek et al., 1996, 1999; Jamroz et al., 1998, 2002; Jorgensen et al., 1996) and are assumed to correlate with dietary composition. SCFA can inhibit *Salmonella* growth when present in undissociated form (Van der Wielen et al., 2001, 2002), hence, provides a potential indicator to determine which of the 10 HFFS could provide a potential combination to formulate an alternative molting diet. Langhout and Schutte (1996) concluded that the concentration in cecal chyme were decreased when chickens were fed on diet containing high-methylated citrus pectin while low methylated citrus pectin had no effect. Tsukahara and Ushida (2000) demonstrated that feeding chickens with a plant protein-based diet generated a higher

concentration of SCFA than a diet of animal protein, it was concluded that the difference in SCFA was due to a higher concentration of dietary fiber component in plant based diet.

Although it is suggested that chickens are poor fermenters of dietary fibers our result indicated that extensive fermentation by cecal microorganisms can occur, by virtue of a significantly higher production of SCFA production from HFFS fermented with cecal microorganisms compared to HFFS without cecal microorganisms. The quantity of SCFA production by specific HFFS was observed. These values may represent the quantity of SCFA that can be accumulated in the cecal microenvironment of poultry in the time specified. It is apparent that results presented in these studies show that SCFA are the main end-products of the fermentation of the GI tract of poultry and the ceca may be the main site for this activity as is shown in other species including ruminants and pigs. Acetic acid is produced in the greatest magnitude with lesser quantities of propionic and butyric acid and trace amount of other acid. Alfalfa, soybean and soybean-based HFFS exhibited consistently high SCFA production. Alfalfa is widely used as animal feed and as a high fiber feed source, it is relatively high in protein, and one of the slowest rates of passage through the avian system (Matsushima, 1972; Sibbald, 1979; Garcia et al., 2000). It is well balanced in amino acids and rich in vitamins and gives carcasses their desirable yellow color (Sen et al., 1998; Ponte et al., 2004). Alfalfa is extremely advantageous due to the fermentation properties by cecal microflora that are capable of limiting *in vitro* growth of *Salmonella* Typhimurium when

alfalfa is present and has been shown to limit *in vivo Salmonella* Enteritidis colonization in laying hens (Woodward et al., 2005; McReynolds et al., 2005).

In an *in vivo* study Donalson et al. (2005) have shown that alfalfa mix with layer ration as a molting diet proved to be effective in molt induction and increased post molt performance and post molt egg production when compared with conventional feed withdrawal method. Among the 5 treatments used (100% layer ration, feed withdrawal, 100% alfalfa, 90% alfalfa + 10% commercial layer ration, 70% alfalfa + 30% commercial layer ration), birds fed with 100% alfalfa consumed the least amount of feed. This may suggest a slower passage rate which correlates with increased fermentation and a feeling of satiety (Sibbald, 1979). Other implications may include an increase in fermentation in GI tract and a subsequent reduction in pathogen by possible increased in SCFA accumulation. It has been demonstrated that whole wheat is retained for a longer time in the gizzard to allow proper grounding compared to pellets, which easily disintegrate (Enberg et al., 2004; Bjerrum et al., 2005). This allows more surface area for microorganisms to more extensively ferment feed substrates.

An examination of the digesta from different morphological regions of the GI tract of poultry was observed (Annison et al., 1968), results indicated that VFA were present in the highest concentration in the ceca, including acetic, propionic and butyric acid. Further investigation demonstrated that VFA was not influenced by age (14-20 wk) and when cacectomy was performed on birds there was a drop in VFA production. This suggested that the ceca may play a role in GI tract fermentation in poultry. Supporting evidence demonstrated that cacectomy leads to reduction in digestibility coefficient for

crude fiber including wheat and maize (Halnan, 1949). Furthermore, Thornburn and Wilcox (1965) reported that cacectomy of young cockrel showed a reduction in droppings dry matter and an overall feed digestibility.

PCR-based DGGE

It was assumed that bacterial composition were obtained from both cecal inoculum and HFFS during *in vitro* fermentation, however, it was difficult to determine the percentage microbial contribution from either source. In Trial 1, amplicon profile of bacteria from cecal inoculum and HFFS fermentation *in vitro* revealed a developmental progression of microbial diversity (Figure 19). Bacterial diversity was divided into four distinct groups, A91, A81, A1 and W1 formed a cluster with a 65% correlation, whereas S1, H1, G1 and B1 formed a cluster with a 73% correlation. A71 and NF1 formed a group with an 85% similarity coefficient; C1 was segregated from the other HFFS with a 45% similarity. The highest level of similarity (89.5%) was observed with A91 and A81 which formed a sub-group in the aforementioned cluster. A71 and NF1 also demonstrated high levels of similarity coefficient ((85%).

In Trial 2, cecal inoculum and HFFS were also divided in the basis of microbial diversity (Figure 20). Three distinct groups were formed with H2, W2 and G2 exhibiting an 85% similarity in microflora banding patterns, A92, A82, A2, A72, NF2 and B2 forming a large cluster with an 84% correlation and S2 and C2 forming a group with an 89% similarity coefficient. The overall band patterns for microbial similarity in Trial 2 (72%) was much higher than trial 1 (35%). All the alfalfa-based HFFS exhibited a higher similarity coefficient in Trial 2 than Trial 1 with a band pattern that revealed a 90%

similarity, A92 and A82 formed a sub-group showing a 94% microbial similarity coefficient.

Historically, the isolation and characterization of commensal bacteria has been based on traditional culturing techniques. These techniques used to profile a bacterial community demonstrated a bias, which select for some organisms based on nucleic acid sequencing which tend to eliminate others. These modern approach analyzed the structure of bacterial communities by determining the characteristic features of the microbial DNA extracted from the samples (Tannock, 2001; Knarreborg et al., 2002; Gong et al., 2002; Lan et al., 2002; Zhu et al., 2002; Zhu and Joerger, 2003; Amit-Romach et al., 2004). These techniques have shown that 90% of the bacteria community in the chicken GI tract ecosystem is representative of previously unknown species (Lan et al., 2002).

Recent studies have demonstrated the efficacy of DGGE as a tool to examine complex microbial community (Ferris and Ward 1997; Heuer et al., 1997; Muyzer and Smalla, 1998). A difference in amplicon pattern and similarity scores of 16S rDNA when amplified may determine microbial variation. One factor that must be taken into consideration for these techniques is the choice of polymerase enzyme for amplification. The use of different primer pairs for PCR and other bias that are inherently related to these techniques can also be a factor.

The current *in vitro* study indicated that bacterial population was altered on the basis of different high fiber feed substrate fermented with cecal inocula. Therefore, diet could be a major determinant factor in the bacterial diversity of the GI tract. Since this

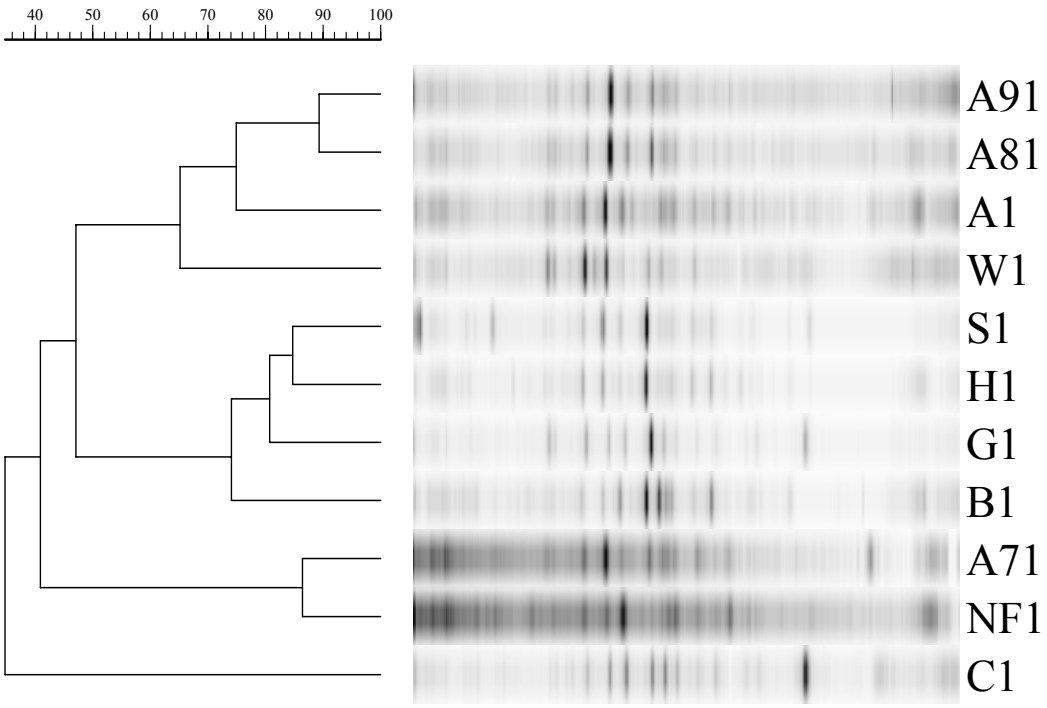


FIGURE 19: Relative similarity of band patterns (Trial 1) from leghorn cecal inoculum and different feed substrates during *in vitro* fermentation. Band patterns are indicated by their grouping on the dendrogram and the percentage coefficient (bar), >= 92% are very related or the same. 85 – 91 % are similar. 80 – 84 are somewhat similar. <= 79% are unrelated. Soybean meal (S1), soybean hull (H1), beet pulp (B1), wheat middling (W1), ground sorghum (G1), cotton seed meal (C1), 100% alfalfa meal (A1), 90% alfalfa + 10% commercial layer ration (A91), 80% alfalfa + 20% commercial layer ration (A81), 70% alfalfa + 30% commercial layer ration (A71) and no feed + inoculum (NF1).

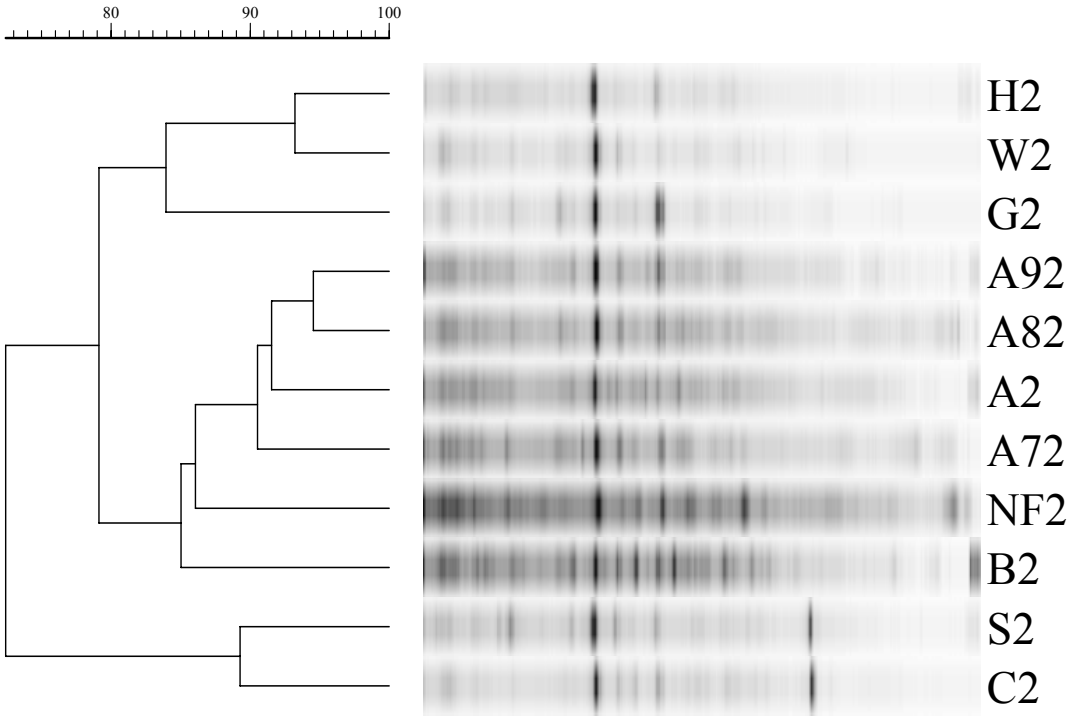


FIGURE 20: Relative similarity of band patterns (Trial 2) from leghorn cecal inoculum and different feed substrates during *in vitro* fermentation. Band patterns are indicated by their grouping on the dendrogram and the percentage coefficient (bar), >= 92% are very related or the same. 85 – 91 % are similar. 80 – 84 are somewhat similar. <= 79% are unrelated. Soybean meal (S2), soybean hull (H2), beet pulp (B2), wheat middling (W2), ground sorghum (G2), cotton seed meal (C2), 100% alfalfa meal (A2), 90% alfalfa + 10% commercial layer ration (A92), 80% alfalfa + 20% commercial layer ration (A82), 70% alfalfa + 30% commercial layer ration (A72) and no feed + inoculum (NF1).

represents the cecal microenvironment it gives an idea of the changes in the microbial diversity of poultry fed on a wide range of dietary fiber. For the most part microbial amplicon patterns of alfalfa-based HFFS in both trials demonstrated high levels of microbial similar coefficient. Therefore, suggests that the microbial population may be similar and could explain the significantly higher SCFA production for alfalfa-based HFFS. A previous report indicated that both the source of feed and local feed amendment, changed the bacterial profile of the intestinal microbial community significantly whereas bacterial profile of birds fed on identical feed regimens exhibited minimal variation from each other (Hume et al., 2003; Apajalahti et al., 2001; Ricke et al., 2004b). Therefore dietary ingredients may possibly represent a shift in microbial community from harmful to non-harmful bacteria (Gibson and Roberfroid, 1995).

