

5-2020

Investigating the potential of Sodium Butyrate to Control Salmonella Enteritidis in Poultry

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Investigating the potential of Sodium Butyrate to Control *Salmonella* Enteritidis in Poultry

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

by

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ABSTRACT

Salmonella Enteritidis (SE) is a major poultry associated food borne pathogen that causes enteric illnesses in humans. Despite using various pre-harvest and post-harvest intervention strategies to reduce Salmonellosis, SE infection is still an extensive problem in the poultry industry with increased incidences since SE has developed multiple strategies to adapt in the chicken intestinal tract particularly in the ceca. Therefore, reducing SE in the intestine of chickens would reduce contamination of poultry derived foods and minimize the risk of human infection. Short chain fatty acids such as butyrate are microbial metabolites known to modulate inflammatory response. In this dissertation, the effect of sodium butyrate on the colonization of SE in primary chicken enterocytes and chicken macrophages was investigated. In addition, the effect of sodium butyrate on the proteome of primary chicken enterocytes and chicken macrophages cells infected with SE was investigated. A primary chicken enterocytes culture model was developed and the effect of sub-inhibitory concentrations (SICs) of sodium butyrate on SE adhesion and invasion of primary chicken enterocytes and chicken macrophages was determined. The effect of sodium butyrate on the expression of SE virulence genes and selected inflammatory genes in chicken macrophages challenged with SE was studied using RT-qPCR. Also, the tryptic peptides of sodium butyrate treated primary chicken enterocytes and chicken macrophages in the presence or absence of SE infection were analyzed using tandem-mass spectrometry and the differentially regulated proteins were evaluated. The two SICs of sodium butyrate against SE were 22 mM and 45 mM respectively. The two SICs of sodium butyrate reduced SE adhesion by ~ 1.7 Log colony forming unit (CFU)/mL and ~ 1.8 Log CFU/mL and invasion by ~ 2 Log CFU/mL and ~ 2.93 Log CFU/mL respectively in primary chicken enterocytes ($P < 0.05$). Sodium butyrate did not significantly affect the adhesion of SE to chicken macrophages. However, 45 mM sodium butyrate reduced invasion

by ~ 1.7 Log CFU/mL as compared to control ($P < 0.05$). The expression of inflammatory genes (*Il1 β* , *Il8* and *Mmp9*) was significantly downregulated by sodium butyrate as compared to control ($P < 0.05$). Proteomic analysis revealed that sodium butyrate significantly downregulated the expression of proteins involved in glycolysis and GTP binding proteins in uninfected chicken macrophages and primary chicken enterocytes. Sodium butyrate treatment in SE infected chicken macrophages downregulated proteins associated with actin cytoskeletal rearrangements as well as intracellular SE growth and replication. Additionally, sodium butyrate treatment in SE infected chicken macrophages upregulated protein modulating innate immune response and bacterial infection. Additionally, sodium butyrate in SE infected primary chicken enterocytes downregulated ($P < 0.05$) proteins involved in pro-inflammatory immune response and phagocytosis, intracellular trafficking of SE and host actin cytoskeleton rearrangements. In contrast, sodium butyrate treatment in SE infected cells upregulated ($P < 0.05$) protein involved in bacterial killing during pro-inflammatory signaling pathway. Collectively, sodium butyrate reduced proteins involved in glycolysis, pro-inflammatory immune response, host actin cytoskeleton changes and intracellular trafficking of SE. The results suggest that sodium butyrate could be utilized in reducing colonization of SE in chickens as a pre harvest intervention for potentially decreasing enteric illnesses in humans.

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ACKNOWLEDGEMENTS

I am immensely thankful to my daughter Shaila Bansal for providing me emotional support and love during my Ph.D. program.

I am indebted to my husband Mohit Bansal for always standing next to me in my arduous and good times, without his continuous support in my research and at home, this journey would not be completed.

I would like to express my deepest gratitude and sincere thanks to my major advisors and mentors Dr. Narayan C Rath and Dr. Xiaolun Sun for their excellent guidance, support and motivation throughout my Ph.D. program at University of Arkansas. Without them, this laborious journey of my Ph.D. would not have seen its final day. They both played a pivotal role in developing my scientific skills by sincerely participating in my research projects and raising different questions about science on a daily basis.

I am extremely grateful to my all committee members Dr. Annie Donoghue, Dr. Abhinav Upadhyay and Dr. Sami Dridi for providing me scientific direction, experiences and valuable advices that helped me immensely throughout my research.

I would like to thank Dr. Rohana Liyanage for his valuable support in mass spectrometry work of my research.

Special thanks and heartily appreciation go to my father, Vijay Kumar Gupta and my mother, Shashi Prabha Gupta for their unfailing care, support, prayer and sacrifice. My father was the only one, who always made me realize my potential and motivate me to achieve my goal. My mother was always there for me as an emotional support and her love and care always increase my confidence. I am also grateful to my sister Alisha Gupta and my brother Anmol Gupta for always keeping my spirit high.

I would like to extend my gratitude to Dr. Indu Upadhyaya, Dr. Komala Arsi, Dr. Basanta Wagle, and Dr. Sandip Shrestha for their valuable suggestions, guidance and help during my laboratory work and writing dissertation.

I would like to acknowledge Lara Baker for travel arrangements during my conferences. I am also grateful to Scott Zornes and Sonia Tsai for their technical support. I am thankful to whole USDA/ARS unit for providing me an opportunity to work with them and funding my research. I am also thankful to Graduate School for providing me bridge funding when I have no other source of funding available to support my living.

I would like to thank all of my friends Dr. Bhuvan Pathak, Shilpi Agrawal, Dr. Deepti Samarth, Sumana Venkat, Sipra Rath, Vishal Dhiman, Lisa Pineira, Laura Florick, Justin, and Breanna who helped me directly and indirectly in pursuing my goals.

At last not but least, I am whole-heartedly thankful to my God, who always built positive energy and courage in me by his unconditional blessings.

DEDICATION

This work is dedicated to my parents and my husband

Vijay Kumar Gupta, Shashi Prabha Gupta

and

Mohit Bansal

TABLE OF CONTENTS

I. Introduction/ Review of Literature	1
1.1 <i>SALMONELLA</i> AND FOOD SAFETY CONCERNS	2
1.2 EPIDEMIOLOGY OF SE AND ROUTE OF TRANSMISSION IN POULTRY	3
1.3 MECHANISM OF SE COLONIZATION AND PATHOGENESIS IN BROILERS.....	4
1.3.1 Initial colonization of intestinal epithelium.....	4
1.3.2 Invasion of intestinal epithelium	4
1.3.3 Survival of <i>Salmonella</i> in intestinal macrophages	5
1.3.4 Gene specific for SE colonization.....	5
1.4 CHICKENS AS A HOST OF <i>SALMONELLA</i>	6
1.5 SALMONELLOSIS IN HUMANS.....	8
1.6 SIGNIFICANCE OF PRE-HARVEST STRATEGIES FOR CONTROLLING SE IN POULTRY	9
1.7 GUT METABOLIC PRODUCTS.....	11
1.8 MECHANISM OF ACTION OF BUTYRATE	11
1.9 BUTYRATE IN CHICKENS.....	13
1.10 REFERENCES	15
II. Sodium butyrate as a potential anti-inflammatory compound to reduce <i>Salmonella</i> Enteritidis colonization in chickens	27
2.1 ABSTRACT	28
2.2 INTRODUCTION.....	30
2.3 MATERIAL AND METHODS	33
2.3.1 Primary chicken enterocytes cell culture.....	33
2.3.2 The effect of common chicken gut chemicals on morphology and viability of primary chicken enterocytes	34
2.3.3 Bacterial strains and culture conditions.....	35
2.3.4 Determination of sub-inhibitory concentrations of sodium butyrate	35
2.3.5 Effect of SICs of sodium butyrate on cell viability of primary chicken enterocytes and chicken macrophages	36
2.3.6 Effect of SICs of sodium butyrate on SE adhesion to and invasion of primary chicken enterocytes and chicken macrophages.....	36
2.3.7 Effect of SIC of sodium butyrate on the expression of virulence genes of SE.....	37

2.3.8	Effect of SICs of sodium butyrate on the expression of inflammatory genes of chicken macrophages challenged with SE.	38
2.3.9	Statistical Analysis	38
2.4	RESULTS.....	39
2.4.1	The effect of common chicken gut chemicals on morphology and viability of primary chicken enterocytes	39
2.4.2	Sub-inhibitory concentrations of sodium butyrate against SE	39
2.4.3	The effect of SICs of sodium butyrate on cell viability of primary chicken enterocytes and chicken macrophages	40
2.4.4	The effect of SICs of sodium butyrate on SE adhesion to and invasion of primary chicken enterocytes and chicken macrophages.....	40
2.4.5	The effect of sodium butyrate on the expression of SE virulence genes	41
2.4.6	The effect of sodium butyrate on the expression of inflammatory genes in chicken macrophages challenged with SE	41
2.5	DISCUSSION.....	42
2.6	CONCLUSIONS	45
2.7	REFERENCES	46

III. Butyrate influences the global protein expression of *Salmonella* Enteritidis infected chicken macrophages62

3.1	ABSTRACT	63
3.2	INTRODUCTION	65
3.3	MATERIALS AND METHODS	66
3.3.1	Chicken Macrophage cell line	66
3.3.2	Effect of sodium butyrate on viability of chicken macrophages	67
3.3.3	Bacterial strains and culture conditions.....	67
3.3.4	Proteomic sample collection, preparation and in-gel protein digestion.....	68
3.3.4.1	Mass spectrometry analysis	69
3.3.5	Statistical Analysis	70
3.4	RESULTS.....	70
3.4.1	Effect of sodium butyrate on the proteome of chicken macrophages.....	70
3.4.2	Effect of SE on the proteome of chicken macrophages.....	72
3.4.3	Effect of sodium butyrate on the proteome of chicken macrophages infected with SE	73
3.5	DISCUSSION.....	74
3.6	CONCLUSIONS	79
3.7	REFERENCES	81

IV.	Sodium butyrate modulates chicken intestinal epithelial proteins responsible for <i>Salmonella</i> Enteritidis colonization and replication	101
4.1	ABSTRACT	102
4.2	INTRODUCTION	104
4.3	MATERIALS AND METHODS	106
4.3.1	Primary chicken enterocyte cell culture	106
4.3.2	Effect of SIC of sodium butyrate on viability of primary chicken enterocytes	107
4.3.3	Bacterial strains and culture conditions	107
4.3.4	Proteomic sample collection, preparation and In-gel protein digestion	107
4.3.4.1	Mass spectrometry analysis	108
4.3.5	Statistical Analysis	110
4.4	RESULTS	110
4.4.1	Effect of sodium butyrate on the proteome of primary chicken enterocytes	110
4.4.2	Effect of SE on the proteome of primary chicken enterocytes	111
4.4.3	Effect of sodium butyrate on the proteome of primary chicken enterocytes infected with SE	112
4.5	DISCUSSION	113
4.6	CONCLUSIONS	117
4.7	REFERENCES	119
V.	CONCLUSIONS	135

LIST OF TABLES

Table 2.1	List of primers used for RT-qPCR analysis of SE genes.	53
Table 2.2	List of primers used for RT-qPCR analysis of host immune response genes	55
Table 3.1	Differentially regulated proteins in chicken macrophages by sodium butyrate (SB) treatment	87
Table 3.2	Pathways downregulated by SB treatment in chicken macrophages	89
Table 3.3	Pathways upregulated by SB treatment in chicken macrophages	89
Table 3.4	Differentially regulated proteins in chicken macrophages after <i>Salmonella</i> Enteritidis (SE) infection.....	90
Table 3.5	Pathways downregulated by SE infected chicken macrophages.....	92
Table 3.6	Pathways upregulated by SE infected chicken macrophages.....	92
Table 4.1	Differentially regulated proteins in primary chicken enterocytes by sodium butyrate (SB) treatment	124
Table 4.2	Pathways downregulated by SB treatment in primary chicken enterocytes.	125
Table 4.3	Differentially regulated proteins in primary chicken enterocytes after <i>Salmonella</i> Enteritidis (SE) infection.....	125
Table 4.4	Pathways downregulated by SE infected primary chicken enterocytes	126
Table 4.5	Pathways upregulated by SE infected primary chicken enterocytes	126
Table 4.6	Differentially regulated proteins by SB treatment in SE infected primary chicken enterocytes	127
Table 4.7	Pathways downregulated by SB treatment in SE infected primary chicken enterocytes.....	129
Table 4.8	Pathways upregulated by sodium butyrate treatment in SE infected primary chicken enterocytes	129

LIST OF FIGURES

- Figure 2.1 The effect of various chemicals commonly encountered in chicken gut environment on morphology of primary chicken enterocytes, Bar=30µm. **A:** Control **B:** Calcitriol **C:** Aflatoxin B₁ **D:** dbcAMP **E:** DON **F:** LPS **G:** Sodium butyrate **H:** PMA **I:** Trans Retinoic Acid **J:** Thiram (magnification ×100).56
- Figure 2.2 The effect of test chemicals on cell viability of primary chicken enterocytes. Sodium butyrate (SB) does not affect cell viability of primary chicken enterocytes. Data presented as mean absorbance and error bar represents SEM (n=6). Bar with different letters represents a statistical difference at $P<0.05$57
- Figure 2.3 The effect of various concentrations (0, 363, 181.5, 90.75, 45, 22 and 11 mM) of SB against SE for the determination of subinhibitory concentrations based on growth curve analysis ($P<0.05$).....58
- Figure 2.4 The effect of SICs of SB on cell viability of primary chicken enterocytes (A) and chicken macrophages (B) using MTT assay. Data presented as mean absorbance and error bar represents SEM (n=6). Bar with different letters represents a statistical difference at $P<0.05$58
- Figure 2.5 The effect of SICs of SB on SE adhesion (A, C) to and invasion (B, D) of primary chicken enterocytes (A, B) and chicken macrophages (C, D). Data presented as mean Log CFU/mL and error bars represents SEM (n=6). Bars with different letters represents a significant difference at $P<0.05$59
- Figure 2.6 The effect of SIC 45 mM of SB on the expression of SE genes crucial for virulence and intestinal colonization. Data presented as relative fold change normalized to endogenous control, *16S* rRNA. *indicates significant change in the expression of genes at $P<0.05$60
- Figure 2.7 The effect of SICs (22 mM and 45 mM) of SB on the expression of inflammatory genes [*Il1b* (A), *Il8* (B), *Mmp9* (C)] in chicken macrophages challenged with SE. Data presented as fold change normalized to *Gapdh* and error bars represents SEM (n=6). Bars with different letters represents a significant difference at $P<0.05$61
- Figure 3.1 Effect of SIC of sodium butyrate (SB) 45 mM on viability of chicken macrophages using MTT assay. Chicken macrophages were treated with SB at 45 mM for 4 h. Data presented as mean absorbance and error bar represents SEM (n=6). Bar with different letters represents a statistical difference at $P<0.05$93

Figure 3.2	SB treatment in chicken macrophages induced down and upregulated proteins in different biological processes	93
Figure 3.3	Signaling pathway analysis by STRING showed pathways downregulated by treatment of SB in chicken macrophages.....	94
Figure 3.4	Signaling pathway analysis by STRING showed pathways upregulated by treatment of SB in chicken macrophages	95
Figure 3.5	<i>Salmonella</i> Enteritidis (SE) infection in chicken macrophages induced down and upregulated proteins in different biological processes	96
Figure 3.6	Signaling pathway analysis by STRING showed pathways downregulated by SE infection in chicken macrophages.....	97
Figure 3.7	Signaling pathway analysis by STRING showed pathways upregulated by SE infection in chicken macrophages	98
Figure 3.8	SB treatment in SE infected chicken macrophages induced down and upregulated proteins in different biological processes	98
Figure 3.9	Signaling pathway analysis by STRING showed pathways downregulated by SB treatment in SE infected chicken macrophages.....	99
Figure 3.10	Signaling pathway analysis by STRING showed pathways upregulated by SB treatment in SE infected chicken macrophages.....	100
Figure 4.1	The effect of SIC of sodium butyrate (SB) 45 mM on cell viability of primary chicken enterocytes using MTT assay. Primary chicken enterocytes were treated with SB at 45 mM for 4 h. Data presented as mean absorbance and error bar represents SEM (n=6). Bar with different letters represents a statistical difference at $P<0.05$	130
Figure 4.2	SB treatment in primary chicken enterocytes induced down and upregulated proteins in different biological processes	130
Figure 4.3	Signaling pathway analysis by STRING showed pathways downregulated by treatment of SB in primary chicken enterocytes	131
Figure 4.4	Signaling pathway analysis by STRING showed pathways upregulated by treatment of SB in primary chicken enterocytes	131
Figure 4.5	<i>Salmonella</i> Enteritidis (SE) infection in primary chicken enterocytes induced down and upregulated proteins in different biological processes	132
Figure 4.6	Signaling pathway analysis by STRING showed pathways downregulated by SE infection in primary chicken enterocytes.....	132

Figure 4.7	Signaling pathway analysis by STRING showed pathways upregulated by SE infection in primary chicken enterocytes	133
Figure 4.8	SB treatment in SE infected primary chicken enterocytes induced down and upregulated proteins in different biological processes	133
Figure 4.9	Signaling pathway analysis by STRING showed pathways downregulated by SB treatment in SE infected primary chicken enterocytes	134
Figure 4.10	Signaling pathway analysis by STRING showed pathways upregulated by SB treatment in SE infected primary chicken enterocytes	134

CHAPTER I

Introduction/ Review of Literature

1.1 *SALMONELLA* AND FOOD SAFETY CONCERNS

Salmonella is a major food borne pathogen that causes severe gastroenteritis in humans resulting in 1.35 million illnesses, 26,500 hospitalizations, 420 deaths, and an annual economic loss of \$4.43 billion every year in the United States (CDC, 2019). Contaminated poultry meat and its byproducts, including raw and uncooked eggs are the major sources of *Salmonella* enterica serotype Enteritidis (SE) infection in humans (Kollanoor-Johny et al., 2012). Per capita consumption of poultry meat, poultry byproducts, and eggs, which comprise a major portion of American's diet, has significantly increased from 1960 to forecast 2020 (National Chicken Council, 2019), therefore, microbiological safety of these foods are of paramount importance. In 2018, the incidence of *Salmonella* infection, 18.3 cases per 100,000 population, has increased by ~9% as compared to 2015-2017 average annual data (Marder et al., 2018). According to a U.S. Centers for Disease Control and Prevention (CDC) reports multistate *Salmonella* outbreaks linked with backyard poultry resulted in 1003 infection from 49 states with 175 hospitalizations and 2 deaths (CDC, 2018).

SE is a major zoonotic pathogen causing food-borne zoonoses from animals to humans worldwide (Antunes et al., 2016). It is a facultative intracellular enteropathogenic anaerobe that colonizes the gastrointestinal tract and has a broad host range such as chickens, turkeys, swine, cattle and other domestic and wild animals (CDC, 2006; Heredia and García, 2018). In the United States, the incidences of SE infections have not decreased from last decade (McNew et al., 2011); in fact, SE has adapted to live inside poultry without showing any clinical signs (Kollanoor-Johny et al., 2012; Marder et al., 2018) and spread of the pathogen in environment, which transmits infection to humans and further increases food safety concerns. Salmonellosis symptoms are mainly characterized by self-limiting gastroenteritis such as diarrhea, fever and abdominal cramps

with an incubation of 4-72 h after infection and illness lasts for 2-7 days (Antunes et al., 2016). In addition, severe infections can occur in young children, elderly and immunocompromised people, which requires immediate treatment (CDC, 2019).

Nair et al. (2018) suggest that 100,000 infections out of 1.2 million illnesses annually in the United States are due to antibiotic resistance of *Salmonella* against ceftriaxone and ciprofloxacin that results in 33,000 and 36,000 illnesses respectively. In addition, recent CDC report suggests that 5% non-typhoidal *Salmonella* strains are resistance to five or more classes of antibiotics, which further increases food safety concerns (CDC, 2013). Therefore, it is very important to reduce food borne Salmonellosis in poultry industry and develop noble methods for alternative to antibiotics.

1.2 EPIDEMIOLOGY OF SE AND ROUTE OF TRANSMISSION IN POULTRY

Chickens serve as the primary reservoir host of SE infection and can disseminate the pathogen in the environment without showing obvious clinical signs (Bakshi et al., 2003; CDC, 2006). The SE transmits in chickens by two possible routes: horizontal and vertical transmission (Gantois et al., 2009; Upadhyaya et al., 2015). SE mainly colonizes caecum of gastro-intestinal tract in chickens resulting in horizontal transmission of pathogen with contamination of eggshell along with feces and carcass during slaughter and poultry processing (Keller et al., 1995). The colonization of SE in the ceca of chickens also leads to vertical transmission of the pathogen by trans-ovarian route, which causes contamination of egg yolk, albumen and shell membranes (De Reu et al., 2006; Gantois et al., 2009).

1.3 MECHANISM OF SE COLONIZATION AND PATHOGENESIS IN BROILERS

1.3.1 Initial colonization of intestinal epithelium

Chickens disseminate SE to the environment by acting as asymptomatic carriers for the pathogen (Kollanoor-Johny et al., 2012, Upadhyaya et al., 2015). SE predominantly colonizes the ceca of chickens thereby leading to contamination of carcasses during slaughter. Despite the presence of significant defensive barriers in intestine, *Salmonella* has developed several strategies to colonize the gastrointestinal tract and penetrated the intestinal epithelium of the host, which has altered host cell physiology (Lhocine et al., 2015).

1.3.2 Invasion of intestinal epithelium

Salmonella interacts with the host by expressing an array of bacterial proteins, which help in its invasion of intestinal epithelium (Lhocine et al., 2015). Once SE attaches to the intestinal epithelium, it uses the Type III Secretion System (T3SS) for its pathogenesis and colonization of host intestinal tissues. SE encodes two different *Salmonella* pathogenicity islands I (SPI-1) and II (SPI-2), associated with T3SS that function at distinct times during infection (Haraga et al., 2008). The SPI-1 associated with T3SS transports bacterial effector proteins that helps in invasion of bacteria through the plasma membrane and results in induction of inflammatory response in the intestine (Ly and Casanova, 2007; Wemyss and Pearson, 2019). SPI-1 plays a crucial role in colonization and invasion of *Salmonella* in the host intestinal epithelial cells to induce the production of inflammatory cytokines and antimicrobial peptides such as *IL-1 β* , *IL-12*, *IL-18*, and α -defensins and cathelicidins along with activation of macrophages and recruitment of neutrophils (Gart et al., 2016). Therefore, SE colonization in host intestine

competes with healthy gut microbiota and results in an inflammatory immune response for its pathogenesis (Gart et al., 2016).

1.3.3 Survival of *Salmonella* in intestinal macrophages

After internalization into the host intestinal epithelial cells, SE survives and replicates in an acidic modified phagosome called as *Salmonella*-containing vacuole (SCV) (Larock et al., 2015; Wemyss and Pearson, 2019). Once *Salmonella* internalizes into host cells, it encodes SPI-2 that plays a potential role in causing systematic infection and intracellular pathogenesis in the host (Foley et al., 2013). The SPI-2 associated with T3SS transports effector proteins (SifA, SopD2 and SseJ) helps in intracellular survival of bacteria in macrophages and the establishment of systemic disease (Haraga et al., 2008; Foley et al., 2013). *Salmonella* interferes with NADPH oxidase complex inside the phagocytic macrophages, which prevents superoxide production, thus, allowing the bacterium to survive inside the macrophages.

1.3.4 Gene specific for SE colonization

Previous literatures show colonization of SE in the gastrointestinal tract of host resulting in the secretion of effector proteins, which leads to the alteration in host cells physiology and cytoskeletal changes, inflammatory response, membrane ruffling and autophagy. After entering gastro-intestinal tract of host, SE uses its flagella to access the epithelial layer of the small intestine and fimbriae for initial cell attachment (Gart et al., 2016; Wemyss and Pearson, 2019), and uses adhesion proteins such as SiiE and BapA that allow the bacteria to adhere firmly to the intestinal epithelium (Fàbrega and Vila, 2013). The T3SS encoded on SPI-1 helps to transfer an array of bacterial effector proteins such as SopE2, SipA, SopA, SopB, SopE2, and SopD from bacterial cell into the cytoplasm of host cell causing actin rearrangement, membrane ruffling,

non-phagocytic cellular uptake of bacteria into the host cell membrane and internalization of bacteria in a membrane bound compartment such as *Salmonella* containing vacuole (SCV) (Ly and Casanova, 2007; Wemyss and Pearson, 2019). SE causes localized inflammation in the intestinal tract of host for its pathogenesis inside the host. Bacterial effector proteins SopE2, SopE and SopB activates Rho GTPases which induce mitogen activated protein kinase (MAPK) pathways including the ERK, JNK and p38 pathways to further activate nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) leading to the production of pro-inflammatory cytokine such as interleukin-8 (IL-8) which contribute to intestinal inflammation. In addition, Rho GTPase activates Caspase 1 in macrophages with the release of cytokines such as IL-1 β and IL-18 which further induce inflammatory response in the intestinal mucosa (Haraga et al., 2008; Wemyss and Pearson, 2019). SipA activates caspase 3 in host cells and promotes polymorphonuclear leukocytes migration across the intestinal epithelium and plays a direct role in inflammation (Larock et al., 2015). However, the SPI-2 associated with T3SS transports effector proteins (SifA, SopD2, PipB and SseJ) help bacterial survival in macrophages and to the establishment of systemic disease (Haraga et al., 2008).

1.4 CHICKENS AS A HOST OF *SALMONELLA*

SE has been known as the most common genetically homogeneous serotype among all *Salmonella* serovars (Shah et al., 2011). Although there are few genomic diversities, the clinical isolates of the *Salmonella* serotype have the potential to vary in their growth characteristics, lipopolysaccharide (LPS) production, and survival through vertical and horizontal transmission inside egg albumen (Clavijo et al., 2006; Jain and Chen, 2007; Yim et al., 2010). SE isolates show great variation in virulence potential in chickens to cause mortality and colonize in the intestinal tract resulting in systemic spread of diseases in the vital organs such as liver and spleen

and subsequently colonize reproductive tract of chickens (Gast and Benson, 1995). Previous reports concerning on-farm investigation of chickens showed that if one chicken in the farm is exposed to SE, the whole chickens flock can be at risk of colonization of the pathogen (Berrang et al., 1997; Foley et al., 2008). This is due to *Salmonella*'s ability to rapidly colonize the gastrointestinal tract of chickens (G C Mead, 2005).

Shah et al. (2011) reported that SE is invasive in young and adult chickens. In young chickens, SE infection leads to systemic disease (Duchet-Suchaux et al., 1995; Castillo-Olivares and Wood, 2004). The infected chicks show symptoms of anorexia, depression, ruffled feathers, huddling together, reluctance to move, drowsiness, dehydration, white diarrhea, stained and pasted vents, and stunted growth (McIlroy et al., 1989). However, adult chickens after SE colonizes in their intestine, they act as asymptomatic carriers and, persistently sheds the pathogen in the environment (Castillo-Olivares and Wood, 2004; Golden et al., 2008). Severe infection of SE in chickens results in clinical salmonellosis with severe mortality; however, less severe infection of SE does not results in any clinical signs and the birds remain healthy acting as carriers (Gast and Benson, 1995; Desmidt et al., 1997; Immerseel et al., 2004).

For initiating a successful microhabitat in chickens, SE should control its hostile strategies to colonize chickens and adapt at different temperatures, oxidation-reduction potentials, organic and inorganic nutrient environments, anti-microbial substances and host immune response (Slauch et al., 1997). SE colonize the intestinal tract of host by its virulence properties such as motility, adhesion and invasion of intestinal epithelial cells of host, which are critical factors for its perseverance inside the host. SE is a virulent pathogen that use various mechanisms such as resistance against lytic action of complement (Ho et al., 2011), increased activity of siderophores (Holden and Bachman, 2015), virulence plasmid (Silva et al., 2017) and

antimicrobial resistance (Nair et al., 2018). However, SE has to adapt new strategies to survive inside the host and avoid encounter with normal flora of the chicken gut (Mon et al., 2015). SE tends to colonize in the intestinal mucosa and utilizes nutrients and oxygen for its survival, which reduces the possibility of colonization of obligate anaerobes inside the host (Lawley and Walker, 2013). Likewise, *Salmonella* has been reported for utilizing an unusual source of carbon such as gluconate during the colonization process in the host (Dandekar et al., 2012; Steeb et al., 2013; Diacovich et al., 2017). Therefore, SE persistence in the chickens is very complicated and involves regulation of various genetic loci particularly those required for nutrition metabolism, virulence and stress tolerance (Potts et al., 2019).