In a study conducted by Zhu et al., (2002) TGGE used 16S rRNA genes amplicon for comparisons, the band comparison indicated that 243 different sequences were identified from cecal contents of chickens of various age (1 d, 1, 2, 4 & 6 wks old). The sequence represented approximately 50 phylogenic groups and subgroups of bacteria, however, approximately 89% of the total sequencing represented only 4 phylogenic groups, this may suggest a skew in the of amplicon band patterning towards those 4 phylogenic groups. It was concluded that unsuspected microorganisms (aerobic) were detected and sequencing originating from for predominant bacteria such as related lactic acid producing bacteria (*Bifidobacterium* spp) were underrepresented. Also, the low sensitivity level of the silver staining techniques obscured amplicon visibility. Limitations of this molecular technique may include comigration of amplicon band

especially if present in low concentration (Wintzingerode et al., 1997; Muyzer and Smalla, 1998). In addition, 16S rDNA amplicon sequences that exhibit identical G-C ratio may migrate at the same denaturant level resulting in an inaccurate evaluation of genotypic diversity. Differences in amplicon band patterns for different compartment in the GI tract (jejuna, ilea and colon) of young leghorn chickens was demonstrated by Hume et al., (2003). Changes were evident on microbial content on the basis of age. These results were similar to microfloral diversity in porcine GI tract (Simpson et al., 1999). Similar band patterns were observed based on anatomical segment of GI tract.

The microorganisms that inhabit the ceca could be of significant importance to competitive exclusion mixture since they are unique and presumably adapted to this particular microenvironment and could form a strong barrier that are capable of inhibiting *Salmonella* in the ceca. Earlier studies have shown that the microorganisms of the ceca is relative stable, some dietary changes such as low and high protein levels have little influence on its microbial population (Barnes, 1972; Takahashi et al., 1982). The current *in vitro* study was conducted to simulate the efficacy of cecal microorganisms of chickens to ferment various HFFS. It appears that cecal microorganisms may be able to ferment HFFS to produce SCFA. SCFA such as acetate, propionate and butyrate (Jamroz et al., 1998) are important to limiting *Salmonella* colonization and is believed to correlate with dietary fiber metabolism in ceca. Microbial amplicon patterns from DGGE results indicate that the cecal microorganisms can be altered by changes in diets. These changes may be beneficial to the poultry industry since, feed regimen can be manipulated for required performances. The use of amplicon visualization can be a

potentially useful tool to investigate microbial diversity in relationship to dietary shift in poultry.

CHAPTER VI

FERMENTATION AND MICROBIAL RESPONSE OF *SALMONELLA* ENTERITIDIS CHALLENGED HENS FED ALFALFA CRUMBLE DIET

Introduction

The microbial composition in the gastrointestinal (GI) tract of chickens is not extensively defined compared to what is known about microorganisms in ruminants (Ricke and Pillai, 1999). There is the perception that the role of microorganisms in chickens is not as important in fermentation mechanism as the rumen microflora in ruminants (Józefiak et al., 2004). The ceca are the major fermentation organs in GI tract of chickens and contain the largest number of bacteria (Annison et al., 1968; Barnes et al., 1972, 1973; Barnes, 1979). A full compliment of normal microflora that constitutes microbial complexity in adult chickens is considered resistant to enteropathogens (McNab, 1973). It has been suggested that the poultry digestive microbial ecology is altered during dietary stress, such as molting, leaving the GI tract vulnerable to pathogen infection and colonization (Durant et al., 1999; Holt, 2003; Ricke, 2003a). Durant et al. (1999) reported that induced molted hens via feed withdrawal exhibited reductions in lactate, short chain fatty acids (SCFA) and total SCFA while crop pH decreased significantly in molted hens compared to fully fed hens. These conditions ultimately reduce protective microflora in GI tract and facilitate *Salmonella* colonization (Ricke et al., 2003a). Therefore, dietary regimens that promote SCFA production and maintain GI tract microenvironment pH could be a priority to eliminate incidence of *Salmonella* invasion. Fermentable high fiber diets such as alfalfa have been examined as potential

molting dietary approaches that can retain normal microbial flora, hence, reduce proliferation of *Salmonella* (Woodward et al., 2005) in GI the tract of molting hens. The use of alfalfa as a single dietary source could provide microbial fermentative products including SCFA (Kass et al., 1980; Ricke et al., 2003a; Woodward et al., 2005) needed for retaining beneficial microorganisms (Van der Wielen et al., 2000; Kaplan and Hutkins, 2000), also developing bacteriostatic effect on enteric bacteria including *Salmonella*.

Molecular techniques have been used to compare bacterial diversity in chicken cecal microenvironment and for potential probiotic sources (Ricke and Pillai, 1999; Zhu et al., 2002; Hume et al., 2003; Ricke et al., 2004b), however, some DNA isolation techniques have their limitation regarding amplification and cloning, and tend to favor the detection of certain bacteria and nucleic acid sequences. The PCR-based Denaturing Gradient Gel Electrophoresis (DGGE) has been widely used in the last two decades and facilitates unique visualization of PCR products (amplicons) representing predominant diversity of digestive bacteria (Muyzer et al., 1993; Lee et al., 1996; Murray et al., 1996). Hume et al reported that molecular-based DGGE techniques allowed visualization of changes in cecal bacterial communities during molt induction by feed withdrawal and hens supplemented with high levels of dietary zinc, low calcium and alfalfa. Results indicate similarities between amplicon band patterns for fully fed hens and those given dietary zinc, low calcium and to a lesser extent those fed alfalfa high fiber diets, however, less similarity was observed in feed withdrawal hens. Ricke et al. (2004b) also utilized molecular-based DGGE to detect changes in cecal and crop microbial community of

molted hens fed on zinc acetate, zinc propionate compared to feed withdrawal and fully fed hens. However, these studies involved profiled the cecal population only once at the end of the molt period and did not characterize microbial populations during initial introduction of the molt diet. The specific objectives of this study were (1) to determine the SCFA production profile of fecal and cecal samples of *Salmonella* Enteritidis (SE) challenged laying hens fed a 100 % alfalfa crumble molting diet; (2) to follow the microbial populations before and after *Salmonella* Enteritidis (SE) challenge by determining the microbial DGGE profile of fecal and cecal samples of SE challenged laying hens fed a 100 % alfalfa crumble molting diet.

Materials and methods

Preparation of inoculum

Salmonella Enteritidis poultry isolate strain (phage type 13A), was selected for resistance to novobiocin[1] and naladixic acid (Sigma Aldrich Co., St. Louis, MO) (NO: NA) has been previously described by McReynolds et al. (2005) was used in the study. The media used to culture the resistant isolate contained 25 μg of NO and 20 μg of NA per mL. Bacteria were sub-cultured overnight 3 times in tripticase soy broth (Becton, Dickerson and Company, Sparks, MD) and were serially diluted in sterile phosphate-buffered saline to approximately 10^6 cells per mL. The inoculum was plated on brilliant green agar (Becton, Dickerson and Company, Sparks, MD) (BGA) and incubated at 37 ^{o} C overnight to confirm the CFU/mL.

Experimental design

Fecal and cecal contents were collected from a 15 single comb Leghorn hens (>50 wks old) obtained from a local commercial laying flock. Hens were placed in wire layer cages (USDA-ARS, College Station, TX) and acclimatized to their environment for a 2-wk period. One week before the test diet was administered the lighting regime was changed to 8 h light and 16 h dark, which was continued throughout the experiment. Hens were separated into three treatment groups (5 birds each) based on diet: (1) alfalfa (100 % crumble, ALC+); (2) full fed or non-molted (100% commercial layer ration, FF+); and (3) a full feed withdrawal to induce molt (no feed, (FW+). All birds were provided with water *ad libitum*. Treatment diets were fed on Day 0 through Day 12 (end of forced-molt). Feed for the FW groups were removed for the entire 12-day period. All hens were challenged by crop gavage 4 days after the beginning of molt induction with 1 mL of inoculum containing approximately 10^6 cfu of SE (phage type 13A). The experiment was repeated with the same number of birds under similar conditions and was designated Trial 2. Fecal samples were collected at days 4, 6, 8, 11, and cecal day 9 (via necropsy) and analyzed for microbial population shifts by PCR-based 16S rRNA gene amplification and DGGE and SCFA production.

DGGE

Fecal and cecal bacteria genomic DNA was isolated from 1.5 mL of each sample with a QIAamp DNA Mini Kit (QIAgen, Valencia, California) by the method described in the kit. Denaturing gradient gel electrophoresis was conducted according to the method of Muyzer et al. (1993) with modification, using bacterial specific PCR primers

(Integrated DNA Technology, Inc., Coralville, IA) to conserved regions flanking the variable V3 region of 16S rDNA genes. PCR was run with a 50 µL total reaction volume. Primers (50 pmol of each per reaction mixture; primer 2, 5'-ATTACCGCGGCTGCTGG-3', and primer 3 with a 40-base G-C clamp (Sheffield et al., 1989; Muyzer et al., 1993), *5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG-3'*), were mixed with Jump Start Red-Taq Ready Mix (Sigma Aldrich Co., St. Louis, MO) according to kit instructions, with 250 ng (50 ng of DNA pooled from 5 samples each) template DNA. Acetamide (5%, wt/vol) was added to eliminate preferential annealing (Reysenbach et al., 1992), also 10 mg/mL bovine saline albumin (BSA) and deionized water to make up a final 50-µL volume reaction. Amplification was done on a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA) with the following program: 1) denaturation at 94.9 ^{o}C for 2 min; 2) subsequent denaturation at 94.0^{o}C for 1 min; 3) annealing at 67.0 ^{o}C for 45 s; -0.5^{o}C per cycle [touchdown to minimize spurious by-product (Don et al., 1991; Wawer and Muyzer, 1995); 4) extension at 72.0^{o}C for 2 min; 5) repeat step 2 to 4 for 17 cycles; 6) Denaturation at 94^{o}C for 1 min; 7) annealing at 58.0^{o}C for 45 s; 8) repeat step 6 to 7 for 12 cycles; 9) extension at 72.0^{o}C for 7 min; 10) 4.0^{o}C final.

Gel electrophoresis

Polyacrylamide gels (8% vol/vol) acrylamide-bisacrylamide ratio 37.5:1) were cast with a 35 to 60% urea-deionized formamide gradient; 100% denaturing acrylamide was 7 M urea and 40% deionized formamide. Amplified samples (4 µL of pooled template)

were mixed with an equal volume of 2x loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and 7 μL were placed in each sample well (16-well comb). Gels were placed in a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Richmond, CA) for electophoresis in 1x TAE (20 mM Tris (pH 7.4), 10 mM sodium acetate, 0.5 M ETDA) buffer at 59^{o} C for 17 h at 60 V. Gels were stained with SYBR Green 1 (1:10,000 dilution) for 40 min. Amplified fragment pattern relatedness of samples were determined with Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories, Hercules, CA) based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. Dice coefficient (values between 0 and 1) is an arithmetic method, which determines the degree to which banding patterns are alike. Clusters (groups) were determined by sequentially comparing the patterns and the construction of a dendrogram reflecting the related similarities. The amount of similarity was reflected by the relatively closeness or grouping and is indicated by the percentage similarity coefficient bar located above each dendrogram.