1.5 SALMONELLOSIS IN HUMANS

SE infection in humans is manifested by fever, nausea, vomiting, headache, diarrhea (can be bloody), abdominal cramps and is mostly self-limiting. The incubation period of the disease typically ranges from 12 to 72 h, symptoms usually started within 6 h to 6 days after infection and lasts for 4 to 7 days (Roth, 2013). Patients usually recover from infection within a week without any antibiotic treatment, except in extreme cases of severe diarrhea where intravenous fluid therapy is prescribed. However, antibiotic resistant strains of SE cause severe illness and required treatment for a longer period of time (Crum-Cianflone, 2008; Kim et al., 2019).

Affected populations such as infants, children, the elderly and immuno-compromised people are more inclined to the serious outcomes characterized by invasive disease resulting in bacteremia and sometimes death (Skovgaard, 2004). Likewise, some patients can become susceptible to chronic reactive arthritis, osteoarthritis, appendicitis, meningitis, and peritonitis (Skovgaard, 2004; Smoot and Cordier, 2009). Also, SE has been indicated as an activating factor for reactive arthritis and Reiter's syndrome in humans (Dworkin et al., 2001; Chun et al., 2011). In addition,

SE has been also reported to cause extra-intestinal infections such as urinary tract pathologies in humans (Ramos et al., 1996; Sirinavin et al., 1999; Huang and DuPont, 2005; Abbott et al., 2012; Chen et al., 2014; Klosterman, 2014).

1.6 SIGNIFICANCE OF PRE-HARVEST STRATEGIES FOR CONTROLLING SE IN POULTRY

Preharvest intervention strategies associated with food safety for poultry production is a standardized approach to reduce SE transmission from hatcheries and production farms to their transmission inside host (Foley et al., 2011; Alali and Hofacre, 2016). The cecal colonization of SE leads to horizontal transmission of pathogen, due to contamination of eggshells with feces and contamination of chicken carcass during slaughter. In addition, the cecal colonization of SE in chickens also results in contamination of eggs by trans-ovarian route of transmission (Thiagarajan et al., 1994; Gantois et al., 2009; Cox et al., 2012; Dawoud, 2015; Pande et al., 2016). SE may also spread to chickens after oral ingestion of the pathogen from environmental sources through contaminated equipment, feed, water, and insects. That results in contamination of eggs by colonization of ovary and oviducts but the detection of SE in eggs can be extremely difficult until the bacterial count reaches $>10^7$ CFU (Upadhyaya, 2015; Akil and Ahmad, 2019). During processing of carcasses in the slaughterhouses there are always chances of transmission of SE from chicken body surfaces to the meat. Likewise, manual evisceration of gastrointestinal organs causes splattering of the pathogen, which further increases contamination of edible chicken meat and meat byproducts (Hagren et al., 2005; Jayathilakan et al., 2012). Hence, chicken should be transported to slaughterhouses with least contamination of major food borne pathogens such as SE (Vandeplas et al., 2010). A reduction in SE colonization in the intestinal

tract of chickens can potentially leads to decline in contamination of poultry meat, eggs and poultry byproducts (Kollanoor-Johny et al., 2012).

Different approaches have been investigated to reduce SE colonization in chickens which includes feeding chicken with competitive exclusion bacteria (Stern et al., 2001; Revollo et al., 2006; Kim et al., 2020), antibiotics (Chadfield and Hinton, 2004), bacteriophages (Fiorentin et al., 2005; Atterbury et al., 2007; Nabil et al., 2018), vaccines (Inoue et al., 2008; Bearson et al., 2019; Wilde et al., 2019), plant derived compounds (Kollanoor-Johny et al., 2012; Darre et al., 2014; Upadhyaya et al., 2015; Johny et al., 2017) and organic acids (Xiong et al., 2016; Wu et al., 2018). However, limited antimicrobial efficacy, toxicity concerns, and adverse effect on production parameters of various chemicals have necessitated the exploration of new antimicrobial compound for controlling *Salmonella* colonization in chickens.

Antibiotics are used for treatment of specific bacterial diseases in livestock animals including poultry and act as growth promoters when added in feed at lower doses (Rabsch et al., 2003). However, extensive use of antibiotics at therapeutic and sub-therapeutic levels has led to development of multi-drug resistance of major food borne pathogens potentially SE, which is a major public health concern worldwide in food and poultry industries.

Similarly, vaccination strategies have been used to control SE in chickens (Dórea et al., 2010; Berghaus et al., 2011), however, fully effective commercial vaccine against SE in chickens are still in progress. This is because SE lives in a state of commensalism with chickens, which lead to inadequate immune response in vaccinated birds (Bailey, 1993; Inoue et al., 2008; Bearson et al., 2019; Wilde et al., 2019).

Therefore, exploring natural compounds is trending to circumvent safety concerns against chemical compounds for reducing SE colonization in chickens (Kollanoor-Johny et al., 2012; Ricke, 2014; Forkus et al., 2016). The gut metabolic products have been investigated in this PhD dissertation for reducing SE colonization in chickens is discussed below.

1.7 GUT METABOLIC PRODUCTS

Short chain fatty acids such as butyrate are microbial metabolites produced from microbial fermentation of undigested carbohydrates and dietary fibers in the colonic lumen of monogastric animals and birds (Vinolo et al., 2011; Sun and O’Riordan, 2013; Tan et al., 2014). Short chain fatty acids in their undissociated form can cross bacterial membranes and maintain intracellular homeostasis (Van Deun et al., 2008; Cummings et al., 1987). The principal short chain fatty acids synthesized from carbohydrates and amino acids fermentation are butyrate, acetate and propionate, produced in the ratio of 15%, 60% and 25% respectively, which are essential for gut health and wellbeing of host (Canani et al., 2011; LeBlanc et al., 2017). Butyrate is a microbial metabolite that plays a protective role against enteric pathogens (Sun and O’Riordan, 2013). Butyrate plays a crucial role in maintenance of gut homeostasis and used as a rich energy source of intestinal epithelial cells (Lamas et al., 2019; Venegas et al., 2019). Butyrate also helps in maintenance of integrity of gut epithelial barrier and modulates inflammatory response in intestine (Meijer et al., 2010; Ashida et al., 2012).

1.8 MECHANISM OF ACTION OF BUTYRATE

Butyrate maintains gastrointestinal health, has therapeutic role in inflammatory bowel disease (IBD) since it serves as main energy source for colonocytes, enhances epithelial barrier integrity, and inhibits inflammation (Hamer et al., 2008; Kasubuchi et al., 2015). Canani et al.

(2011) had reported that butyrate plays a therapeutic role in intestinal and extra-intestinal diseases of humans.

Histone deacetylases (HDACs) along with histone acetyltransferases (HATs) maintains histone acetylation, which regulate gene expression by making conformational changes in chromatin with the modification of acetyl group of lysine residues located in the N-terminal tail of histones and results in determining the accessibility of transcription factors (Rundlett et al., 1996; Kurdistani and Grunstein, 2003). Histone deacetylases act by removing acetyl groups from lysine residues resulting in formation of condensed and transcriptionally silent chromatin and suppress transcription, whereas, HATs induce transcriptional activation (Wolffe, 1996). Butyrate represents a class of epigenetic substances such as histone deacetylase inhibitors (HDI), acts as HDAC inhibitor and has binding ability towards various specific G protein-coupled receptors (GPCRs, de Clercq et al., 2016). The histone deacetylase inhibitors block this action, and further affecting gene expression analysis (Marks et al., 2000; Bedford and Gong, 2018).

Butyrate suppresses nuclear factor kappa B (NF- κ B) and the production of pro-inflammatory cytokines and promotes the production of anti-inflammatory cytokines (Place et al., 2005; Park et al., 2007). Butyrate also interferes with I κ B kinase (IKK) by inhibiting phosphorylation of I κ B- α or I κ B- β , which further suppresses NF- κ B pathway and reduces reactive oxygen species level (ROS, Moeinian et al., 2013). Zhou et al. (2017) reported that sodium butyrate has significant role in restoring dysbiosis of gut microbiota in specific pathogen free male mice. In addition, Elce et al. (2017) showed that butyrate helps in modulation of immune response in human intestine by regulating pro-inflammatory cytokines and recommended to use butyrate as a therapeutic agent for the treatment of inflammatory disorders in human intestine.

1.9 BUTYRATE IN CHICKENS

Previous *in-vivo* studies have shown butyric acid possessing unappetizing odor and constantly changing volatile nature, due to this reason, nowadays sodium butyrate has been specifically used for applied poultry research as it serves as an energy source of colonocytes after entering in the intestine of chickens (Wu et al., 2018). When sodium butyrate is added in the diet of chickens, it helps in the development of intestinal mucosa and regulates growth of intestinal microbiota resulting in healthy gut flora (Hu and Guo, 2007; Smulikowska et al., 2009; Wu et al., 2018). Sodium butyrate also possess anti-inflammatory property in chickens by increasing antimicrobial host defense peptides and downregulates expression of inflammatory mediators such as IL-6, IL-8, IFN- γ , TGF- β and IL-1 β (Sunkara et al., 2011; Xu et al., 2016) and maintains growth performance by regulating the immune response in broiler chickens (Zhang et al., 2011). In addition, partially protected sodium butyrate-based feed additives and microencapsulated form of sodium butyrate reduce SE infection and colonization in broiler chickens (Immerseel et al., 2004; Fernández-Rubio et al., 2009). Canani et al. (2011) reported that butyrate regulates trans-epithelial fluid support, which enhances inflammation of intestinal mucosa and intestinal motility.

Previous *in-vitro* studies showed that butyrate treatment modulates antibacterial activity of chicken monocytes against SE and supplementation of 0.1% butyrate in feed significantly increase host defense peptides gene expression in the intestinal tract of chickens (Sunkara et al., 2011). Zhou et al. (2014) showed that butyrate possesses immunomodulatory activity in chickens and has potential to reduce inflammation and restore gut homeostasis. Van Immerseel et al. (2003) suggested that the invasion of SE is decreased after exposure of bacteria with media supplemented with butyric acid and propionic acid in an avian intestinal epithelial cell line and increased with media containing acetic acid. In addition, Gantois et al. (2006) reported that butyrate specifically

downregulates *Salmonella* Pathogenicity Island 1 gene expression. Despite the multiple beneficial effects of butyrate on improving intestinal development and gut barrier function, there is limited understanding on the efficacy of butyrate in reducing SE infection in chickens and its potential mechanism(s) of action.

Based on the previous published literature and preliminary research, the hypothesis of this dissertation is that sodium butyrate modulates inflammatory host response and *Salmonella* virulence; hence, the following objectives are investigated.

1. The effect of sodium butyrate on SE adhesion and invasion of primary chicken enterocytes and chicken macrophages. In addition, the effect of sodium butyrate on the expression of virulence genes of SE and inflammatory genes in chicken macrophages infected with SE was studied.
2. The effect of sodium butyrate on the proteome of chicken macrophages infected with SE.
3. The effect of sodium butyrate on the proteome of primary chicken enterocytes infected with SE.

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CHAPTER II

Sodium butyrate as a potential anti-inflammatory compound to reduce *Salmonella* Enteritidis colonization in chickens

2.1 ABSTRACT

Salmonella Enteritidis (SE) is a facultative intracellular pathogen, that colonizes the chicken gut in high numbers leading to contamination of carcasses during processing. A reduction in chicken intestinal colonization by SE could result in reduced carcass contamination thereby reducing the risk of enteric illnesses in humans. Hence, the objective of the present study was to determine the effect of sub-inhibitory concentrations (SICs; concentrations below minimum inhibitory concentrations that do not affect bacterial growth) of sodium butyrate on the adhesion and invasion of SE in primary chicken enterocytes and in chicken macrophages cells. In addition, the effect of sodium butyrate on the expression of SE virulence genes and selected inflammatory genes were investigated in chicken macrophages challenged with SE.

The two SICs of sodium butyrate against SE were 22 mM and 45 mM respectively. The SICs of sodium butyrate did not affect the viability and proliferation of primary chicken enterocytes and chicken macrophage cells ($P > 0.05$). The two SICs of sodium butyrate reduced SE adhesion by ~ 1.7 Log CFU/mL (27%) and ~ 1.8 Log CFU/mL (29%) respectively ($p < 0.05$). The SE invasion was reduced by ~ 2 Log CFU/mL (46.8%) and ~ 2.93 Log CFU/mL (62.6%) respectively in primary chicken enterocytes ($p < 0.05$). Sodium butyrate did not significantly affect the adhesion of SE to chicken macrophages. However, 45mM sodium butyrate reduced invasion by ~ 1.7 Log CFU/mL (41%) as compared to control ($P < 0.05$).

Exposure to sodium butyrate did not change the expression of SE genes coding for motility (*flgG*, *prot6E*), invasion (*invH*), type 3 secretion system (*sipB*, *pipB*), survival in macrophages (*spvB*, *mgtC*), cell wall and membrane integrity (*tatA*), efflux pump regulator (*mrr1*) and global virulence regulation (*lrp*) ($P > 0.05$). However, few genes contributing to type 3 secretion system components (*ssaV*, *sipA*), adherence (*sopB*), macrophage survival (*sodC*) and

oxidative stress (*rpoS*) were upregulated. The expression of inflammatory genes (*Il1 β* , *Il8* and *Mmp9*) was significantly downregulated by sodium butyrate as compared to control ($P < 0.05$).

The results suggest that sodium butyrate could potentially be used as an anti-inflammatory compound to reduce SE colonization in chickens, however, *in-vivo* experiments are necessary to validate these results.

Keywords: Primary chicken enterocytes, chicken macrophages, sodium butyrate, anti-inflammatory, *Salmonella*, gene expression

2.2 INTRODUCTION

In the United States, *Salmonella* Enteritidis (SE) is one of the major bacterial pathogens responsible for causing food borne illnesses in humans (Kohli et al., 2018). Contaminated poultry meat and eggs are the major sources of SE infection in humans (Kollanoor-Johny et al., 2012). Hoffmann and Anekwe, (2015) reported that non-typhoidal *Salmonella* ranked first in annual cost of illness and cause an annual economic burden of \$4.43 billion per year impacting government and food industry. The incidence of *Salmonella* infection has increased by ~9% in 2018 as compared to 2015-2017 data (Marder et al., 2018). The U.S. Centers for Disease Control and Prevention (CDC) recently reported multistate *Salmonella* outbreaks linked with backyard poultry in which 1003 people were infected from 49 states resulting in 175 hospitalizations and 2 deaths (CDC, 2018). Moreover, among 1.2 million illnesses annually, approximately 100,000 infections are due to antibiotic resistance of *Salmonella* against potential drugs such as ceftriaxone and ciprofloxacin which causes an annual illness of 33,000 and 36,000 respectively in the United States (Nair et al., 2018). *Salmonella* isolates resistant to five or more classes of antibiotics have been previously reported (CDC, 2013).

Chickens disseminates SE in the environment by acting as asymptomatic carrier for the pathogen (Upadhyaya, 2015). The SE predominantly colonizes in the cecum of chickens thereby leading to contamination of carcasses during slaughter. Despite the presence of significant defensive barriers in intestine, *Salmonella* has developed several strategies to colonize the gastrointestinal tract and penetrate the intestinal epithelium of the host. *Salmonella* interacts with the host through an array of different bacterial proteins which help in their invasion of intestinal epithelium (Lhocine et al., 2015). In the intestinal lumen, *Salmonella* uses flagella and fimbriae for cell attachment (Gart et al., 2016) and adhesion proteins such as SiiE and BapA to attach to

the intestinal epithelium (Fàbrega and Vila, 2013). *Salmonella* injects bacterial effector proteins such as SipA, SopA, SopB, SopD and SopE2 into host epithelial cells by utilizing Type III secretion systems (T3SS) encoded on *Salmonella* pathogenicity island-1 (SPI-1) for cytoskeletal rearrangement and bacterial engulfment (Wemyss and Pearson, 2019). SPI-1 plays a crucial role in colonization and invasion of *Salmonella* into host intestinal epithelial cells which further induces secretion of inflammatory cytokines and antimicrobial peptides such as IL-1 β , IL-12, IL-18, α -defensins and cathelicidins along with activation of macrophages and recruitment of neutrophils (Gart et al., 2016). Therefore, *Salmonella* induces an inflammatory immune response in host intestine which allows it to compete with commensal microbiota and to effectively colonize in the host gut (Hallstrom and McCormick, 2011; Fàbrega and Vila, 2013; Gart et al., 2016).

In addition, evidence exists that the pathogen survives in chicken macrophages and disseminates systemically thereby contaminating meat and eggs (Foley et al., 2013). Internalization of *Salmonella* causes formation of *Salmonella* containing vacuoles (SCVs) which induces expression of Type III secretion systems (T3SS) encoded on *Salmonella* Pathogenicity Island-2 (SPI-2) through various effector proteins for bacterial replication and intracellular survival such as SpiC, PipB, SseJ, SifA, SspH and SopD2 (Ly and Casanova, 2007; Foley et al., 2013; Wemyss and Pearson, 2019). *Salmonella* interferes with NADPH oxidase complex inside the phagocytic macrophages, which prevents superoxide production and allows the bacteria to survive inside macrophage cells.

Different approaches have been investigated to reduce SE colonization in chickens which include feeding chickens with competitive exclusion bacteria (Stern et al., 2001; Revollo et al., 2006; Kim et al., 2020) antibiotics (Chadfield and Hinton, 2004), bacteriophages (Fiorentin et

al., 2005; Atterbury et al., 2007; Nabil et al., 2018), vaccines (Inoue et al., 2008; Bearson et al., 2019; Wilde et al., 2019), plant derived compounds (Darre et al., 2014; Johny et al., 2017; Kollanoor-Johny et al., 2012; Upadhyaya et al., 2013, 2015) and organic acids (Wu et al., 2018; Xiong et al., 2016). However, limited antimicrobial efficacy, toxicity, palatability concerns, or adverse effect on production parameters necessitates the exploration of new antimicrobial compound for controlling *Salmonella* colonization in chickens.

Short chain fatty acids such as butyrate are microbial metabolites synthesized from fermentation of dietary fibers in the colonic lumen. Previous studies have shown that invasion of SE is influenced by short chain fatty acids in avian intestinal epithelial cells (Van Immerseel et al., 2003) and partially protected sodium butyrate based feed additives and microencapsulated forms of sodium butyrate reduced SE colonization in broiler chickens (Immerseel et al., 2004; Fernández-Rubio et al., 2009). Follow up in-vitro studies have shown that butyrate treatment modulates antibacterial activity of chicken monocytes against SE and in feed supplementation of 0.1% butyrate significantly increasing host defense peptides gene expression in the intestinal tract of chickens (Sunkara et al., 2011). In a recent study, the supplementation of dietary sodium butyrate in feed promoted growth, intestinal development by increasing length of villi in ileum with mucus secretion and improved morphological structure and biological function in broiler chickens. In addition, the sodium butyrate at higher doses (800mg/kg) modulated antioxidant capacity, decreased malondialdehyde concentration in the jejunal mucosa by regulation of intestinal microbial community in broilers chickens (Wu et al., 2018). Despite the multiple beneficial effects of butyrate, there is limited understanding on the efficacy of butyrate in reducing SE infection in chickens and its potential mechanism(s) of action.

Therefore, the objective of this study was to investigate the effect of sodium butyrate on SE adhesion and invasion of primary chicken enterocytes and chicken macrophages. In addition, the effect of sodium butyrate was studied on the expression of virulence genes of SE and inflammatory genes in chicken macrophages challenged with SE.

2.3 MATERIAL AND METHODS

2.3.1 Primary chicken enterocytes cell culture

Day-old male broiler chicks (Cobb 500) were obtained from Cobb-Vantress, Fayetteville, AR and were housed overnight (brooding temperature of ~90°F with *ad-libitum* water) as approved by Institutional Animal Care and Use Committee, University of Arkansas. Chicks were euthanized by cervical dislocation and small intestines were collected in a petri-dish containing Dulbecco's modified minimum essential medium (DMEM F-12; HiMedia Laboratories Pvt. Ltd, Mumbai, India) enriched with 1X antibiotic antimycotic solution (Sigma-Aldrich, St Louis, MO, USA), 1X sodium pyruvate solution (Sigma-Aldrich), gentamicin solution (Sigma-Aldrich), 10 mM glutamine solution (Thermo Fisher Scientific, Carlsbad, CA) as described earlier (Rath et al., 2018). Intestinal segments were rinsed three times with DMEM F-12 and squeezed to harvest villi from intestinal segments in petri plate containing DMEM F-12 medium. Harvested intestinal villi were centrifuged at 300 g for 10 min to form a pellet. The pellet was resuspended in 0.1% Streptomyces hyaluronidase (Sigma-Aldrich) and incubated for 60 min at 37°C in a humidified 5% CO₂ incubator. The intestinal villi were centrifuged at 300 g for 10 min and further digested with 0.025% Trypsin: cell dissociation solution (Sigma-Aldrich) in the ratio of 1:9 for 15 min at 37°C in a humidified 5% CO₂ incubator. Dissociated cells were layered over Histopaque-1119 (Sigma-Aldrich) for 30 min at 400 g for density gradient centrifugation. Cells layer at the interface of gradient medium was collected, suspended in DMEM F-12 and centrifuged at 300 g for 10 min.

Cell clusters were resuspended in DMEM F-12 culture medium containing growth factors such as 10% heat inactivated fetal bovine serum (Thermo Fisher Scientific), 1X Insulin Transferrin Selenium (Sigma-Aldrich), 1X Epithelial cell growth supplement (EpiCGS, Sigma-Aldrich), 20 ng/mL epidermal growth factor (EGF, Thermo Fisher Scientific) for 24-48 h in a humidified 5% CO₂ incubator at 37°C till it reached semi-confluency (50%). Enterocytes were dissociated with Accutase (Sigma-Aldrich) to perform cell culture assays.

Chicken macrophages (HTC cells; a naturally transformed cell line) were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Thermo Fisher Scientific) (Rath et al., 2003) containing 10% fetal bovine serum, 1X antibiotic antimycotic solution, 1X sodium pyruvate solution, gentamicin solution, 10 mM glutamine solution at 37°C for 24-48 h in a humidified 5% CO₂ incubator. The cells were cultured to semi-confluency of 50% followed by dissociation with Accutase to perform appropriate cell culture assays.

2.3.2 The effect of common chicken gut chemicals on morphology and viability of primary chicken enterocytes

The effect of chemicals commonly encountered in the gut such as mycotoxins (Aflatoxin B₁; 4-deoxy Nivalenol (DON)), endotoxin (*Salmonella* Typhimurium Lipopolysaccharide, LPS), metabolic activators (dibutyl cyclic-AMP (dbcAMP); Phorbol 12-myristate 13-acetate (PMA); Sodium butyrate (SB)), vitamins (calcitriol and trans retinoic acid) and pesticide (thiram) on the viability of primary chicken enterocytes was investigated using -[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sakurazawa and Ohkusa, 2005) and morphological parameters (Rath et al., 2018). All the aforementioned chemicals were purchased from Sigma-Aldrich and the tested concentration was 1 µg/mL. Primary chicken enterocytes (10⁴ cells per well) were seeded in 96-well plate (Costar, Corning Incorporated, Corning, NY) for 24 h at 37°C

in a humidified, 5% CO₂ incubator to form a monolayer. The chicken enterocytes were incubated with each chemical separately for 24 h. For the morphological changes, the treated enterocytes were observed under a BX Olympus microscope at 100 X and photographed with a Cool Snap Pro camera (Media Cybernetics Inc. MD, USA). For testing the cell viability of primary chicken enterocytes in response to chemicals, the MTT reagent (10 µL) was added to the treated chicken enterocytes and incubated at 37°C in a humidified, 5% CO₂ incubator for 2 h. Following addition of 100 µL isopropanol (Sigma-Aldrich) the plate was further incubated at room temperature in dark for 1 h. The absorbance was measured at 570 nm by using spectrophotometric microplate reader (Benchmark; Bio-Rad Laboratories, Hercules, CA, USA).

2.3.3 Bacterial strains and culture conditions

Salmonella Enteritidis GFP 338 (SE) was cultured in 10 mL of tryptic soy broth (TSB; Hardy Diagnostics CRITERION™, Santa Maria, CA, USA) at 37°C for 18 h. Following subculture in 10 mL TSB for another 10 h, the culture was centrifugated at 2500 g for 10 min. The pellet was suspended in sterilized phosphate buffer saline (PBS, pH 7) and used as the inoculum. The enumeration of SE counts in inoculum was made by plating serial 5-fold dilutions on brilliant green agar (BGA; Difco Laboratories, Detroit, Michigan, USA) and the plates were incubated at 37°C for 24 h for bacterial enumeration.

2.3.4 Determination of sub-inhibitory concentrations of sodium butyrate

The sub-inhibitory concentrations (SICs) of sodium butyrate against SE was determined as described previously (Upadhyaya et al., 2015). Briefly, twofold dilutions of sodium butyrate (363, 181.5, 90.75, 45, 22 and 11 mM) in TSB were prepared in sterile 96-well polystyrene tissue culture plate. The SE (~6.0 Log CFU) was added to each well except negative controls and the plate was incubated at 37°C for 24 h under aerobic condition. The growth of SE was

determined by measuring absorbance using spectrophotometric microplate reader (Benchmark; Bio-Rad Laboratories, Hercules, CA, USA) at 570 nm. The two highest concentrations of sodium butyrate that did not inhibit SE growth after 24 h of incubation were determined as the SIC for the present study.

2.3.5 Effect of SICs of sodium butyrate on cell viability of primary chicken enterocytes and chicken macrophages

The effect of SICs of sodium butyrate on cell viability was performed as per standard protocol using MTT assay (Jung et al., 2005; Sakurazawa and Ohkusa, 2005). Primary chicken enterocytes and chicken macrophages were grown (10^3 cells per well) using 96 well plate for 24 h at 37°C in a humidified, 5% CO₂ incubator. Monolayers of the chicken enterocytes or chicken macrophages were incubated with SICs of sodium butyrate for 2 h at 37°C in a humidified, 5% CO₂ incubator and the MTT assay was performed as described above.

2.3.6 Effect of SICs of sodium butyrate on SE adhesion to and invasion of primary chicken enterocytes and chicken macrophages

The effect of SICs of sodium butyrate on SE adhesion to and invasion of primary chicken enterocytes cell culture and chicken macrophages was performed using attachment and invasion assays as described earlier (Wagle et al., 2017) with minor modifications. Primary chicken enterocytes or chicken macrophages (10^5 cells per well) were seeded into 6-well plates (Costar) containing DMEM F-12 with 10% FBS and incubated for 48 h at 37°C in a humidified, 5% CO₂ incubator to form a monolayer. A mid-log phase (10 h) culture of SE was inoculated on the primary chicken enterocytes and chicken macrophages (~6 Log CFU/mL; multiplicity of infection 10:1) in the presence or absence of SICs of sodium butyrate. For the adhesion assay, an

infected monolayer was incubated for 2 h followed by rinsing with PBS three times. The cells were lysed by treating with 0.1% Triton-X 100 for 20 min. The number of adhered SE was determined by dilution and plating of cell lysate on Brilliant green agar (BGA) plates followed by incubation at 37°C for 24 h under aerobic condition.

For the invasion assay, infected monolayers after an incubation of 2 h with SE were rinsed with PBS three times, followed by incubation with gentamicin (100 µg/mL, Sigma-Aldrich) at 37°C in a humidified, 5% CO₂ incubator for additional 2 h to kill the extracellular bacteria. The cells were washed with PBS three times and lysed by 0.1% Triton-X 100. The cell lysate was diluted and plated on BGA plates for enumeration of invaded SE.

2.3.7 Effect of SIC of sodium butyrate on the expression of virulence genes of SE.

The effect of SIC of sodium butyrate on the expression of SE virulence genes was determined using real-time quantitative PCR (RT-qPCR) as described previously (Upadhyaya et al., 2015; Sun and Jia, 2018; Bansal et al., 2019). SE was cultured to mid-log phase with or without SIC of sodium butyrate in TSB at 37°C for 10 h. The total RNA was extracted using TRIzol reagents (Thermo Fisher Scientific) as per manufacturer's protocol. Complementary DNA (cDNA) synthesis was made using M-MLV kit (Thermo Fisher Scientific). Messenger RNA (mRNA) expression of SE genes was determined using SYBR Green PCR Master mix (Bio-Rad Laboratories, Inc., CA, USA) in a 384-well real-time PCR System (Model 7500 Fast Step One Plus system-Applied Biosystems, Thermo Fisher Scientific) and normalized to endogenous control, *16S* rRNA. The primers used in this study (Upadhyaya et al., 2015) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). Relative gene expression analysis of candidate gene was determined based on comparative critical threshold ($\Delta\Delta C_T$) method.