SCFA concentration

The fecal and cecal concentration of SCFA from triplicate samples was determined by gas-liquid chromatography as previously described by Corrier et al. (1990). The analyses were conducted with a gas chromatograph equipped with flame ionization detector and peak profiles integration-quantification integrator (Shimadzu Corp., Columbia, MD). Each sample peak profile was integrated and quantified relative to an internal standard of methyl-butyric acid placed in the same sample. Analyses were

conducted at an oven temperature of 200^{o}C and a flow rate of 85 mL/min. The concentration of each acid was expressed in μmoles/mL.

Statistical analysis

Data from concentrations of SCFA were analyzed using the one-way ANOVA subjected to linear regression using PC-SAS software version 9.0 (SAS Institute Inc., Cary, NC) (2001). Differences between means were determined using least square means and Tukey's honest significance test. Statistical variation was also estimated by the standard error of the mean. All statistical analyses were considered significant at $P < 0.05$.

Results and discussion

Fecal and cecal short chain fatty acid response

In the current study fecal SCFA production was evaluated on days 4, 6, 8 and 11 (Figure 21). Results from fecal samples demonstrated that acetate production occurred in the greatest proportion (Figure 21a and b). Propionate (Figure 21c and d) and butyrate (Figure 21e and f) were next in order of magnitude whereas only trace amounts of isovalerate, valerate and isobutyrate (data not shown) were present. The concentration of total SCFA (Figure 21g and h) for feed withdrawal molted birds were significantly lower ($P < 0.05$) when compared to alfalfa crumble and full fed groups. In Trial 1, acetate concentrations for FW+ hens (day 4: 1.75; day 6: 8.86; day 11: 6.96 μmoles/mL) were significantly lower compared to ALC+ (days 4: 26.65; day 6: 38.67; day 11: 42.9 μmoles/mL) and FF+ hens (day 4: 24.03; day 6: 22.42; day 11: 82.6 μmoles/mL). In Trial 2 concentrations of acetate were significantly higher in ALC+ (day 8: 47.05; day

11: 30.87 μmoles/mL) compared to FW+ hens (day 8: 4.0; day 11: 12.84 μmoles/mL). Except for day 8 (0.21 μmoles/mL) in ALC+ hens and FF+ hens (0.93 μmoles/mL), the concentration of propionate for ALC+ (day 4: 6.11; day 6: 9.54; day 11: 8.5 μmoles/mL) and FF+ hens (day 4: 9.58; day 6: 7.28; day 11: 6.62 μmoles/mL) were similar but were significantly higher compared to FW+ hens (day 4: 0.43; day 6: 1.36; day 11: 0.53 μmoles/mL). In Trial 2, days 8 and 11 for both ALC+ and FF+ hens showed no difference but were significantly higher compared to these same days for FW+ hens. In general, butyrate concentration was minimal and was not detected in FW+ hens in Trial 1. Butyrate production (Trial 2) exhibited similar trends except day 4 (8.8 μmoles/mL) for FW+ hens, which was significantly higher than all other days in the 3 treatment groups. In general, total SCFA concentration for ALC+ and FF+ hens were significantly higher compared to FW+ treatment group. As indicated in individual SCFA day 11 (96.39 μmoles/mL) in Trial 1 exhibited the highest concentration. In Trial 2, day 4 (59.78 μmoles/mL) in ALC+ hens showed the highest total SCFA, which showed no difference from day 4 (56.4 μmoles/mL) in FW+ hens.

SCFA concentrations were also determined in cecal contents on day 12 (Figure 22). In both trials, the FF+ and ALC+ treatment groups appear to produce significantly higher acetate, propionate, butyrate and total SCFA in the ceca than the FW+ hens. In both trials FF+ and ALC+ hens exhibited significantly higher SCFA in ceca compared to FW+ treatment group. Acetate concentration (Trial 1) for ALC+ hens (66.48 μmoles/mL) showed no difference from FF+ hens (77.28 μmoles/mL), which were

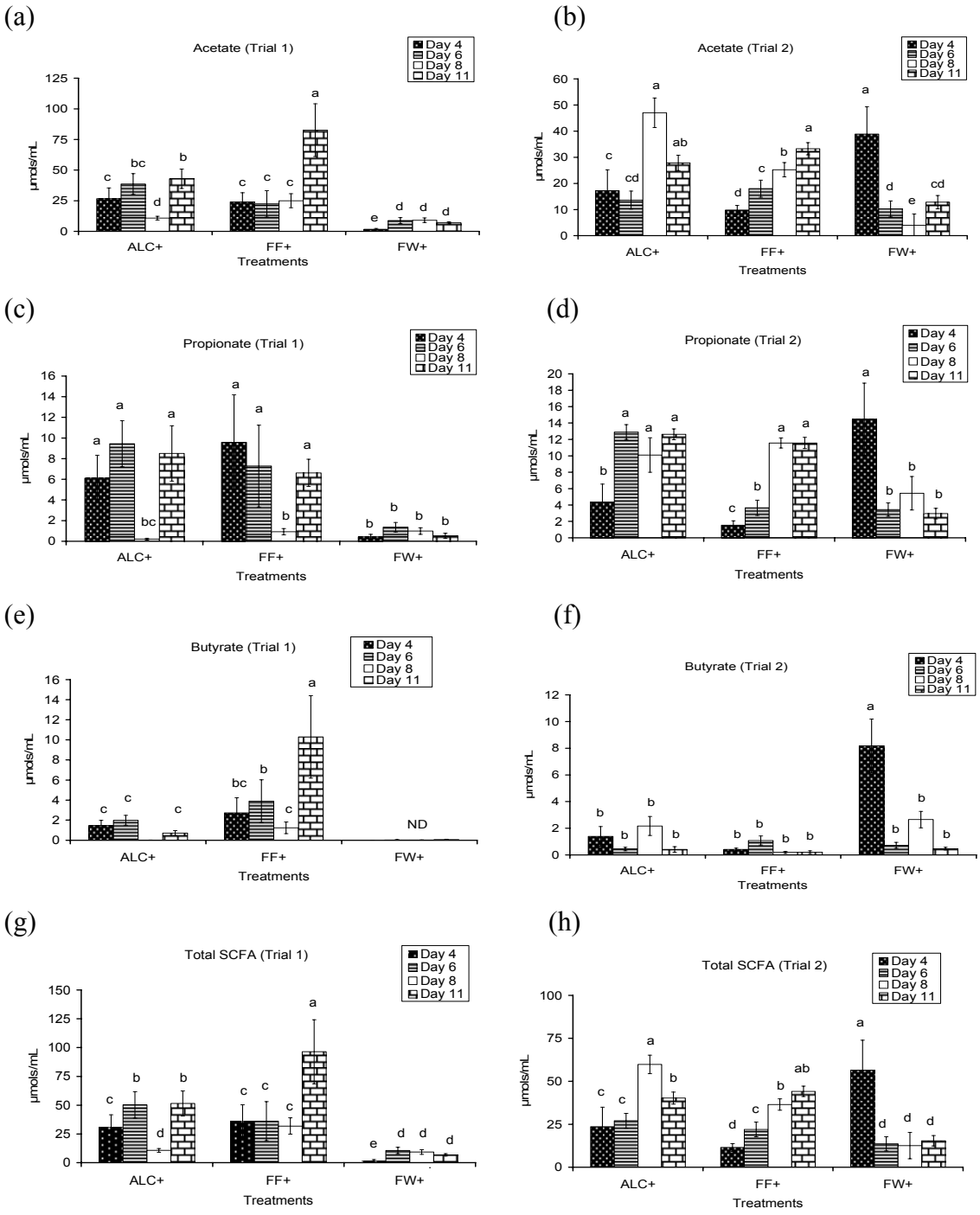


FIGURE 21: Production of short chain fatty acids from fecal samples in *Salmonella* Enteritidis-challenged laying hens fed an alfalfa diet during molt. Error bars indicate standard error of the mean. [a-e]Means within Trial 1 without a common letter differ significantly *(P* < 0.05). [a-e]Means within Trial 2 without a common letter differ significantly *(P* < 0.05). FF+ = full-fed (non-molted); ALC+ = alfalfa; FW+ = feed withdrawal. ND = not determined. Day 4 represent pre-challenged sample. days 6, 8 and 11 represent post-challenged.

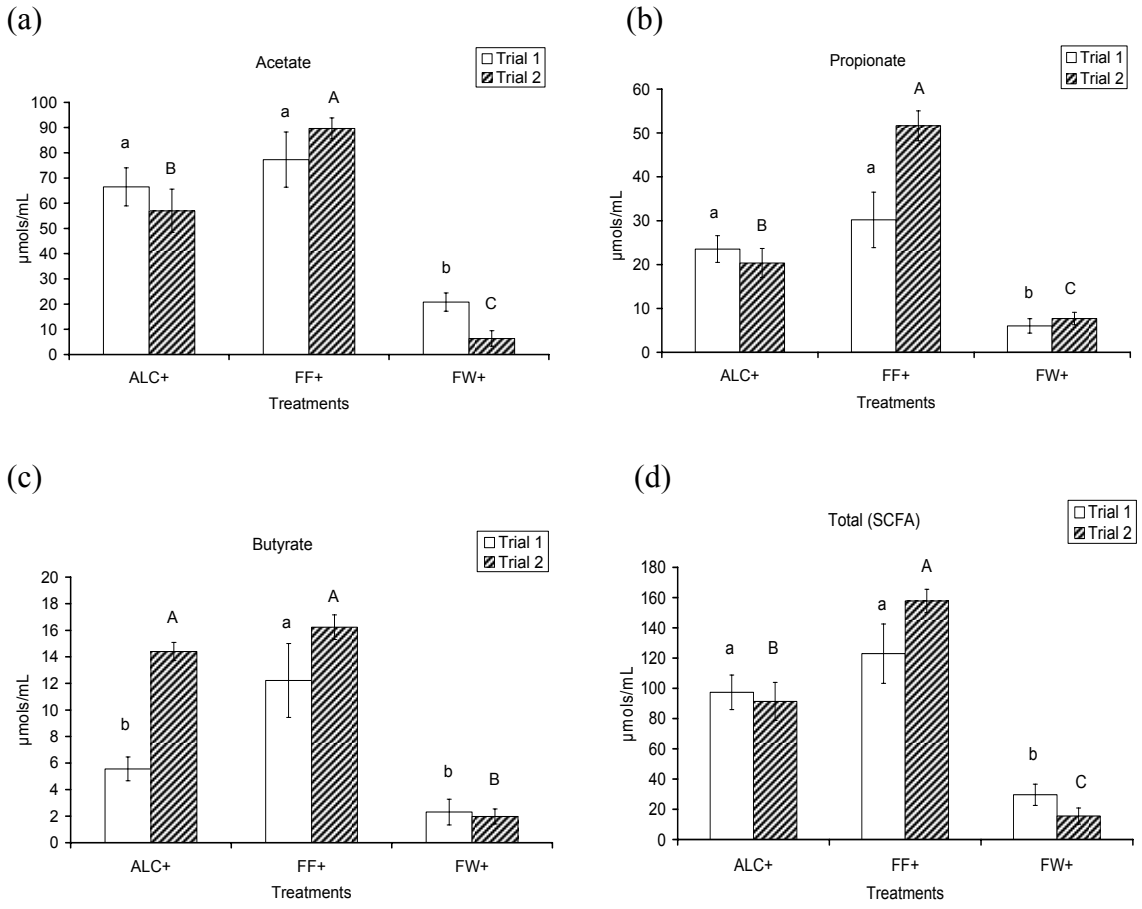


FIGURE 22: Production of short chain fatty acids from cecal samples in *Salmonella* Enteritidis-challenged laying hens fed an alfalfa diet during molt. Error bars indicate standard error of the mean. [a-b]Means within Trial 1 without a common letter differ significantly *(P* < 0.05). [A-C]Means within Trial 2 without a common letter differ significantly *(P* < 0.05). FF+ = full-fed (non-molted). ALC+ = alfalfa. FW+ = feed withdrawal. Cecal samples collected on day 12 of molt via necropsy (post-challenged).

significantly higher compared to FW+ hens (20.97 μmoles/mL). In Trial 2, acetate concentration from FF+ hens (89.65 μmoles/mL) was significantly higher than ALC+ hens (56.99 μmoles/mL), however both were significantly higher than FW+ hens (6.34 μmoles/mL). Propionate concentrations in Trial 1 showed no difference between ALC+ (23.53 μmoles/mL) and FF+ hens (30.91 μmoles/mL) but were significantly different compared to FW+ hens (6.02 μmoles/mL). In Trial 2, FF+ hens (51.62 μmoles/mL) were significant higher than ALC+, however, ALC+ was significantly higher than FW+ hens (7.71 μmoles/mL). Total SCFA exhibited similar trend to acetate, in Trial 2, the highest total SCFA was shown in FF+ hens (122.93 μmoles/mL) which were significantly higher than ALC+ hens (97.38 μmoles/mL), both treatment group (FF+ and ALC+) were significantly higher than FW+ hens (29.61 μmoles/mL). The overall trend of SCFA in cecal and fecal samples demonstrated similar patterns.