2.3.8 Effect of SICs of sodium butyrate on the expression of inflammatory genes of chicken macrophages challenged with SE.

The effect of sodium butyrate on the expression of inflammatory cytokine genes in chicken macrophages was performed using RT-qPCR, as described earlier (Sun and Jobin, 2014). Chicken macrophages (5×10^5 cells per well) were seeded in 6-well plate and incubated at 37°C in a humidified, 5% CO₂ incubator for 48-72 h. A mid-log SE culture was inoculated on HTC cells (~6 Log CFU/mL; multiplicity of infection 10:1) in presence or absence of SICs of sodium butyrate followed by incubation for 4 h at 37°C in a humidified, 5% CO₂ incubator. Following incubation, total RNA was isolated from chicken macrophages using TRIzol reagents (Thermo Fisher Scientific) as per manufacturer's protocol. Complementary DNA (cDNA) synthesis was made using M-MLV kit (Thermo Fisher Scientific). Messenger RNA (mRNA) expression of inflammatory mediators was determined using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Inc., CA, USA) in a 384-well RT-qPCR System and normalized to endogenous control, *Gapdh*. The primers of each gene were designed from Primer 3 software (National Center for Biotechnology Information, Bethesda, MD) and obtained from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 2).

2.3.9 Statistical Analysis

The CFU counts of SE were logarithmically transformed (Log CFU) to maintain homogeneity of variance (Byrd et al., 2001). For all assays, triplicate samples were used, and the experiment was repeated two times. For cell culture assays and RT-qPCR gene expression for host immune response data were analyzed using One-way ANOVA in Graph-pad 7 Software. Treatment means were separated by Tukey's multiple comparisons test. The changes in expression of SE genes in response to sodium butyrate were analyzed by using Student's t-test

for comparisons between treatment and controls. Probability of $P < 0.05$ was set for statistical significance.

2.4 RESULTS

2.4.1 The effect of common chicken gut chemicals on morphology and viability of primary chicken enterocytes

Figure 1 shows the effect of sodium butyrate, PMA, retinoic acid, dbcAMP, LPS, Aflatoxin B₁, calcitriol, DON and thiram on morphology of primary chicken enterocytes. Aflatoxin B₁, DON and thiram showed cytotoxic effect on chicken primary cells, whereas, PMA, calcitriol and retinoic acid showed minimal effect on cellular morphology. Similarly, sodium butyrate, LPS and dbcAMP affected cell morphology. The chicken enterocytes became wider and elongated after treatment with LPS, dbcAMP and sodium butyrate as compared to control.

Figure 2 shows the effect of test chemicals on cell viability of primary chicken enterocytes. The cell viability of primary chicken enterocytes was severely affected ($>50\%$) after treatment with DON and thiram and reduced the viability by 75.7% and 88.40% respectively ($P < 0.05$). Similarly, the viability was moderately affected (25-50%) with dbcAMP, LPS, Aflatoxin B₁ and calcitriol and reduced the viability by 26.7%, 27.7%, 36.2%, and 36.45% respectively ($P < 0.05$). However, sodium butyrate, PMA and retinoic acid did not affect the cell viability as compared to control ($P > 0.05$).

2.4.2 Sub-inhibitory concentrations of sodium butyrate against SE

Figure 3 shows the effect of various concentrations of sodium butyrate on growth of SE. The three concentrations of sodium butyrate that did not inhibit growth of SE as compared to

control were 11, 22 and 45 mM ($P>0.05$). Based on these results, we selected the two highest SICs (22 mM and 45 mM) of sodium butyrate for further studies.

2.4.3 The effect of SICs of sodium butyrate on cell viability of primary chicken enterocytes and chicken macrophages

The effect of SIC's of sodium butyrate on cell viability of primary chicken enterocytes and chicken macrophages is shown in Figure 4. The control had an absorbance of ~ 0.5 in chicken enterocytes and presence of two SICs of sodium butyrate does not affect cell viability (Fig. 4A; $P>0.05$). Similar results were observed with chicken macrophages wherein the presence of SICs of sodium butyrate did not significantly affect the viability of chicken macrophages (Fig. 4B).

2.4.4 The effect of SICs of sodium butyrate on SE adhesion to and invasion of primary chicken enterocytes and chicken macrophages

Figure 5 shows the effect of SICs of sodium butyrate on SE adhesion to and invasion of primary chicken enterocytes and chicken macrophages. Approximately 6.3 Log CFU/mL SE adhered on primary chicken enterocytes (Fig. 5A). The SICs (22 mM and 45 mM) of sodium butyrate significantly reduced adhesion of SE to primary chicken enterocytes by ~ 1.7 (27%) and ~ 1.8 Log CFU/mL (29%) respectively as compared to control. Similarly, the invaded SE counts in controls were ~ 5 Log CFU/mL and the two SICs (22 and 45 mM) of sodium butyrate reduced invasion of SE by ~ 2 Log CFU/mL (46.8%) and ~ 2.93 Log CFU/mL (62.6%) respectively ($P<0.05$) (Fig. 5B). In the chicken macrophages, ~ 6 Log CFU/mL SE adhered (Fig. 5C) and ~ 4 Log CFU/mL invaded the cells (Fig. 5D). The presence of 22 mM sodium butyrate did not reduce SE adhesion to and invasion of chicken macrophages ($P>0.05$). In contrast to 22 mM, 45

mM sodium butyrate significantly reduced invasion of SE by ~ 1.7 Log CFU/mL (41%) as compared to controls ($P < 0.05$).

2.4.5 The effect of sodium butyrate on the expression of SE virulence genes

The effect of SIC (45mM) of sodium butyrate on the expression of SE genes essential for virulence and intestinal colonization is shown in Fig 6. The expression of SE genes crucial for motility (*flgG*, *prot6E*), invasion (*invH*), type 3 secretion system (*sipB*, *pipB*), survival in macrophages (*spvB*, *mgtC*), cell wall and membrane integrity (*tatA*), efflux pump regulator (*mrrI*), and global virulence regulation (*lrp*) was not affected by sodium butyrate ($P > 0.05$). However, few genes contributing to type 3 secretion system components (*ssaV*, *orf245*, *sipA*), adherence (*sopB*), motility (*fimD*), exo/endonuclease activity (*xthA*), and oxidative stress (*rpoS*) were upregulated ($P < 0.05$). Similarly, genes such as *hflK* and *ompR* important for integrity of cell wall and cell membrane, metabolism (*ssrA*), macrophage survival (*sodC*), and lipopolysaccharide biosynthesis (*rfbH*) were slightly upregulated by sodium butyrate treatment.

2.4.6 The effect of sodium butyrate on the expression of inflammatory genes in chicken macrophages challenged with SE

The effect of sodium butyrate on expression of inflammatory genes (*Il1 β* , *Il8* and *Mmp9*) is shown in Figure 7 (A-C). The expressions of *Il1 β* , *Il8*, *Mmp9* were significantly up regulated with SE challenge by 328.4, 141.2 and 41.2 folds respectively as compared to uninfected chicken macrophages. Presence of 22 mM and 45 mM sodium butyrate reduced *Il1 β* gene expression by 41.74% and 76.7% respectively ($P < 0.05$) (Fig. 7A). Similarly, 22 mM and 45 mM sodium butyrate reduced *Mmp9* gene expression by 84.2% and 95.6% respectively ($P < 0.05$) (Fig. 7B). There was no change in the expression of *Il8* gene after treatment with 22 mM sodium butyrate

($P>0.05$); however, 45 mM sodium butyrate reduced its expression by 35% ($P<0.05$) (Fig. 7C). However, there was no significant change on the expression of *Il18*, *Il10*, *Il6*, *iNos2*, *Il12 β* and *Il12 α* after treatment of SE infected chicken macrophages with sodium butyrate 45 mM ($P>0.05$).

2.5 DISCUSSION

Enterocytes plays a vital role in the absorption of nutrients and acts as a protective barrier against many pathogenic and non-pathogenic microbes present in the intestinal lumen (Chougule et al., 2012; Rath et al., 2018). Primary chicken enterocytes could be considered as an *in-vitro* model for screening of various chemicals that influence intestinal physiology and play a crucial role in immunopathology. However, primary chicken enterocytes are not commercially available. Therefore, we developed an *in-vitro* cell culture model and tested the response of the primary enterocytes to various chemicals commonly encountered in chicken gut. Our results (Fig. 1, 2) revealed that the *in-vitro* cell culture model exhibited a dose-dependent, physio-chemical response to the tested chemicals and has the potential to study intestinal physiology and potential host microbial interactions.

Short-chain fatty acids including butyrate are fermentation products of undigested carbohydrates produced in the ceca or colon of animals. Butyrate has been considered as a primary energy source for growth of intestinal epithelial cells (Clausen and Mortensen, 1995; Józefiak et al., 2004) and possess antimicrobial activity against invading pathogens in intestinal lumen (Schulthess et al., 2019). Butyric acid has been Generally Recognised as Safe (GRAS) for its use in foods (Butyric acid- 21CFR182.60, Food and Drug Administration (FDA), 2017). Butyrate supplementation in diet of broiler chickens maintains physiological function of intestinal mucosa and gut health (Hu and Guo, 2007; Smulikowska et al., 2009; Wu et al., 2016,

2018). In addition, Fernandez Rubio et al. (2009) also reported that butyrate supplementation (0.92 g/Kg) in the diet of broiler chickens reduces SE infection. However, there is limited information on the effect of butyrate on SE virulence, colonization factors and host response. Therefore, we tested the anti-virulence, anti-colonization potential of sodium butyrate against SE. In addition, we investigated the effect of sodium butyrate on host genes participating in inflammatory response.

We tested the anti-*Salmonella* potential of sodium butyrate at sub-inhibitory concentrations. These concentrations refer to compound concentrations that do not affect bacterial growth/cell viability but potentially modulate the expression of genes and/or proteins in the host or microbial system (Upadhyay et al., 2012; Upadhyaya et al., 2013, 2015; Wagle et al., 2017; Viedma et al., 2018). Our results suggest that sodium butyrate at SICs does not affect the growth of *Salmonella* (Fig. 3A) and cell viability of primary chicken enterocytes and chicken macrophages (Fig. 4). Yan and Ajuwon, (2017) also reported that butyrate (1mM) promotes tight junction protein expression and intestinal homeostasis by restoring LPS induced impairment of intestinal barrier in porcine intestinal epithelial cell.

Despite the presence of significant defensive barriers in intestine, SE has developed several strategies to colonize gastrointestinal tract of host and invade the intestinal epithelium (Foley et al., 2013; Lhocine et al., 2015). The SE directly interacts with mucosal barrier to colonize and promote its internalization inside host. Our results from primary chicken enterocytes and chicken macrophages cell culture assay (Fig. 5) revealed that sodium butyrate significantly reduced SE adhesion to and invasion of chicken enterocytes (Figure 5A, 5B) which are critical for pathogenesis of bacterium and reduced SE invasion of chicken macrophages which helps in replication and intracellular survival of pathogen (Fig. 5D). Van Immerseel et al.

(2003) had also reported that incubation of SE with 20 mM or 30 mM butyrate in Luria Bertoni medium for 4 h reduce invasion of SE in avian intestinal epithelial cells.

Several genes facilitate the attachment, invasion and translocation of *Salmonella* through the host epithelium by secreting bacterial effector proteins. For example *flgG*, *fimD* and *prot6E* are genes critical for motility (Gantois et al., 2008; Clavijo et al., 2006, De Buck et al., 2004); whereas *lrp* codes for virulence regulation (Baek et al., 2009); *invH* contributes to invasion (Pati et al., 2013); *sopB*, *sopE*, *sopE2*, *sipB*, *pipB*, *ssaV*, *orf245*, *sipA*, *sipC*, *sipD* are critical for type three secretion system function (Ly and Casanova, 2007; Foley et al., 2013; Wemyss and Pearson, 2019; Haraga et al., 2008; Raffatellu et al., 2005). Genes *sodC*, *spvB* and *mgtC* facilitate macrophage survival (Beth et al., 1998; Choi et al., 2019), whereas, *rpoS* gene is important for stress tolerance (Shah et al 2012) and *rfbH* critical for lipopolysaccharide biosynthesis (Gantois et al., 2008). Our gene expression analysis revealed that there was no effect of 45 mM SB on the expression of SE genes including *flgG*, *prot6E*, *invH*, *sipB*, *pipB*, *lrp*, *tatA*, *mrr1*, *spvB* and *mgtC* ($P>0.05$). However, few SE genes in our study were slightly upregulated by SB treatment such as *fimD*, *ssaV*, *orf245*, *sipA*, *sopB*, *xthA*, *ssrA*, *sodC* and *rfbH* (Fig. 6). Previous investigations by Gantois et al. (2006) have observed that exposure of 10 mM butyrate with *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium downregulates the expression of 19 genes encoding for SPI1 effector proteins and invasion including *invF*, *invE*, *invB*, *pipC* and *sopB*. Immerseel et al. (2004) had also reported that expression of *hilA* gene associated with invasion of *Salmonella enterica* serovar Enteritidis was reduced after exposure with 2 mM caproic acid, capric acid, and caprylic acid. However, in our study the majority of genes were either not affected or slightly overexpressed. This could be due to differences in the doses of butyrate tested, strain variation, time of incubation and other unknown factors.

Since SE gene expression data was not conclusive of the anti-*Salmonella* mechanism of action of butyrate, we investigated the effect of sodium butyrate on host inflammatory response. SE infection damages intestinal mucosal barrier and increases susceptibility to intestinal inflammation, which leads to activation of pro-inflammatory pathways. Macrophages are activated in the sub-epithelial region and induce the production of *IL1 β* , *IL8*, *IL6*, *IL23* and *IL12*, which further attracts and activates neutrophils to reduce bacterial infection (Zha et al., 2019). Our RT-qPCR results revealed that sodium butyrate significantly reduced inflammatory cytokines and *Mmp9* in chicken macrophages (Fig. 7) indicating that since butyrate reduced inflammation in chicken macrophages, it could reduce *Salmonella* invasion process. Bedford and Gong, (2018) had also described that anti-inflammatory properties of butyrate could be mediated by the reduction of pro-inflammatory cytokine expression such as interferon gamma (*IFN-g*), tumor necrosis factor- α (*TNF- α*), interleukin-1 β (*IL1 β*), *IL6* and *IL8*.