In general total SCFA production in ceca from FF+ and ALC+ hens nearly doubled compared to fecal SCFA concentration in FF+ and ALC+ groups. However, SCFA for FW+ hens in the ceca and fecal samples were consistently low within the time span in this study, except for day 4 (prechallenged) in Trial 2. For fecal samples (Trial 1), it was observed that acetate, butyrate and total SCFA was significantly higher in day 11, than on other days for FF+ hens, while days 8 and 11 saw a shift to higher concentrations of SCFA for FF+ and ALC+ hens. The overall trend indicates that SCFA in FF+ hens were either similar or slighter higher than the ALC+ group, which corresponded with the cecal samples. Both cecal and fecal samples showed consistently less SCFA production in FW+ hens. Given the numerical differences observed in SCFA concentrations in fecal

compared to cecal samples, they remained similar in trend, which showed that FF+ was either not different or significantly higher than in the ALC+ group but was consistently lower in SCFA concentration for FW+ hens. Based on the trends for both cecal and fecal SCFA production it appears that fecal SCFA can represent trends that would be observed with cecal SCFA production and therefore could provide a reasonable noninvasive indicator for cecal fermentation.

These results indicated that induced molted hens by means of feed deprivation could influence production of SCFA in the ceca. Corrier et al. (1997) observed (day 6 of molt) that feed-withdrawal total SCFA (89.9 μmoles/mL), acetate (46.9 μmoles/mL), and propionate (20.4 μmoles/mL) concentrations in the cecal contents were nearly half the concentration of the respective SCFA from non-molted hens. Moore et al. (2004) observed no differences in acetic acid production among treatments when testing a zinc in layer ration combination diet in an induced molt, however, they observed that the hens molted by feed removal produced less propionate, butyrate and total SCFA than the other treatment groups. In the 3 trials Moore et al. (2004) observed that the concentrations of propionate were ($P < 0.05$) lower in the ceca of molted hens (feed withdrawal) than in ceca of nonmolted hens (fully fed). In addition total SCFA were significantly higher in fully fed hens compared to feed withdrawal hens in Trial 3. In an alfalfa meal molt induction study Woodward et al. (2005) noted that cecal acetate (75-115 μmoles/mL) and propionate (33-47 μmoles/mL) production in fully fed hens nearly doubled compared to feed deprivation with alfalfa molted hens SCFA somewhat intermediate. Woodward et al. (2005) suggested that the intake of alfalfa mash was

inconsistent with decreased intake in alfalfa, generally decreased SCFA production and lowered effectiveness in preventing SE establishment. A reduction in particle size could encourage increase surface area leading to and consequently facilitating increased fermentation of materials by microorganisms in the ceca (Engberg et al. (2002), resulting in increased concentration of various SCFA in the cecal contents. Based on these results feed removal appeared to be accompanied by a decreased fermentation capacity and a reduction of SCFA producing bacteria present in the ceca.

The concentration of SCFA production in the GI tract has been considered an indicator of fermentation capacity of the gut microorganisms. It has been proposed that dietary fiber can be utilized preferentially by *Lactobacillus* and *Bifidobacteria* species (Kaplan and Hutkins, 2000), which leads to the production of lactic acid and SCFA. These effects result in the maintenance of normal microbial population and lower pH, which prevented the establishment of *Salmonella* in the GI tract (Fuller and Turvey, 1971; Juven et al., 1991). The prevention of *Salmonella* colonization in the ceca is positively correlated with increased SCFA concentrations and decreased pH (Barnes et al., 1979; Nisbet et al., 1994; Corrier et al., 1995).

PCR-based DGGE

DGGE results from two trials in the current study indicated that variations in microbial populations were influenced by treatments. Full-fed and ALC+ treatment groups exhibited higher percentage similarity coefficients (>90%) in Trial 2 than in Trial 1. Microbial constituents and ratios are expected to shift with each batch of hens as environmental exposures to variable microbial populations occur within time. These

shifts in microbial populations would be reflected by modifications in DGGE profiles, as well as by bird batch variations in SCFA concentrations. In Trial 1 (Figure 23), the amplicon profile of bacterial diversity was divided into four distinct groups; 4FF+, 8ALC+, 11FF+ formed a group with an 82.1 % correlation; 6FW+, 4FW+, 4ALC+, 6ALC+ formed a group with an 84 % correlation; 6FF+, 8FF+ formed a group with a 92 % similarity coefficient and 11ALC+, 12ALC+Ce, 12FF+Ce exhibited a 68.1 % coefficient similarity. Three feed withdrawal samples (8FW+, 12FW+Ce and 11FW+) were segregated from the other with 66.7, 61.4 and 54.3 % similarity, respectively. These indicated a relatively lower similarity coefficient percentage.

The highest level of similarity (95.5%) was observed early in the study (prechallenged) with 4FW+ and 4ALC+, which formed a sub-group. The similarity correlation grouping largely indicated that FF+ and ALC+ treated hens showed greater similarity, compared to FW+ hens that were more segregated indicating lower similarity coefficient which may suggest that a depletion of feed in the GI tract could lead to a decrease in microbial population (Durant et al., 1999). In Trial 2 (Figure 24), four distinct groups were formed; 11FW+ and 12FW+Ce with an 82.3 % similarity microflora band pattern, 12FF+Ce and 12ALC+Ce formed a 95.5 % correlation similarity. Two large clusters were observed, 4ALC+, 6FW+, 6ALC+, 8FW+, 4FW+ with a 68.3 % similarity coefficient while 6FF+, 8ALC+, 8FF+, 4FF+, 11FF+ and 11ALC+ formed a 67.7 % similarity. 11FF+ and 11ALC+ formed a sub-group of 98.0 % similarity. In Trial 1, the similarity of ALC+ and FW+ treatment groups (Figure 23)

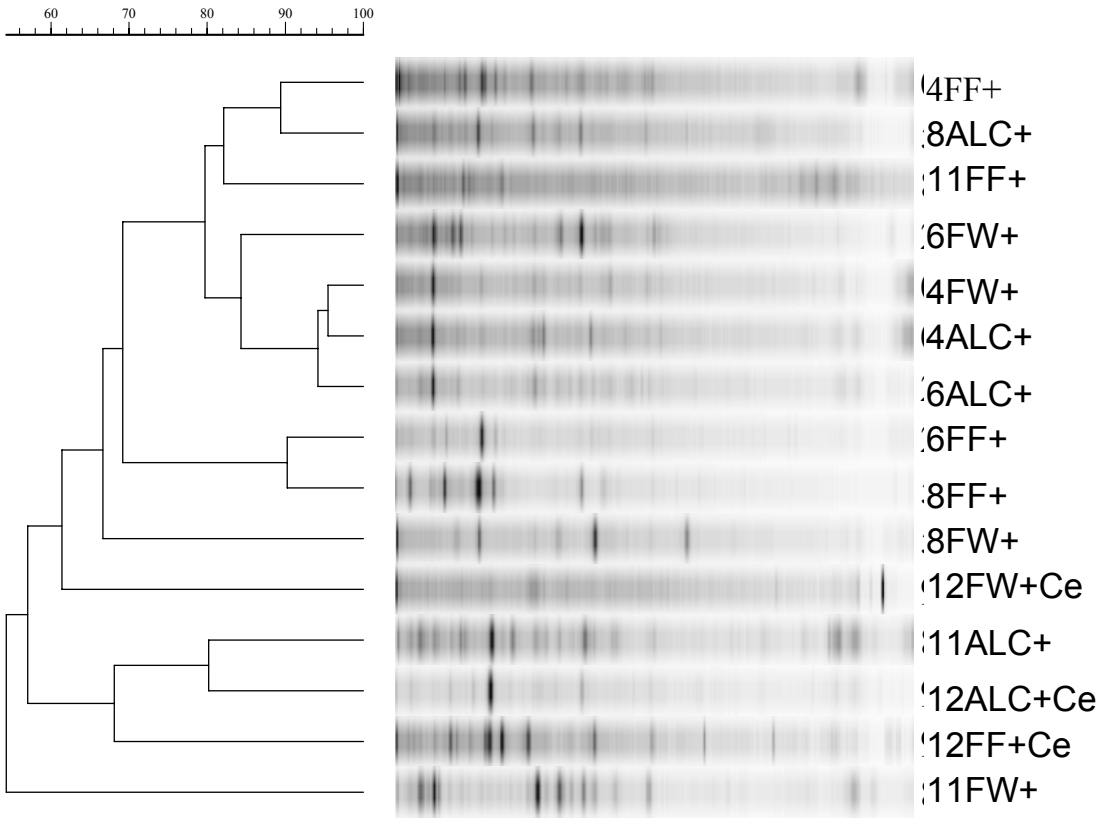


FIGURE 23: Relative similarity of band patterns from *Salmonella* Enteritidis challenged laying hens fed an alfalfa diet during molt (Trial 1). Band patterns are indicated by their grouping on the dendrogram and the percentage coefficient (bar), >= 92% are very related or the same. 85 – 91 % are similar. 80 – 84 are somewhat similar. <= 79% are unrelated. Numbers before treatment groups represent days of molt. Day 4 represent pre-challenged sample; days 6, 8 and 11 represent fecal samples, post-challenged. Day 12 represent cecal samples collected via necropsy, post-challenged. FF+ = full fed (non-molted). ALC+ = alfalfa. FW+ = feed withdrawal. Ce = cecal samples.

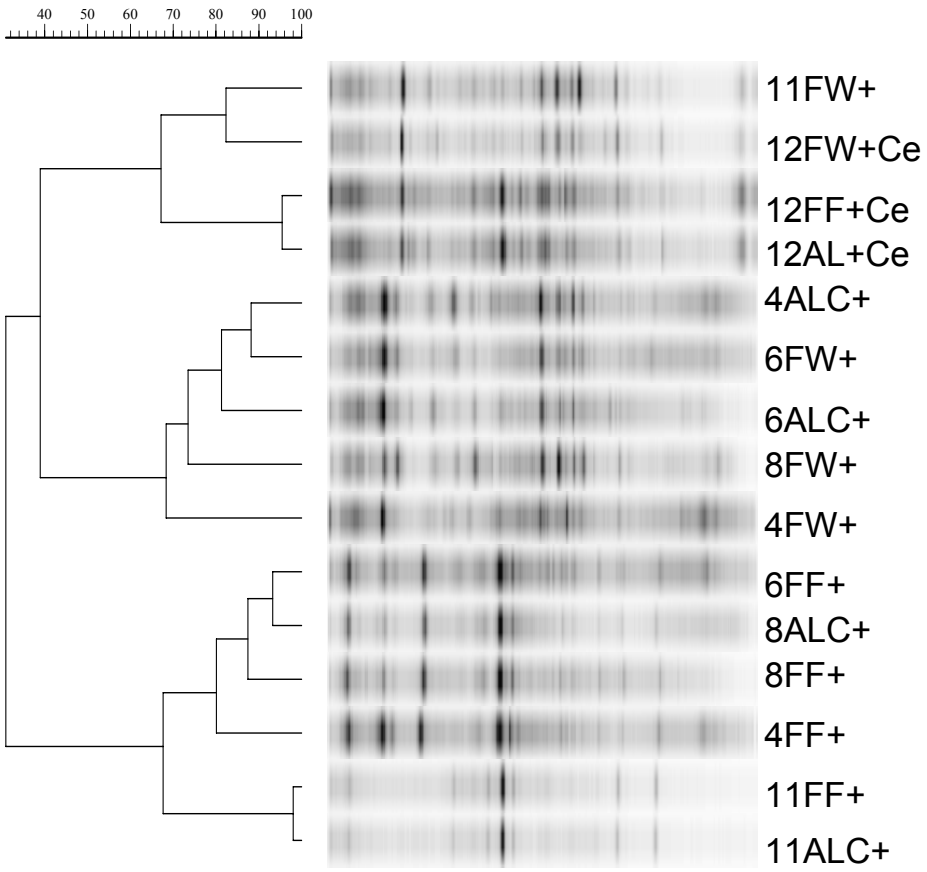


FIGURE 24: Relative similarity of band patterns from *Salmonella* Enteritidis challenged laying hens fed an alfalfa diet during molt (Trial 2). Band patterns are indicated by their grouping on the dendrogram and the percentage coefficient (bar), >= 92% are very related or the same. 85 – 91 % are similar. 80 – 84 are somewhat similar. < = 79% are unrelated. Except for 4-day which represents pre-challenged samples, numbers before treatment groups represent days post SE-challenged. Days 4, 6, 8 and 11 represent fecal samples while Day 12 cecal samples collected via necropsy. FF+ = full fed (non-molted). ALC+ = alfalfa. FW+ = feed withdrawal. Ce = cecal samples.

for pre-challenged hens showed a 95.5 % similarity (Day 4), however, as molting progressed their similarity coefficient began to shift by Day 6 (84 %) and eventually to 57.0 % in the ceca at the end of the experiment (Day 12 necropsy). A comparison of FF+ and ALC+ treatment groups at the initiation of the study (Day 4) demonstrated a 79.7 % similarity and at culmination of molting (Day 12 necropsy) cecal samples for both FF+ and ALC+ demonstrated an 80.2 % similarity coefficient. This observation may suggest stability in band patterns between the FF+ and ALC+ treatment groups.

In Trial 2, (Figure 24) ALC+ and FF+ hens (Day 4) showed a 30.9 % amplicon similarity coefficient however, a 95.4 % similarity for both was observed at Day 12 in cecal samples. The data further suggested that there might be a greater microbial population shift between the ALC+ and the FW+ treatment groups compared to ALC+ and FF+ treatment groups. The overall amplicon band patterns of microbial similarity in Trials 1 and 2 indicated that hens molted with 100 % alfalfa crumble diet were similar to the control group (Full Fed; 100% commercial layer ration). Greater similarity observed in ALC+ and FF+ hens could be linked to the increased fermentative capacity of microorganisms on feed materials present in the GI tract , which leads to SCFA production that potentially retain microbial populations. In general amplicon band patterns of microbial similarity for feed withdrawal were in similar groupings or was segregated which indicates distinct unrelatedness to ALC+ and FF+ especially in Trial 1 (Figure 23).

Microbial diversity in the GI tract may be influenced by variation in diet, however there are difficulties encountered to cultivate microorganisms to provide a true picture of

a full compliment of bacterial population (Ricke and Pillai, 1999). In addition molecular technique for DNA isolation may contribute to a less than precise result for amplicon specificity and visualization; therefore, it can be a daunting task to identify minimal shifts in microbial population (Ricke and Pillai, 1999). In the current study dendrogram (Figure 23) indicated that cecal samples for FF+ and ALC+ (Trial 1) were grouped with a 70% similarity compared to cecal samples for FW+ hens, which was segregated and showing a 61% similarity exhibiting amplicon bands. In the second trial (Figure 24) cecal samples for FF+ and ALC+ formed a sub-group with a 95% similarity and collectively shared a 66% similarity with FW+ (cecal sample) and day 11 FW+ fecal sample (Figure 24). In trial 2, an inconsistent amplicon band pattern was demonstrated in cecal and fecal groups at days 4 and 6 but as the molt progressed there was a shift which demonstrated a level of consistency from days 8 and 11 fecal samples which was similar to cecal samples for the FF+ and ALC+ groups. This suggest that FF+ and ALC+ appeared to be more capable in retaining cecal microbial populations needed to maintain cecal microenvironmental integrity in combination with SCFA to inhibit enteropathogens such as *Salmonella*. Similar SCFA patterns were identified in fecal samples (Trial 2) where a shift to higher concentrations was observed from day 8 onwards for FF+ and ALC+ hens. A comparison of the results which exhibit a consistent trend in SCFA and DGGE molecular technique showing greater similarity coefficient within FF+ and ALC+ treatment groups indicate that alfalfa not only appear to retain protective microflora during molting but apparently derive sufficient fermentative

product needed to inhibit enteropathogens from cecal environment of hens compared to FW+ hens.

The GI tract of chickens constitutes a wide diversity of microorganisms that can be altered by stressors, performance enhancers, locality, and dietary shifts (Nurmi and Rantala, 1973; Gibson and Roberfroid, 1995; Apajalahti et al., 2001; Hume et al., 2003; Ricke et al., 2004a). Changes in dietary composition of the GI tract of poultry brought about by molt induction by feed deprivation are believed to reduce microbial population. In a previous study (Hume et al., 2003) PCR-based DGGE was conducted to determine changes in digestive microbial communities in cecal contents of control (non-molted) and molted Leghorn hens, fed different dietary regimen (low calcium, low calcium and low zinc, alfalfa diets and feed withdrawal). Results from amplicon band patterns showed that molted hens fed low calcium and zinc diet formed an 89.5 % similarity group whereas a 68% similarity was observed with alfalfa feed regimen. Amplicon band patterns from molted hens on feed withdrawal demonstrated lowest similarity of all treatments groups. This suggests that control hens and those given dietary zinc and calcium and to a lesser degree alfalfa prevented the depletion of microbial population compared to complete feed withdrawal. Recently, Ricke et al. (2004b) employed DGGE to analyze cecal and crop microbial profiles of molted hens fed on zinc acetate or zinc propionate compared to feed withdrawal and fully fed hens. Results from amplicon band patterns revealed over 85 % similarity coefficient in cecal microbial profile between zinc acetate fed hens and zinc propionate fed hens, in addition cecal bacterial populations showed changes as a result of feed removal in fully fed hens. Providing molting hens

with a low nutrient alternative dietary regimen over feed withdrawal presumably were more influential in retaining microbial community in GI tract. Alternative molting diet that retains normal microflora is believed to be instrumental in reducing *Salmonella* colonization in the GI tract ecology of chickens (Seo et al., 2001; Woodward et al., 20005; McReynolds et al., 2005, 2006).

Fecal samples for DGGE were analyzed on days 4, 6, 8 and 11 compared to day 12 for cecal sample. It can be difficult to make a feasible comparison because fecal samples were assessed on four days during the trial while cecal samples were on only day 12. In trial 1 (Figure 23), fecal samples for Days 4, 6 and 8 exhibited greater similarity for FF+ and ALC+ hens compared to FW+ hens. Fecal sample for day 11 were segregated. Cecal samples for FF+ and ALC+ (Trial 1) were grouped with a 70% similarity compared to cecal samples for FW+ hens, which was segregated and showing a 61% similarity exhibiting amplicon bands. In trial 2, days 4 and 6 showed inconsistencies in amplicon band patterns, however a shift, which demonstrated a level of consistency from days 8 and 11 fecal samples was similar to cecal samples for the FF+ and ALC+ groups. Cecal samples for FF+ and ALC+ formed a sub-group with a 95% similarity and collectively shared a 66% similarity with FW+ (cecal sample) and day 11 FW+ fecal sample (Figure 24). Fewer inconsistencies were observed among fecal and cecal samples, however for the most part cecal and cecal samples for FF+ and ALC+ hens exhibited greater similarity compared to FW+. Therefore, the data suggested that the increased similarity trends observed in fecal and cecal samples demonstrated that DGGE fecal is consistent

with cecal DGGE and like SCFA could be used as a non invasive indicator of cecal DGGE.

Alfalfa as a dietary fiber may affect the GI tract by supporting microbial activities, decreasing rate of passage, metabolites and digestive efficacy (Bach Knudsen, 2001; Wenk, 2001). Recently, certain dietary components including polysaccharides were identified as having the potential to be utilized as prebiotics (Verstegen and Schaafsma, 1999; Cummings and MacFarlane, 2002). The effectiveness of the ALC+ diet to reduce cecal *Salmonella* colonization may rely on its ability to retain a normal microflora in the intestines (Barnes et al., 1980; Corrier et al., 1995; Nisbet et al., 1994; Nurmi and Rantala, 1973; Van der Wielen et al., 2001, 2002; Woodward et al., 2005). The result of the current study could explain that changes in the GI tract microenvironment such as those occurring during feed deprivation in molted hens create a more SE colonization susceptible state. Based on our results FF+ and ALC+ in both fecal and cecal contents revealed significantly higher SCFA than FW+ hens, which is also reflected in the greater similarity in have a limiting effect of SE colonization in the GI tract microenvironment of microbial amplicon band patterns of FF+ and ALC+ treatment groups when compared to the FW+ hens. In addition it appears that fecal SCFA yield may be a good representation of cecal SCFA production and therefore could provide a reasonable indicator for cecal fermentation. Furthermore, like SCFA the data suggested that the increased similarity trends observed in fecal and cecal samples demonstrated that DGGE fecal is consistent with cecal DGGE and could be used as a noninvasive indicator of cecal DGGE. Our observation in the study showed that DGGE can be used to visualize

microbial diversity of fecal and cecal samples before and after SE challenged during the time span of molting induction. Dietary trends at the initial stages of molt indicated minor inconsistencies but as molt progressed DGGE and SCFA for both fecal and cecal reflected consistencies with day 12 (cecal samples), where FF+ and ALC+ hens exhibited greater similarity and fermentation products compared to FW+ hens. A comparison of the SCFA responses and DGGE amplicon band patterns support the concept of alfalfa diet being able to sustain the compliment of microorganisms needed to inhibit *Salmonella* invasion.

CHAPTER VII

VIRULENCE GENE EXPRESSION IN *SALMONELLA* ENTERITIDIS CHALLENGED HENS FED ALFALFA CRUMBLE DIET

Introduction

The incidents of human gastroenteritis caused by *Salmonella* Enteritidis (SE) have been a major subject of tremendous concerns in the food animal industry (Mead, 2004). Feed withdrawal as a method of molting laying hens has been partially implicated in these incidents (Ricke, 2003a; Holt, 2003). To induce molting, feed withdrawal has been the primary method of choice to achieve a rapid and economical new egg-laying cycle (Brake, 1993; Holt, 1995). USDA-Animal and Plant Health Inspection Service (2000) stated that there was a doubling of environmental SE numbers in molted versus non-molted flocks. *Salmonella* infections in laying hens lead to the production of SE positive eggs after molt (Humphrey et al., 1993). While poultry is not the only source of SE transmission to humans, it is by and large the most common when the source of the infection can be traced, and eggs are the usual culprits (Patrick et al., 2004).

Feed withdrawal compromises the birds' immune system making them susceptible to infection by a number of microorganisms including *Salmonella* (Holt, 2003). Feed withdrawal associated stress causes increased susceptibility to SE infection in the gastrointestinal (GI) tract (Holt, 1993, 2003; Corrier et al., 1997; Durant et al., 1999; Ricke, 2003a) which is usually marked by increased intestinal shedding and colonization in the internal organs such as, the liver, spleen, and ovary (Holt, 1993; Holt et al., 1995; Thiagarajan et al., 1994). *Salmonella* has the capability to survive various environmental

stress situations, nutrient deprivation, osmotic shock, heat stress and pH changes (Foster and Spector, 1995), which significantly determine their survival and virulence potential (Archer, 1996). Stress conditions associated with dietary shifts such as molting elevate the level of *Salmonella* invasion of internal organs of poultry (Holt, 1993; Holt et al., 1995; Thiagarajan et al., 1994). The penetration of *Salmonella* pathogens in major tissues is essential for invasion and pathogenesis through the intestinal epithelium and further into the internal organs (Jones et al., 1994; Giannella et al., 1973). Genes required for *Salmonella* pathogenesis are located on *Salmonella* Pathogenicity Island 1 (SPI1) at centisome 63 on the chromosome (Galán and Sansonetti, 1996). HilA, a transcriptional activator encoded by SPI1, coordinately regulate the expression of invasion genes in *Salmonella* in response to environmental conditions including stress and low nutrients (Bajaj et al., 1996). *HilA* expression as a *hilA-lacZY* transcriptional operon fusion when grown in SE in crop contents of fully fed birds corresponded with *in vivo* response with gene expression in SE grown in filtered-sterile crop contents molted hens nearly doubled compared to the crop of molted hens (Durant et al., 1999). Therefore, a greater *hilA* expression could potentially have an impact on virulence and colonization of SE in the GI tract of chickens.