2.6 CONCLUSIONS

In this study, we developed a primary chicken enterocyte cell culture model that acted as a platform for studying a range of chemicals to modulate intestinal physiology and affect gut integrity. We identified that sodium butyrate, at sub-inhibitory concentration, significantly reduced colonization potential of SE by reducing attachment and invasion capacity. Moreover, sodium butyrate exerted its anti-inflammatory effect on chicken macrophages (challenged with SE) by downregulation of inflammatory cytokine genes. Even though these results from our study were notable, further studies are needed to validate the anti-inflammatory effect of sodium butyrate in chickens in correspond to reduction in SE colonization.

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Table 2.1 List of primers used for RT-qPCR analysis of SE genes

Genes	Function	Primer	Sequence (5'-3')
<i>flgG</i>	Motility	Forward	5'-GCGCCGGACGATTGC-3'
		Reverse	5'-CCGGGCTGGAAAGCATT-3'
<i>prot6E</i>	Motility	Forward	5'-GAACGTTTGGCTGCCTATGG-3'
		Reverse	5'-CGCAGTGA CTGGCATCAAGA-3'
<i>fimD</i>	Motility	Forward	5'-CGCGGCGAAAGTTATTTCAA-3'
		Reverse	5'-CCACGGACGCGGTATCC-3'
<i>invH</i>	Invasion	Forward	5'-CCCTTCCTCCGTGAGCAAA-3'
		Reverse	5'-TGGCCAGTTGCTCTTTCTGA-3'
<i>sipB</i>	Type 3 secretion system	Forward	5'-GCCACTGCTGAATCTGATCCA-3'
		Reverse	5'-CGAGGCGCTTGCTGATTT-3'
<i>pipB</i>	Type 3 secretion system	Forward	5'-GCTCCTGTTAATGATTTTCGCTAAAG-3'
		Reverse	5'-GCTCAGACTTAACTGACACCAAATAA-3'
<i>orf245</i>	Type 3 secretion system	Forward	5'-CAGGGTAATATCGATGTGGACTACA-3'
		Reverse	5'-GCGGTATGTGGAAAACGAGTTT-3'
<i>sipA</i>	Type 3 secretion system	Forward	5'-CAGGGAACGGTGTGGAGGTA-3'
		Reverse	5'-AGACGTTTTTGGGTGTGATACGT-3'
<i>ssaV</i>	Type 3 secretion system	Forward	5'-GCGCGATACGGACATATTCTG-3'
		Reverse	5'-TGGGCGCCACGTGAA-3'
<i>spvB</i>	Survival in macrophages	Forward	5'-TGGGTGGGCAACAGCAA-3'
		Reverse	5'-GCAGGATGCCGTTACTGTCA-3'
<i>mgtC</i>	Survival in macrophages	Forward	5'-CGAACCTCGCTTTCATCTTCTT-3'
		Reverse	5'-CCGCCGAGGGAGAAAAAC-3'
<i>sodC</i>	Survival in macrophages	Forward	5'-CACATGGATCATGAGCGCTTT-3'
		Reverse	5'-CTGCGCCGCGTCTGA-3'
<i>tatA</i>	Cell wall and cell membrane integrity	Forward	5'-AGTATTTGGCAGTTGTTGATTGTTG-3'
		Reverse	5'-ACCGATGGAACCGAGTTTTTT-3'
<i>hflK</i>	Cell wall and cell membrane integrity	Forward	5'-AGCGCGGCGTTGTGA-3'
		Reverse	5'-TCAGACCTGGCTCTACCAGATG-3'
<i>ompR</i>	Cell wall and cell membrane integrity	Forward	5'-TGTGCCGATCTTCTTCCA-3'
		Reverse	5'-CTCCATCGACGTCCAGATCTC-3'
<i>mrr1</i>	Efflux pump regulator	Forward	5'-CCATCGCTTCCAGCAACTG-3'
		Reverse	5'-TCTCTACCATGAACCCGTACAAATT-3'
<i>lrp</i>	Virulence regulation	Forward	5'-TTAATGCCGCGTGCAA-3'
		Reverse	5'-GCCGAAACCAAATGACACT-3'
<i>sopB</i>	Adherence	Forward	5'-GCGTCAATTTTCATGGGCTAAC-3'
		Reverse	5'-GGCGGCGAACCCATAAACT-3'
<i>xthA</i>	Exo/endonuclease activity	Forward	5'-CGCCCGTCCCATCA-3'
		Reverse	5'-CACATCGGGCTGGTGTTTT-3'

Table 2.1 (Cont.)

Genes	Function	Primer	Sequence (5'-3')
<i>rpoS</i>	Oxidative stress	Forward	5'-TTTTTCATCGGCCAGGATGT-3'
		Reverse	5'-CGCTGGGCGGTGATTC-3'
<i>ssrA</i>	Metabolism	Forward	5'-CGAGTATGGCTGGATCAAAACA-3'
		Reverse	5'-TGTACGTATTTTTTGC GGGATGT-3'
<i>rfbH</i>	Lipopolysaccharide biosynthesis	Forward	5'-ACGGTCGGTATTTGTCAACTCA-3'
		Reverse	5'-TCGCCAACCGTATTTTGCTAA-3'
<i>16S</i>	16S rRNA	Forward	5'-CCAGGGCTACACACGTGCTA-3'
		Reverse	5'-TCTCGCGAGGTCGCTTCT-3'

Table 2.2 List of primers used for RT-qPCR analysis of host immune response genes

Genes	Primer	Sequence (5'-3')
<i>Il1β</i>	Forward	5'-GCATCAAGGGCTACAAGCTC-3'
	Reverse	5'-CAGGCGGTAGAAGATGAAGC-3'
<i>Il8</i>	Forward	5'-CCTCCTGCCTCCTACATTCA-3'
	Reverse	5'- ATCTCCAGCTCCTTTCACGA-3'
<i>Mmp9</i>	Forward	5'-CCAAGATGTGCTCACCAAGA-3'
	Reverse	5'-CCAATGCCCAACTTCTCAAT-3'
<i>Il12α</i>	Forward	5'-CAAACGAGGCACTCCTGAAG-3'
	Reverse	5'-GGTCTTCGTAGATCCCCTGC-3'
<i>Il12β</i>	Forward	5'-CTGATGAAGCACTGCCAGTTTAC-3'
	Reverse	5'-AAAGCGTGGACCACTCACTC-3'
<i>Il18</i>	Forward	5'-TTGCTTGTGGTTCGTCCAGA-3'
	Reverse	5'-GCTGAATGCAACAGGCATCC-3'
<i>Nos2</i>	Forward	5'-AAACTTCATCCCCCAACCAGC-3'
	Reverse	5'-GTTTCTAGTCGGGCCAGGTG-3'
<i>Il6</i>	Forward	5'-TTCCCCAGGTGGGAGGAATTG-3'
	Reverse	5'-ACAGCCACATCAAAATAGGCGA-3'
<i>Il10</i>	Forward	5'-AGCCTTCACCTTGATGGAGC-3'
	Reverse	5'-TGATGGGTAGTGAGGAGGGG-3'
<i>Gapdh</i>	Forward	5'-GACGTGCAGCAGGAACACTA-3'
	Reverse	5'- CTTGACTTTGCCAGAGAGG-3'

Il: Interleukin; *Mmp9*: Matrix metalloproteinase 9; *Nos2*: Nitric oxide synthase 2

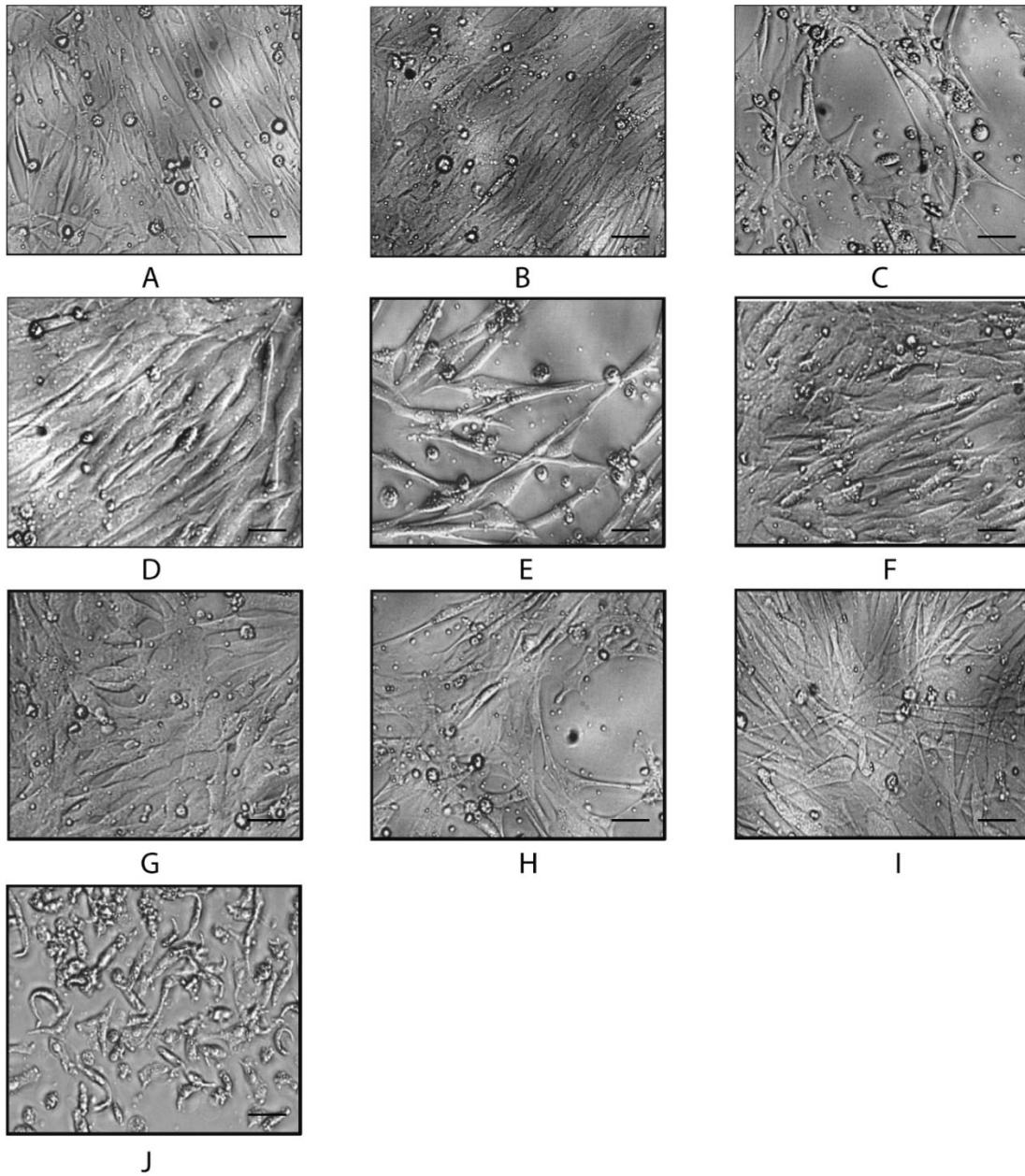


Figure 2.1 The effect of various chemicals commonly encountered in chicken gut environment on morphology of primary chicken enterocytes, Bar=30 μ m. **A:** Control **B:** Calcitriol **C:** Aflatoxin B₁ **D:** dbcAMP **E:** DON **F:** LPS **G:** Sodium butyrate **H:** PMA **I:** Trans Retinoic Acid **J:** Thiram (magnification $\times 100$).

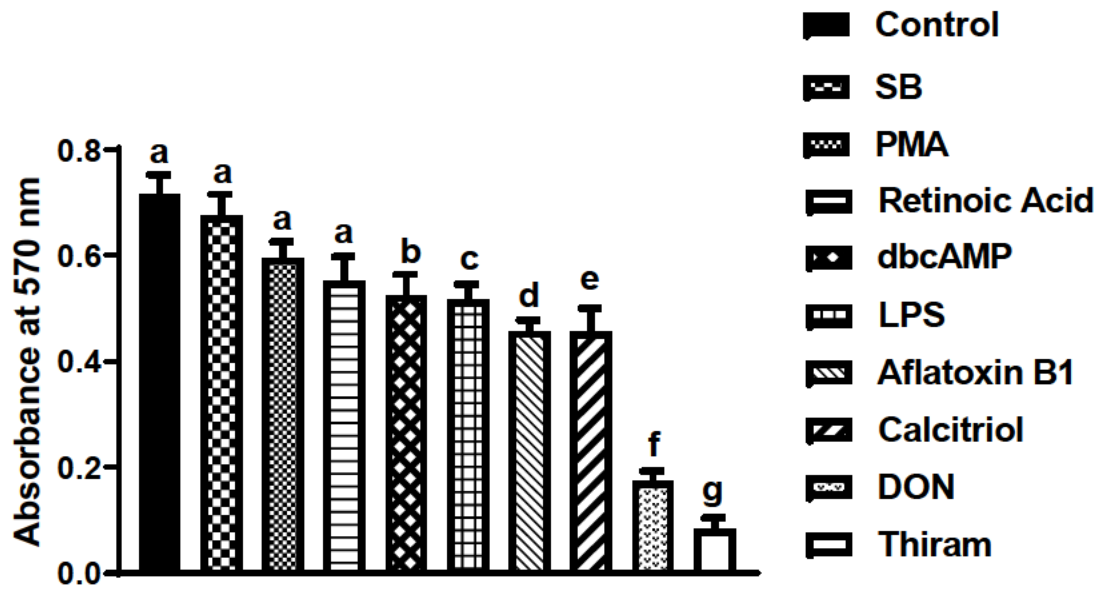


Figure 2.2 The effect of test chemicals on cell viability of primary chicken enterocytes. Sodium butyrate (SB) does not affect cell viability of primary chicken enterocytes. Data presented as mean absorbance and error bar represents SEM (n=6). Bar with different letters represents a statistical difference at $P < 0.05$.