Alternative molting methods are known to reduce *Salmonella* invasion in the GI tract of experimental hens in poultry (Seo et al., 2001; Holt, 2003; Ricke, 2003a; Park et al., 2004; Woodward et al., 2005; McReynolds et al., 2005). Fermentable high fiber diets such as alfalfa have been examined as potential molting approaches that retain normal microbial flora and reduce proliferation of *Salmonella* (Woodward et al., 2005). When

fed in high concentration, alfalfa has been shown to decrease body weight and egg production in laying hens (Heywang, 1950; Mourão et al., 2006) as well as being instrumental in causing ovarian regression and restoration of optimal post egg production, when compared to molting via feed withdrawal (Donalson et al., 2005; Landers et al., 2005 a,b). The use of alfalfa as a single dietary source appears to support sufficient microbial fermentation (Hungate, 1966; Kass et al., 1980; Ricke, 2003a; Woodward et al., 2005) to restrict SE colonization (Woodward et al., 2005). The use of alfalfa crumble and pellets to feed layer hens has shown to derived benefits by increasing feed conversion ration of birds (Kilburn and Edwards, 2001). Furthermore, Nir et al. (1994b) remarked that alfalfa crumble has shown to be more suitable for chicken digestive tract compared to mash with uniform particle size. The objectives of this study were to enumerate SE colonization in fecal, cecal and internal organs and compare the level of virulence gene expression (*hilA*) of experimentally challenged laying hens fed molted different dietary regimen.

Materials and methods

Experimental design

Experiments 1 and 2 were conducted using Single Comb White Leghorn hens (SCWL) over 50 weeks old, obtained from a local commercial laying flock. The hens were placed in wire layer cages and were given free access to water and unmedicated corn-soybean meal based mashed layer ration that met National the Research Council recommendations for nutrients (1994). The alfalfa crumble diet used in the study was considered high in crude fiber (24.1%), with moderate crude protein (17.5%) and is low

in metabolizable energy (1200 kcal/kg; NRC, 1994). Feed samples and fecal samples (1 g) were collected and examined for salmonellae. Samples were cultured in tetrathioninate[1] broth and on brilliant green agar (Becton, Dickerson and Company, Sparks, MD) (BGA) plates as was previously described by Andrews et al. (1995). All the hens and feed used in both trials tested negative for *Salmonella.*

Trials 1 and 2

The hens were allowed to acclimatize in the cages for a period of two weeks. The hens were exposed to 8 h light: 16 h dark photo-period one week before changing the diets and removing feed from the FW hens. This light schedule continued for a 12 day period after which the experiment was terminated. Twelve (12) hens were randomly assigned to six treatment groups designated as followed; (1) feed withdrawal SE+ (FW+), (2) full fed SE+ (FF+), (3) 100% alfalfa crumble SE+ (ALC+), (4) feed withdrawal SE- (FW-), (5) full fed SE-, and (6) 100% alfalfa crumble SE- (ALC-). Treatment diets were applied to each treatment on d 1 of the molt, at the same time feed was removed from the feed withdrawal hens. Treatment diets were administered for 12 days, the period of time the FW hens were deprived of feed. Hens in all the treatment groups were given water *ad libitum*. On d 4 of the molt, all the hens in groups 1, 2 and 3 were challenged by crop gavage with 1 mL of inocula containing approximately 10^6 colony forming units (cfu) of nalidixic acid and novobiocin (NA : NO) (Sigma Aldrich Co., St. Louis, MO) resistant SE. Groups, 4, 5 and 6 were not challenged with SE. The SE positive and SE negative hens were placed in separate rooms. Footbaths were located at the doors of all the rooms in the facilities and non-infected hens were cared for before

the infected hens each day. At the conclusion of both trials, all the hens were euthanized and SE colonization was enumerated in the cecal contents, liver, spleen, ovaries. In addition, fecal (days 4 and 8) and cecal samples (necropsy at day 12) were collected post challenged from 5 hens in treatment group 1, 2 and 3 (SE+) for determination of *hilA* expression by PCR.

Bacterial strain

A primary poultry isolate of SE (phage type 13A) from the National Veterinary Services Laboratory (Ames, IA), selected for resistance to NO and NA in the USDA-ARS facility, College Station, TX, was used. The media used to culture the resistant isolate contained 25 µg of NO and 20 µg of NA per mL. The culture was prepared from an overnight culture previously transferred 3 times in trypticase soy broth (Becton, Dickerson and Company, Sparks, MD). The challenge inoculum was prepared by serially diluting the culture in sterile phosphate buffered saline (PBS) to a concentration of approximately 10^6 cfu per mL. The cfu of the challenge inoculum was confirmed by plating on BGA plates.

Necropsy

At the conclusion of both studies, hens were euthanized and the ceca, liver, spleen, and ovary were excised aseptically. Serial dilutions were preformed using 0.25 g of the cecal contents, then 100 µL from each dilution tube was placed onto a BGA plate containing NA and NO and spread plated using a bacterial cell spreader. Plates were incubated for 24 h at 37° C, and the number of cfu were determined and expressed as Log_{10} SE /g cecal contents. Samples of the ceca, liver, spleen and ovary of each hen

were cultured for SE. The organ samples were incubated for 24 h at 41°C in Rappaport-vassiliadis R10 broth (Difco Laborotaries, Detroit, MI). After incubation, the broth was streaked onto NA: NO BGA plates, incubated for an additional 24 h at 37°C. The next day the plates were examined for the presence of SE colonies, and were recorded as either SE negative or SE positive.

Immunomagnetic separation of SE using dynabeads

Anti-*Salmonella* dynabeads (Dynal Biotech ASA, Oslo, Norway) were used in an immunomagnetic separation technique to remove SE from feces (Olsvik et al., 1994) for *hilA* detection by real-time PCR. Fecal and cecal samples were suspended into 1:2 wt/vol) ratio of RNAlater (Sigma Aldrich Co., St. Louis, MO) in Whirl-Pak filter bags (Nasco, Fort Atkinson, WI). Triplicate 1-mL portions were placed into 1.5-mL sterile microcentrifuge tubes with 20 μL of anti-*Salmonella* Dynabeads. Microcentrifuge tubes were transferred to a Dynal MPC-M rack (Dynal Biotech ASA, Oslo, Norway) and samples were processed according to the manufacture's instructions. Dynabeads-bacteria complexes were resuspended in 100 μL of RNAlater and frozen until RNA was extracted.

RNA extraction and primer design

RNA was extracted from the Dynabeads-SE complexes according to the manufactures' instructions (RNeasy Mini Kit, Qiagen, Valencia, CA) *Salmonella* RNA was subjected to reverse transcriptase-PCR (RT-PCR) to obtained cDNA. Primers were designed for *hilA* and 16S rRNA genes using sequences data obtained from the genBank website and optimized using primer express1.0 Software (Perkin-Elmer Applied,

Biosystems, Foster City, CA). Optimized sequences were processed on the NCBI website in order to determine their cross- reactivity with other species of bacteria. Primers used in this study are listed in Table 3 (McClelland et al., 2001).

Reverse transcriptase reaction

To obtain *Salmonella* cDNA for real time-PCR, reverse transcription (RT) reactions were performed using the reagents from the TaqMan OneStep RT-PCR Kit. Each reaction contained 10X RT-PCR buffer, 500 µM of dNTPs Mix, 20U/reaction RNase Inhibitor, 25 mM $MgCl_2$, 200 ng of RNA, 2.0 µL of each primer (2.5 µM), MultiScribe^{TM} RT 1.25/UµL and RNase-free water to a final volume of 20 µL. A positive (+) RT reaction was run in order to ensure proper procedure. The positive reaction contained all of the same components except that in place of RNA sample template a DNA sample supplied with the kit was used as the template. To determine whether RNA samples were contaminated with DNA, two negative (-) RT reactions were run on each RNA sample. One (-) RT reaction contained the same components as the (+) RT reactions, except it lacked the RNA sample template and contained more water to ensure that the final concentration of the remaining components remains the same. The other (-) RT reaction did not contain the RT enzyme to ensure that there was no DNA contamination. All one-step RT reactions were performed on a Gene Amp PCR System (Perkin Elmer, geneAmp PCR Systems, Wellesley, MA) under the following conditions: Incubation at 10 min at 25˚C, RT at 30 min at 45˚C, RT inactivation for 5

TABLE 3: Primer sequences used in real-time PCR reactions (McClelland et al., 2001)

hilA FWD	5’ TATCGCAGTATGCGCCCTT 3’
hilA REV	3’ TCGTAATGGTCACCGGCAG 5’
16srRNA FWD	5’ TGCGCGACCAGGCTAAA 3’
16srRNA REV	3’ TTCACCACTGGCAGGTATTAAGC 5’

min at 95 ^{o}C, three step cycling 1 min at 94˚C 4, 1 min at 53˚C and 1 min at 72˚C for 40 cycles. The samples were then held at 4˚C until the samples could be removed.

Real-time PCR reaction

Real-time PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City CA). Each 20 µL SYBR Green PCR reaction contained 1 µL cDNA, 0.2 µL of each primer, and 10 µL 1 X SYBR® Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City CA), 8.6 µL and PCR water. Thermal cycling conditions were as follows: 48^{o}C for 30 min, 95^{o}C for 10 min, and 40 repeats of 95^{o}C for 15 second and 60^{o}C for 1 min. The same (+) and (-) RT reactions were run for the real-time PCR as for RT reactions.

Gene analysis and expression

Real-time PCR reactions (Bustin, 2000; Orlando et al., 1998; Livak and Schmittgen, 2001; Pierson et al., 2003) were run to determine the expression of *Salmonella hilA*. All samples were run in triplicate. Data were analyzed using the relative quantification method ($2^{-\Delta\Delta CT}$), (Livak and Schmittgen, 2001), which describes the change in expression of the target gene (*hilA*) relative to the reference gene (16S rRNA) from an untreated SE control sample. Data were analyzed by averaging the C_T values (cycle at which each sample amplification curve crosses a specific threshold) for triplicate samples. The resulting ΔC_T value of the target gene (*hilA*) was determined by normalizing to the endogenous control gene 16S rRNA. These samples were subsequently subtracted from the 16S rRNA gene from the untreated SE control sample, prepared according to Fey et al. (2004). The resulting $\Delta\Delta C_T$ was used to calculate

relative expression using the formula $2^{-\Delta\Delta CT}$ (Guilietti et al., 2001; Livak and Schmittgen, 2001; Lehman and Kreipe, 2001).

Statistical analysis

Chi-square analysis was used to determine significant differences between treatment groups for incidences of SE colonization of the ceca, liver, spleen and ovary (Luginbuke and Schlotzhauer, 1987). Data from concentrations of SCFA and gene expression were analyzed using the one-way ANOVA subjected to linear regression using PC-SAS software (SAS Institute Inc., Cary, NC) (2001). Differences between means were determined using least square means and Tukey's honest significance test. Statistical variation was also estimated by the standard error of the mean. All statistical analyses were considered significant at $P < 0.05$.

Results and discussion

SE colonization of the cecal contents

In the present study we evaluated SE colonization in the ceca of molted hens fed alfalfa and feed withdrawal compared to fully fed hens. In both trials, based on chi-square analysis, there were no differences between ALC+ diet and FF+ control in ceca enrichment positives, however the hens in FW+ treatment yielded significantly ($P < 0.05$) higher infectivity rates of SE cfu per gram cecal contents (Figure 25). Trial 1 exhibited a 2 fold ($\log_{10}$ 1.99) SE reduction in colonization for ALC+ hens compared to FW+ ($\log_{10}$ 3.89) SE colonization. In trial 2 a 4 fold ($\log_{10}$ 1.29) SE reduction in colonization for ALC+ hens compared to FW+ ($\log_{10}$ 5.12) SE colonization. These results confirmed that the utility of alfalfa as a molting diet could be effective in

reducing SE colonization in the ceca. Since SE invasion in ceca ultimate mediates to internal organs this would subsequently reduce transfer of SE to the respective organs. In an alfalfa mash study Woodward et al. (2005), saw a 2-7 fold reduction among 4 trials in feed withdrawal hens compared to alfalfa treated hens. Durant et al. (1999) also observed in a 9 day molt induction that feed withdrawal exhibited a 3.5- and 5.5 fold increase SE colonization compared to fully fed hens. In an alfalfa meal and alfalfa combined with standard commercial layer diet molt induction study (McReynolds et al., 2006), a reduction ($P < 0.05$) of SE colonization in the ceca (Trial 1) was observed in the 100% alfalfa meal diet and 70 % alfalfa meal / 30% standard commercial layer diet treatment hens when compared with fully fed hens with Log_{10} values of 0.54, 0.44 and 2.82, respectively. This study (McReynolds et al., 2006) showed that alfalfa not only reduce SE colonization but also was successful in achieving the required physiological changes (weight loss, ovary regression and feed consumption) needed to complete molt induction. In a previous report (McReynolds et al., 2005), alfalfa combined with experimental chlorate product (ECP) and distilled water were used for molt induction, compared to non-fed hens. In non-fed hens given distilled water, 94% (Log_{10} 5.26) of ceca was colonized with SE compared to hens fed alfalfa with a 94% (Log_{10} 4.06) of SE colonization of ceca. However in hens fed alfalfa and ECP a highly significant ($P < 0.05$) SE reduction of 39% (Log_{10} 1.12) was observed in the ceca. It was suggested that a low alfalfa intake was the cause for lower protectively against SE in the ALD treatment group. However, it has been shown that alfalfa combined with ECP effectively reduces infectivity of SE in the ceca of molted hens (McReynolds et al., 2005).

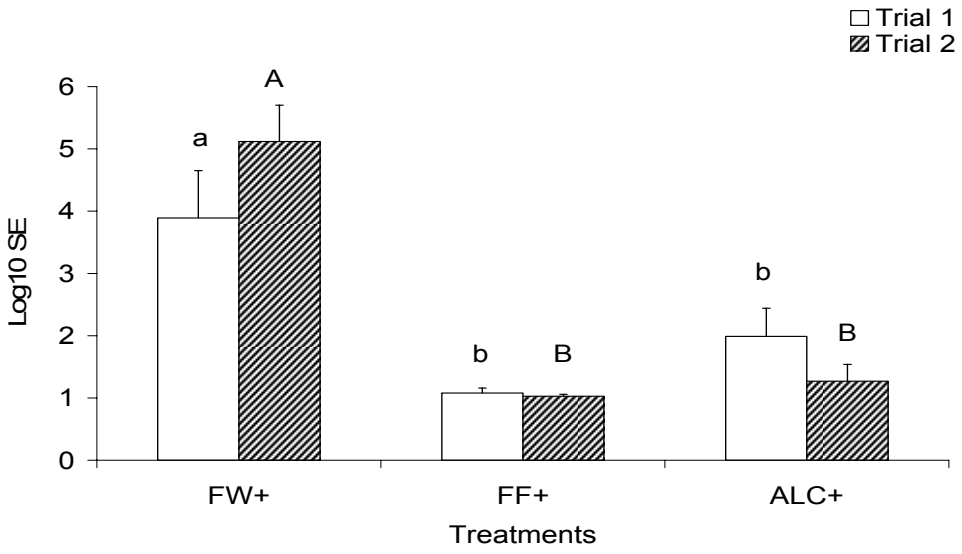


FIGURE 25: *Salmonella* Enteritidis (SE) colonization in the ceca of molted laying hens (Trials 1 and 2). [a-b]Mean values within Trial 1 with no common superscripts differ significantly ($P \leq 0.05$). [A-B]Mean values within Trial 1 with no common superscripts differ significantly ($P \leq 0.05$). FW+ = feed withdrawal challenged with SE. FF+ = Full fed challenged with SE. ALC+ = alfalfa crumble challenged with SE SE+ve

SE colonization in the organs

When the SE colonized the gut, it replicates and disseminates to various organs in the body including but not exclusive to the liver, the spleen and the ovaries (Gast and Beard, 1990; Shivaprasad et al., 1990, Durant et al., 1999). The result of SE colonization in the liver, spleen and ovaries are shown Table 4. In both trials all non-challenged birds were SE negative (data not shown). In Trial 1 of this study, the ALC+ treatment group showed numerically lower SE colonization in their ovaries 8% (1/12), spleen 17% (2/12), and liver 25% (3/12), compared to FW+ hens, (ovaries 33% (4/12), spleen 33% (4/12), and liver 50% (6/12), but these differences were not statistically significant ($P \geq 0.05$). In trial 2 the FW+ hens exhibited 42% SE infectivity rate in the liver, and 63% of the hens were positive in the spleen. In that same trial ALC+ hens exhibited 10% of the SE+ in the spleen and 15% SE+ in the liver. This could be an indication that the alfalfa crumble diet effectively reduced the incidence of colonization in these organs. The ALC+ treatment group exhibited a 25% infectivity rate of SE in the ovaries, compared to 55% in ovaries in FW+ hens; these were not statistically different ($P < 0.05$).

Similar results were observed by Woodward et al. (2005) when they used alfalfa meal to induce a molt. In a 9 day molt induction study (Durant et al., 1999) colonization of SE in spleen and liver exhibited a 5- and 6 fold increase in feed withdrawal hens respectively, compared to fully fed hens while we saw a 4- and 6 fold increased in spleen and liver in Trial 1 and 12- to 2 fold increase (Trail 2) in Feed withdrawal hens. Moore et al. (2004) also observed similar results when they compared the SE colonization in the organs of molting hen to that of fully fed hens. Of the 3 trials conducted (Moore et al.,

2004), Trial 1 exhibited the highest significant differences in non-molted (liver: 0%; spleen: 0%; ovary: 16.7%) compared to feed withdrawal hens (liver: 67.7%; spleen: 58.3%; ovary: 83.3%). In an alfalfa meal and alfalfa combined with standard commercial layer diet molt induction study (McReynolds et al., 2006), results from microbial cultures from internal organs indicated that all 3 treatment groups significantly reduce ($P < 0.05$) SE colonization in liver in the 100% alfalfa (not infected), 90% alfalfa + 10% standard commercial layer diet (ALF90) (3.4 fold reduction), and 70% alfalfa + 30% standard commercial layer diet (ALF70) (3.4 fold reduction) compared to feed withdrawal hens. Furthermore, cultures from spleen also showed a significant reduction on the ALF70 hens and SE was also reduced in the ovaries after birds were fed with ALF70 and ALF90. It is essential to control incidence of *Salmonella* in the poultry facilities since it has been shown that infections have been transmitted vertically to subsequent flock, which tend to prolong contamination (Humphrey, 1999). An effort to reduce the incidence of *Salmonella* contamination has shown to decrease the transovarian method of table eggs.

In this study colonization of SE in ceca, liver, spleen and ovaries exhibited similar trends in both trials for the 3 treatment groups (FF+, ALC+ and FW+). For all trials internal organs were significant higher in FW+ hens, compared to FF+ and ALC+ hens, similar results were also observed in cecal contamination. Studies have shown that feed withdrawal induced molts, significantly compromises the immune system (Holt, 1992a; 1992b) making laying hens susceptible to pathogens including *Salmonella* spp. It was reported that molted hens, which shed significantly more SE in their feces (Holt, 1993;

TABLE 4: Effects of alfalfa diet on colonization of *Salmonella* Enteritidis (SE) in the organs of molted laying hens (Trials 1 and 2). FW+ = feed withdrawal challenged with SE. FF+ = Full fed challenged with SE, ALC+ = alfalfa crumble SE+ve. Values represent the mean of 12 or 20 hens per treatment. A significant difference was found between the number of positive controls and positive internal organs ($^{*}P \leq 0.05$) Significant differences were seen between the number of and positive internal organs ($^{**}P \leq 0.001$).

Treatment[1]	**Trial 1**			**Trial 2**		
	SE Culture Positive Hens[3]			**SE Culture Positive Hens**[3]		
	Liver	Spleen	Ovaries	Liver	Spleen	Ovaries
FW+	6/12	4/12	4/12	8/19	12/19	9/19
FF+	0/12*	0/12	0/12	4/20	1/20**	0/20**
AL+	3/12	2/12	1/12	3/20	2/20**	5/20

Holt et al., 1995), normally show much higher levels of SE invasion in their internal organs including liver, spleen and ovaries (Thiagarajan et al., 1994; Holt et al., 1995; Durant et al., 1999). Holt (1992a) observed that molt by way of feed deprivation significantly depressed the cell-mediated immune response in the hens. In another study, Holt (1992b) demonstrated by flow cytometric analysis of peripheral blood from molted and un-molted hens that the CD4+ cells, the helper T-cell subset, are significantly decreased in molted hens.

HilA response

The relative expression of *Salmonella hilA* genes was examined from fecal (Days 6 and 11) and cecal contents (Day 12 necropsy) of post SE-challenged molted hens. Molt was induced by total FW+ and was used in comparison to hens given ALC+ and hens fed on FF+ as a control (Figure 26). Relative expression of *hilA* was significantly higher ($P < 0.05$) in all days in Trial 2 compared to Trial 1. There was no significant ($P < 0.05$) difference in *hilA* expression between fecal and cecal contents. However, for both trials, fecal and cecal *Salmonella hil*A expression in FW+ hens was significantly ($P < 0.05$) higher than in the ALC+ and FF+ hens. However, there were no significant differences ($P < 0.05$) between *hil*A expression in the ALC+ group compared to FF+ hens. In Trial 2, *hilA* expression in FW+ hens was 3.2-, 4.2-, and 1.9-fold higher for Days 6, 11 and 12 respectively (Figures 26b, d and f), when compared with to alfalfa-molted hens. The results reported here suggest that changes that occur in the ceca during feed withdrawal that could contribute to the survival of SE and enhance virulence and invasiveness in molted hens. In Trial 1, *hilA* expression in the ceca for FW+ hens was nearly 3 fold

higher compared to FF+ hens, also FW+ hens indicated a 2 fold higher *hilA* expression compared to ALC+ hens. These corresponded with 4 fold increased in SE colonization in the ceca of FW+ hens and a 2 fold increase in ALC+ hens. Furthermore, a 6-, 4- and 4 fold increased in SE infections in liver, spleen and ovaries respectively, compared to FF+ hens. In Trial 2, *hilA* expression in the ceca for FW+ hens was 2.3 fold higher compared to FF+ hens, also FW+ hens indicated a nearly 2 fold higher *hilA* expression compared to ALC+ hens. These corresponded with 5 fold increased in SE colonization in the ceca of FW+ hens and a 3.6 fold increase in ALC+ hens. Furthermore, a 2-, 12- and 9 fold increased in SE infections in liver, spleen and ovaries respectively, compared to FF+ hens. The trends in both trials could suggest that *hilA* expression in ceca paralleled those in SE colonization trends in ceca, liver, spleen and ovaries and could be a good indicator of SE colonization and virulence. Except for day 4 in fecal samples, *hilA* expression appears to exhibit similar patterns when noticed in fecal samples (Trial 2 day 4 and both trials day 11) and could also be an indicator of colonization of internal organs. This corresponds to previous *in vitro* studies by Durant et al., (1999) utilizing sterile crop contents to incubate *Salmonella*, where they observed that *hilA* expression was nearly doubled in crop contents from hens undergoing feed withdrawal compared to crop from fully fed hens, and corresponded to a 5- to 6-fold increase in SE colonization in the spleen-liver on molted hens to those of fully fed hens. In addition, there was a 3.5- to 5.5-fold increase of *Salmonella* colonization in the ceca of feed withdrawal birds compared to non-molted hens.