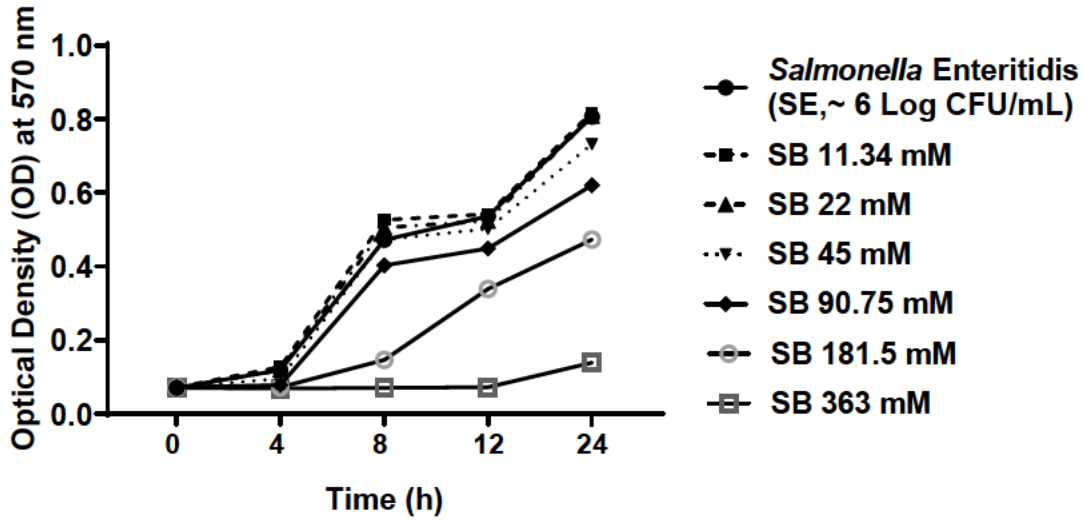


Figure 2.3 The effect of various concentrations (0, 363, 181.5, 90.75, 45, 22 and 11 mM) of SB against SE for the determination of subinhibitory concentrations based on growth curve analysis ($P < 0.05$).

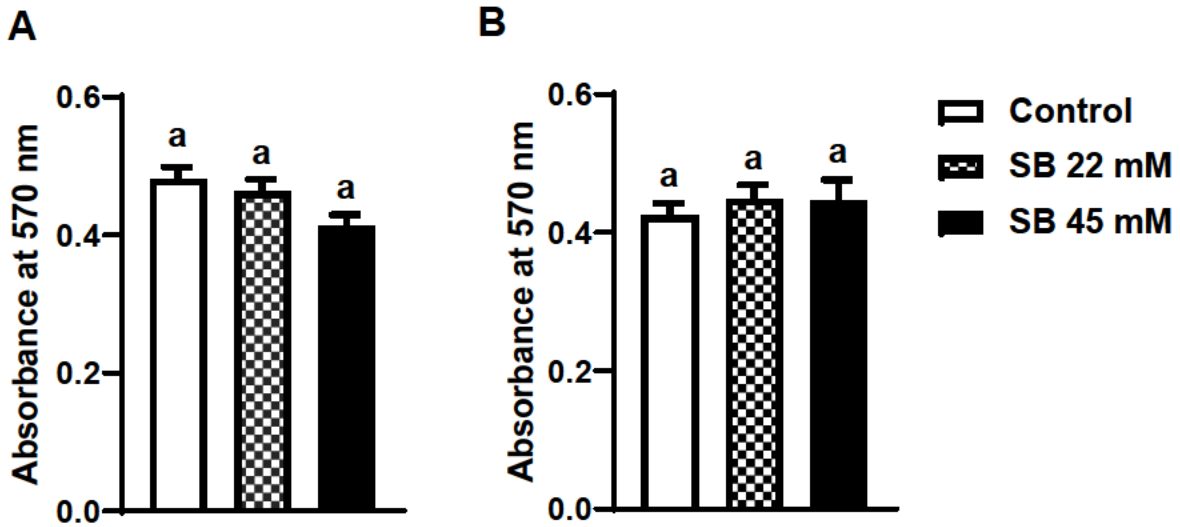


Figure 2.4 The effect of SICs of SB on cell viability of primary chicken enterocytes (A) and chicken macrophages (B) using MTT assay. Data presented as mean absorbance and error bar represents SEM ($n=6$). Bar with different letters represents a statistical difference at $P < 0.05$.

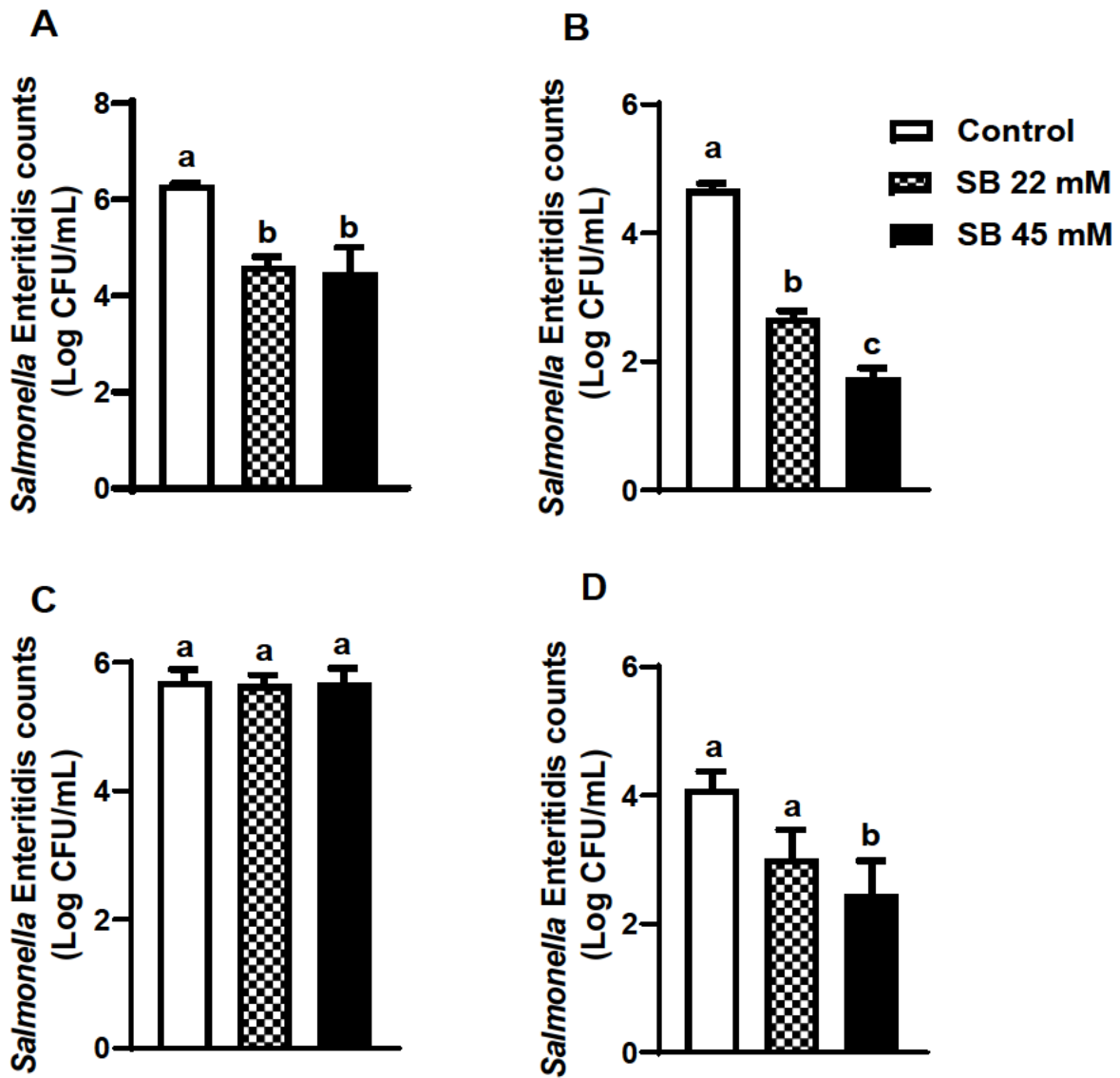


Figure 2.5 The effect of SICs of SB on SE adhesion (A, C) to and invasion (B, D) of primary chicken enterocytes (A, B) and chicken macrophages (C, D). Data presented as mean Log CFU/mL and error bars represents SEM (n=6). Bars with different letters represents a significant difference at $P < 0.05$.

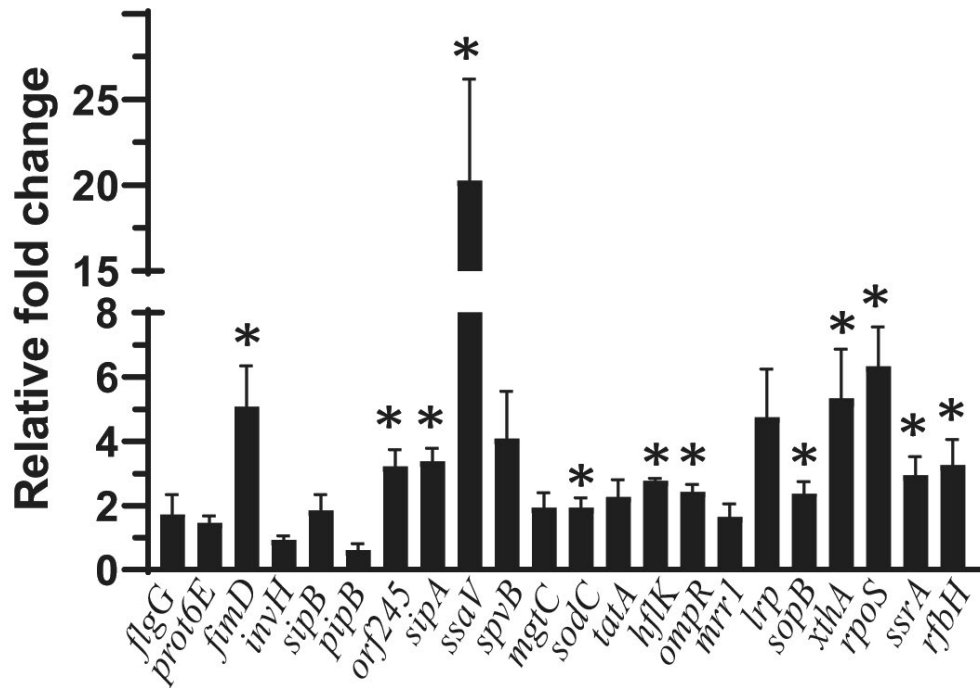


Figure 2.6 The effect of SIC 45 mM of SB on the expression of SE genes crucial for virulence and intestinal colonization. Data presented as relative fold change normalized to endogenous control, *16S* rRNA. *indicates significant change in the expression of genes at $P < 0.05$.

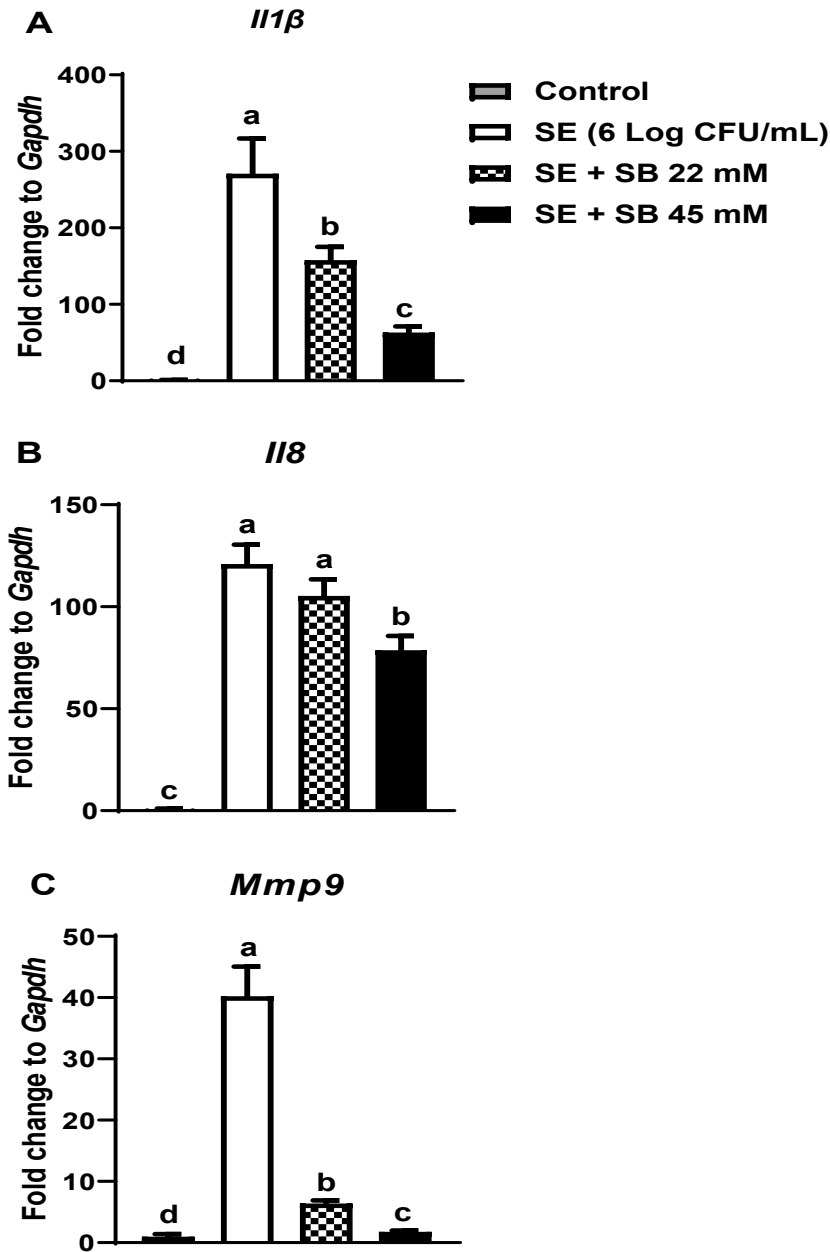


Figure 2.7 The effect of SICs (22 mM and 45 mM) of SB on the expression of inflammatory genes [*Il1b* (A), *Il8* (B), *Mmp9* (C)] in chicken macrophages challenged with SE. Data presented as fold change normalized to *Gapdh* and error bars represents SEM (n=6). Bars with different letters represents a significant difference at $P < 0.05$.