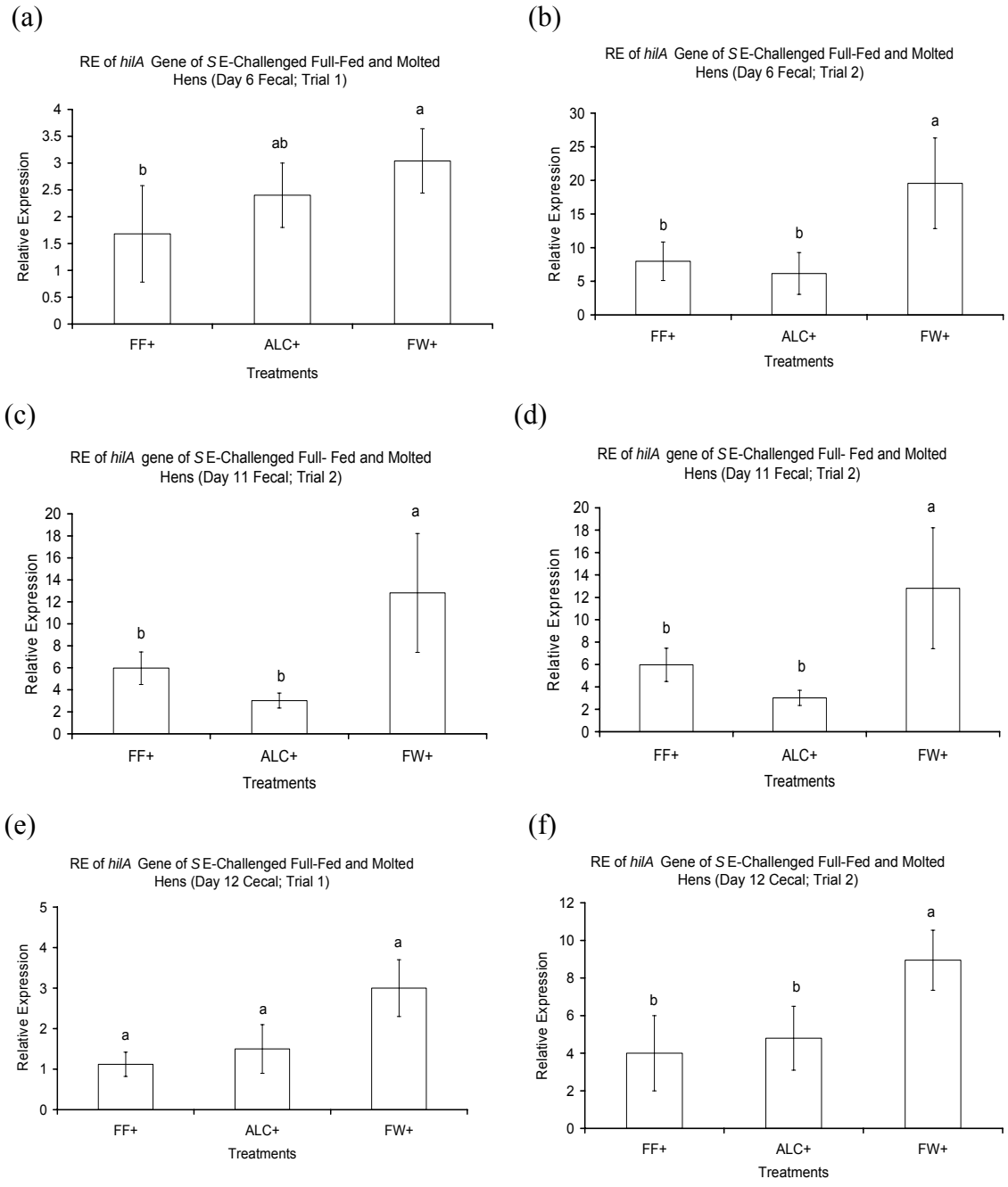


FIGURE 26: Relative expression of *hilA* in *Salmonella* Enteritidis challenged full fed (non-molted) and molted hens. (a) Day 6 fecal samples, Trial 1. (b) Day 6 fecal samples, Trial 2. (c) Day 11 fecal samples, Trial 1. (d) Day 11 fecal samples, Trial 2. (e) Day 12 cecal content, Trial 1. (f) Day 12 cecal contents, Trial 2. Error bars indicate standard error of the mean. $^{a\text{-}b}$Means within Trial 1 without a common letter differ significantly *(P* < 0.05). $^{a\text{-}b}$Means within Trial 2 without a common letter differ significantly *(P* < 0.05). FF+ = full-fed (non-molted). ALC+ = alfalfa. FW+ = feed withdrawal.

It has been demonstrated that molted hens shed significantly more SE in their feces (Holt, 1993; Holt et al., 1995), and generally exhibit much higher levels of SE survival and invasion in their internal organs (Holt et al., 1995). The quantitation of *hilA* expression should be a reasonable indicator of the level of *Salmonella* virulence in the GI tract and internal organs of post challenged hens (Durant et al., 1999; Ricke, 2003a). When Durant et al. (2000) examined the effects of pH, carbon source, amino acid addition and lactate on the expression of *hilA* in *Salmonella* in LB (Luria Bertani) and DLB (Diluted Luria Bertani) broths, *hilA* expression was nearly 3 fold higher in DLB (lower nutrients concentration) and addition of 2 % glucose, fructose and mannose reduced *hilA* expression 1.5 fold. The comparatively low *hilA* expression in ALC+ diet, which paralleled those in FF+ diet, suggests that alfalfa could be employed as a molt ration to minimize SE invasiveness in forced-molt hens. Alfalfa as a dietary fiber may affect the GI tract by supporting microbial activities, decreasing rate of passage, metabolites and digestive efficacy (Bach Knudsen, 2001; Wenk, 2001). Recently, certain dietary components including polysaccharides were identified as having the potential to be utilized as prebiotics (Verstegen and Schaafsma, 1999; Cummings and MacFarlane, 2002). The effectiveness of the ALC+ diet to reduce cecal *Salmonella* colonization may rely on its ability to retain a normal microflora in the intestines (Barnes et al., 1980; Corrier et al., 1995; Nisbet et al., 1994; Nurmi and Rantala, 1973; Van der Wielen et al., 2001, 2002; Woodward et al., 2005).

The result of the current study could further improve our understanding related to changes that occurred in the GI tract ecology such as those created during feed

deprivation. Consequently, alfalfa as a molt induction diet could minimize SE colonization, virulence and susceptibility in molted hens. Our data indicated no difference in SE colonization in the ceca of ALC+ hens compared to FF+ control hens, however the hens in FW+ treatment yielded significantly ($P < 0.05$) higher infectivity rates of SE cfu per gram cecal contents. In both trials *Salmonella* colonization in liver, spleen and ovaries were significant ($P < 0.05$) higher in FW+ hens compared to ALC+ and FF+ treatments groups. The significant increases in *hilA* expression in FW+ is an important indication of SE survival and could influence its capability to migrate from the GI tract to the internal organs. Our study revealed that *hilA* expression was 2.2-fold higher when isolated from cecal contents of FW+ hens (Trial 2) compared to cecal contents of FF+ hens, which corresponded to a 2-, 12- and 9-fold increase in SE colonization in the liver, spleen and ovaries, respectively. Furthermore, *hilA* expression was nearly doubled in cecal contents of FW+ hens (Trial 2) compared to cecal contents of ALC+ hens, which corresponded to a 2.7-, 6- and 1.8-fold increase in SE colonization (FW+ hens) in the liver, spleen and ovaries, respectively. Similar trend in *hilA* expression trends were also observed in fecal contents of FW+ hens compared to FF+ and ALC+. In Trial 2, on day 8 *hilA* expression in fecal contents was 2.2- and 4.3-fold higher in FW+ hens compared to FF+ and ALC+ treatment groups. These comparisons shown in the present study could be an indicator which confirms that SE virulence may be imminent in GI tract microenvironment of feed deprived molted hens. Furthermore, *hilA* expression in ceca and fecal paralleled those of SE colonization trends in ceca, liver, spleen and ovaries and could be a good indicator of SE colonization and virulence.

The comparatively low *hilA* expression in ALC+ diet, which paralleled those in FF+ diet, may suggest that alfalfa can be employed as a molt ration to minimize SE invasiveness in the intestine of forced-molt hens. Based on these results the utility of alfalfa as an alternative molting approach seemed capable of retaining protective microflora and provide the desired fermentative capacity needed during molt to established resistance to enteropathogens such as *Salmonella*. In addition our observation may provide important implications for understanding the necessary steps needed for protection of molted hens against *Salmonella* infections.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

These studies investigated specific environmental stimuli encountered by *Salmonella* at various levels, in order to regulate their pathogenicity and opportunistic behavior in the gastrointestinal (GI) tract of chickens. Comparisons of growth rates, virulence responses, microbial shifts, infectivity rates and fermentative end products were examined to determine the potential ecological preferences of *Salmonella* during colonization, invasion and pathogenesis. *Salmonella* propagated in continuous culture (CC) under low and high dilution rates confirmed that ST was capable of colonizing a nutural environment under limiting glucose conditions. The results indicated that ST consumption of glucose was consistently proportional at low dilution which corresponded to increased ST cell protein concentrations. This suggests that ST is capable of surviving an environment that is exceptionally low in glucose. Therefore, dietary shifts in the GI tract microenvironment such as those created during feed withdrawal in molted hens could support ST invasion.

Relatively higher expression of *hilA* virulence gene was largely observed at low dilution rates, because these dilution rates were comparable to less optimal growth conditions it could be conferred that the potential of ST virulence may occur in the event of feed removal form birds. In addition, Ac: Prop ratio was approximately 10**:**1 at low and medium range dilution rates (0.0125 h^{-1} to 0.27 h^{-1}). This corresponded with the capacity of ST to increase ST cell protein from glucose utilization. This level of Ac:Prop ratio could be related to an increased metabolism which generate excess energy in the

form of ATP needed for bacterial growth. *Salmonella* incubated in CC at various pH shifts demonstrated that cell protein concentration, glucose utilization, Y_{ATP} and Ac:Prop ration were influenced by pH (6.14 to 7.41). These parameters increased and decreased consistently with a corresponding increase and decrease in pH. Relative expressions of *hilA* and *rpoS* were significantly higher at steady states and the highest pH in CC showed a comparatively higher *hilA* expression.

Salmonella virulence gene expression (*hilA*) in fecal and cecal samples of *Salmonella* Enteritidis-challenged laying hens molted on different dietary regimens could explain that changes in the GI tract microenvironment of molted hens, such as those created during feed removal, may encourage virulence and survival SE. The significant increase in *hilA* expression in FW+ hens is an important indication of SE survival. Furthermore, *hilA* expression in ceca and fecal paralleled those of SE colonization trends in ceca, liver, spleen and ovaries and could be a good indicator of SE colonization and virulence. However, the comparatively low *hilA* expression in ALC+ diet, which paralleled those in FF+ diet, may suggest that alfalfa can be employed as a molt ration to minimize SE invasiveness in the intestine of forced-molt hens.

The efficacy of cecal microorganisms of chickens to ferment various high fiber feed substrates (HFFS) was determined. It appears that cecal microorganisms were able to ferment HFFS to produce SCFA. SCFA such as acetate, propionate and butyrate are important to limiting *Salmonella* colonization and are believed to correlate with dietary fiber metabolism in ceca. It appeared that fecal SCFA yield may be a good representation of cecal SCFA production and therefore could provide a reasonable

indicator for cecal fermentation. Like SCFA, the data suggested that the increased percecntge similarity trends observed in fecal and cecal samples demonstrated that fecal DGGE was consistent with cecal DGGE and could be used as a noninvasive indicator of cecal DGGE. Microbial amplicon patterns from DGGE results indicate that the cecal microorganisms can be altered by changes in dietary regimens. These changes may be beneficial to the poultry industry, since feed regimen can be manipulated for required performances. The microbial population amplican band patterns in hens fed alfalfa during molt were comparable to those of full-fed hens demonstrating the utility of alfalfa as an alternative molting diet may be capable of retaining the complimentary number microflora in GI tract needed to resist *Salmonella* invasion.

More extensive use of the continuous culture model to simulate ST growing conditions might help to define specific parameters linked to ST virulence and pathogenesis, and therefore clarify some of the reasons for inconsistence and effectiveness in dealing with *Salmonella* colonization in the GI tract of chickens. Additionally, these *in vitro* results may further explain *Salmonella* colonization and invasion in the GI tract of chickens in response to pH and limiting nutrient conditions, furthermore these results could be beneficial in minimizing the effects of *Salmonella* colonization in the GI tract ecosystem.

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