CHAPTER III

Butyrate influences the global protein expression of *Salmonella* Enteritidis infected chicken macrophages

3.1 ABSTRACT

Salmonella enterica serotype Enteritidis (SE), a gram-negative anaerobe, is one of the leading food borne pathogens. Poultry is the main reservoir for SE and the pathogen invades intestinal epithelial cell and immune cells such as macrophages. Butyrate is a microbiota metabolic product and an important energy source for intestinal epithelial cells. Currently limited knowledge is available on the interaction of SE, butyrate and host response. Therefore, the aim of this study was to understand how butyrate influenced the global protein expression of macrophages infected with SE. A growth curve assay was used to determine sub-inhibitory concentration (SIC) of sodium butyrate against SE strain GFP338. A naturally transformed chicken macrophage cell line (HTC cells) were cultured and the effect of SIC 45 mM sodium butyrate was determined on the proteome of chicken macrophages challenged with SE. The proteins were extracted, and the differentially regulated proteins were analyzed by tandem mass spectrometry. Our results showed that treatment of uninfected chicken macrophages with sodium butyrate downregulated ($P < 0.05$) proteins involved in glycolysis such as Triose-phosphate isomerase (TPI1), Phosphoglycerate mutase (PGAM1) and Phosphoglycerate kinase (PGK1); proteasome complex such as Proteasome subunit alpha type-7 (PSMA7); GTP binding proteins such as GNB1, RHOA and Ras-related protein Rab-18 (Rab18). SE infection in chicken macrophages upregulated proteins involved in cellular homeostasis and bacterial respiration such as ATP synthase subunit alpha (ATP5A1) and Cytochrome-c (CYC) which are important for its pathogenesis in chicken intestinal cells. Interestingly, sodium butyrate treatment in SE infected chicken macrophages downregulated proteins associated with actin cytoskeletal rearrangements such as WD repeat-containing protein-1 (WDR1), Alpha actinin-1 (ACTN1), and Vinculin (VCL); intracellular SE growth and replication (ATPV1A). Additionally, sodium butyrate treatment in SE infected chicken macrophages upregulated protein modulating innate immune

response and bacterial infection such as Vimentin (VIM). The results suggest that sodium butyrate modulates protein expression of SE infected chicken macrophages responsible for invasion, growth and survival.

Keywords: *Salmonella*, butyrate, host response, chicken macrophages, immune response, bacterial infection

3.2 INTRODUCTION

Salmonellosis is the number one food borne bacterial infectious disease (CDC, 2019). *Salmonella* Enteritidis (SE) is a major food borne pathogen causing the enteric disease in human primarily through consumption of contaminated poultry meat, eggs and poultry byproducts (Fearnley et al., 2011; Kollanoor-Johny et al., 2012; Antunes et al., 2016; Koutsoumanis et al., 2019). SE predominantly colonizes in the cecum of chickens and the chickens act as an asymptomatic carrier of SE without showing any clinical signs. The birds shed the pathogen in feces, which leads to contamination of egg yolk and shell membrane and carcass contamination during slaughter (Gantois et al., 2009; Upadhyaya et al., 2015). Therefore, a reduction in SE colonization in the intestinal tract of chickens could potentially decrease contamination of poultry meat, eggs and their byproducts and minimize risk of human health.

Salmonella has developed several strategies to alter host cell physiology for colonizing and penetrating the gastrointestinal tract of host (Larock et al., 2015). After invasion of gastrointestinal tract of the host, SE induces localized inflammation in the intestinal tract for its transmission inside the host (Agbor and McCormick, 2011; Larock et al., 2015). SE effector proteins activate Rho GTPase and induce mitogen activated protein kinase (MAPK) pathways, which produce interleukin-8 (IL-8) and contribute to intestinal inflammation in humans. In addition, Rho GTPase activates Caspase 1 in macrophages to secrete IL-1 β and IL-18, which further induces intestinal inflammatory response (Haraga et al., 2008; Wemyss and Pearson, 2019). Internalization of SE induces the formation of phagosome membrane *Salmonella* containing vacuole (SCV) for its survival and intracellular replication (Gart et al., 2016; Wemyss and Pearson, 2019).

Different approaches have been investigated to reduce SE colonization and persistence in chicken's intestinal tract but with a limited extent of success. Short chain fatty acids such as

butyric acid are microbial metabolites which promotes gastrointestinal health, serving as main energy sources for colonocytes, enhances epithelial barrier integrity, and inhibiting inflammation (Hamer et al., 2008; Kasubuchi et al., 2015). Butyric acid has been Generally Recognised as Safe (GRAS) for use in foods (Butyric acid- 21CFR182.60, Food and Drug Administration (FDA), 2017). We recently found that sodium butyrate was effective in reducing SE colonization and invasion *in-vitro* at a SIC of 45 mM in primary chicken enterocytes as compared to control. Furthermore, we observed that the same SIC of sodium butyrate also reduced the invasion of SE in chicken macrophages. In addition, sodium butyrate at 45 mM significantly downregulate the expression of inflammatory genes (*Il1 β* , *Il8* and *Mmp9*) in chicken macrophages challenged with SE.

Although previous findings revealed the effect of sodium butyrate on SE invasion in chicken macrophages at the transcriptional level, the protein expression of sodium butyrate on SE infected chicken macrophages is lacking. In light of the current state of knowledge, the objective of this study was to determine the effect of sodium butyrate on the proteome of chicken macrophages in the presence of SE.

3.3 MATERIALS AND METHODS

3.3.1 Chicken Macrophage cell line

Chicken macrophages also named HTC cells, a naturally transformed cell line were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Thermo Fisher Scientific, Carlsbad, CA) containing 10% fetal bovine serum (Thermo Fisher Scientific), 1X antibiotic antimycotic solution (Sigma-Aldrich, St Louis, MO, USA), 1X sodium pyruvate solution (Sigma-Aldrich), gentamicin solution (Sigma-Aldrich), 10 mM glutamine solution (Thermo Fisher Scientific) at 37°C for 24-48 h in a humidified 5% CO₂ incubator as described earlier

(Rath et al., 2003). The cells were cultured until semi-confluence followed by dissociation with Accutase (Sigma-Aldrich) to perform appropriate cell culture assays.

3.3.2 Effect of sodium butyrate on viability of chicken macrophages

Chicken macrophages (10^4 cells/well) were seeded in a 96-well plate (Costar, Corning Incorporated, Corning, NY) for 48 h at 37°C in a humidified incubator containing 5% CO₂ to form a monolayer. The chicken macrophages were incubated with 45 mM sodium butyrate for 4 h at 37°C. The cellular viability of chicken macrophages in response to sodium butyrate was determined by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sakurazawa and Ohkusa, 2005). The MTT reagent (10 µL) was added to the chicken macrophages and incubated at 37°C for 2 h. After removing the supernatant, 100 µL isopropanol (Sigma-Aldrich) was added and the plate was incubated at room temperature in dark for 1 h. The absorbance was measured at 570 nm by using spectrophotometric microplate reader (Benchmark; Bio-Rad Laboratories, Hercules, CA, USA).

3.3.3 Bacterial strains and culture conditions

SE GFP 338 was cultured in 10 mL of tryptic soy broth (TSB; Hardy Diagnostics CRITERION™, Santa Maria, CA, USA) at 37°C for 18 h. Following subculture in 10 mL TSB for another 10 h, the culture was centrifugated at 4000 rpm for 10 min. The pellet was suspended in sterilized phosphate buffer saline (PBS, pH 7) and used as the inoculum. The enumeration of SE counts in inoculum was made by plating serial 5-fold dilutions on brilliant green agar (BGA; Difco Laboratories, Detroit, Michigan, USA) and the plates were incubated at 37°C for 24 h for bacterial enumeration.

3.3.4 Proteomic sample collection, preparation and in-gel protein digestion

Chicken macrophages (10^5 cells per well) were seeded into 6-well plate (Costar) containing RPMI 1640 media with 10% FBS and incubated for 48 h at 37°C in a humidified, 5% CO₂ incubator to form a monolayer. A mid-log phase (10 h) culture of SE was inoculated on the chicken macrophages (~6 Log CFU/mL; multiplicity of infection 10:1) in the presence or absence of 45 mM sodium butyrate. Infected macrophages were incubated for 4 h followed by rinsing with 1% FBS RPMI 1640 media twice. The chicken macrophages were then lysed by M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) as described earlier (Rath et al., 2018; Rath et al., 2019).

Cell lysate were subjected to 4-20% gradient SDS Page gel electrophoresis and each sample was run in triplicate. Gel was stained with Coomassie blue and the gel segments were excised and triturated into small pieces followed by washing with 25 mM ammonium bicarbonate (NH₄HCO₃, Thermo Fisher Scientific). Destaining of gel segments was performed by adding 50% Acetonitrile (ACN, Bio-Rad Laboratories, Hercules, CA, USA) in 25 mM NH₄HCO₃ for 1 h followed by decanting all the detaining solution. Subsequently, 100% ACN was added to dehydrate gel pieces and evaporated to the dryness using Labconco centriyap. Reduction of proteins were performed by adding 10 mM Dithiothreitol (DTT, Bio-Rad) in 25 mM NH₄HCO₃ (1.5 mg/mL) to the dried gel pieces and keeping it at 60°C for 1 h. After 1 h, excess DTT was discarded and proceeded to alkylation 55 mM iodoacetamide (Bio-Rad) with 25 mM NH₄HCO₃ (10 mg/mL) at room temperature for 1 h in the dark. Excess iodoacetamide was completely removed and the gel pieces were rinsed with 25 mM NH₄HCO₃ followed by dehydration of gel pieces with ACN. Dehydrated gel pieces were then vacuum dried before adding MS Grade Trypsin (20 ng/mL in 25 mM NH₄HCO₃) and incubated overnight at 37°C. The peptides extracted were dried completely and resuspended in 0.1% formic acid for analyses

by Liquid chromatography tandem mass spectrometry (LC-MS/MS). Three samples were used for each group and data were analyzed individually for each sample.

3.3.4.1 Mass spectrometry analysis

LC-MS/MS was performed by using an Agilent 1200 series micro-flow high-performance liquid chromatography (HPLC) coupled to a Bruker AmaZon SL quadrupole ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA, United States) with a captive spray ionization source as described earlier (Rath et al., 2019, 2018; Karash et al., 2017). Tryptic peptides were separated by using C₁₈ capillary column (150 mm × 0.1 mm, 3.5 μm particle size, 300 Å pore size; ZORBAX SB) with 5-40% gradients of 0.1% formic acid (solvent A) and ACN in 0.1% formic acid (solvent B). Solvent flows at a rate of 4 μL/min over a duration of 300 min each.

LC-MS/MS data were acquired in positive ion mode. Bruker captive electro spray source was operated with a dry gas temperature of 150°C and a dry nitrogen flow rate of 3 L/min with captive spray voltage of 1500 volts. The data acquisition was in the Auto MS (n) mode optimized the trapping condition for the ions at m/z 1000. MS scans were performed in enhanced scanning mode (8100 m/z/second), MS/MS fragmentation scans performed automatically for top 10 precursor ions. The samples were run three times for each group as technical replicates and experiment was repeated two times for analyzing results.

By using Bruker Data Analysis 4.0 software, peaks were picked from LC-MS/MS chromatogram using default peak picking method recommended and to created Protein Analysis Results.xml file. This was used for searching Mascot database. In Mascot search, parent ion and fragment ion mass tolerances were set at 0.6 Da with cysteine carbamidomethylation as fixed modification and methionine oxidation as variable modifications. For the identification of

proteins in cell extracts, Mascot search was performed against Gallus UniProt database. Identification of proteins is with 95% confidence limit and with less than 5% false discovery rate (FDR). FDR was calculated in during the Mascot search by simultaneously searching the reverse sequence database. Uncharacterized Gallus proteins were identified based on gene sequence similarities tentatively. For evaluation of differentially expressed proteins, Mascot.dat files were exported to Scaffold Proteome Software version 4.8 and quantitative differences were determined based on 95% confidence limit. To determine the signaling pathway of proteins, the differentially regulated proteins were analyzed using software such as Protein Analysis through Evolutionary Relationships software (PANTHER) and STRING protein association network.

3.3.5 Statistical Analysis

The CFU counts of SE were logarithmically transformed (Log CFU) to maintain homogeneity of variance (Byrd et al., 2001). In the present study, we used triplicate samples and the experiment was repeated two times. Scaffold Proteome Software version 4.8 (Proteome Software Inc, Portland, OR) was used to analyze Mascot files for the proteomic analysis. Differentially expresses proteins were determined using Student's t-test and probability of $P < 0.05$ was required for statistically significant differences.

3.4 RESULTS

3.4.1 Effect of sodium butyrate on the proteome of chicken macrophages

Figure 1 showed the effect of 45 mM sodium butyrate on cell viability of chicken macrophages. 45 mM sodium butyrate did not inhibit growth of SE as compared to control ($P > 0.05$). Therefore, the highest SIC 45 mM of sodium butyrate was selected to culture chicken macrophages and the global protein expression was analyzed by proteomic assay. A total of 389 proteins were identified when chicken macrophages were treated with 45 mM sodium butyrate.

Quantitative comparison revealed downregulation of 28 proteins and upregulation of 14 proteins in sodium butyrate treated cells compared to control ($P < 0.05$), while 347 proteins were not significantly altered.

Specifically, sodium butyrate treatment in the chicken macrophages downregulated the expression of proteins associated with biological adhesion such as Basigin (BSG) (Table 1, Fig. 2). Sodium butyrate also reduced proteins of biological regulation including Non-specific serine/threonine protein kinase (ATM), Ryanodine receptor type-3 (RyR3), Ras-related protein Rab-18 (RAB18), GTP-binding protein (RHOA) and Inositol-1-monophosphatase (IMPA1). In addition, reduced expression of proteins was related with cellular component biogenesis such as ATM, Centromere protein E (CENPE), RHOA, Prostaglandin E synthase 3 (PTGES3) and BSG as well as cellular process proteins such as ATM, Saccharopine dehydrogenase (SCCPDH), UMP-CMP kinase (CMPK), CENPE, Triosephosphate isomerase (TPI1), Natural killer cell triggering receptor (NKTR), RyR3, RAB18, Proteasome subunit alpha type-7 (PSMA7), RHOA, PTGES3, IMPA1 and BSG. Also, proteins correlated with developmental process such as RHOA, Neuron navigator-3 (NAV3) and BSG and localization such as CENPE, RyR3, RAB18 and RHOA were reduced by sodium butyrate treatment in chicken macrophages. Similarly, proteins related with locomotion such as RHOA and BSG; metabolic process such as ATM, SCCPDH, TPI1, PSMA7 and IMPA1; multicellular organismal process such as NAV3 and BSG; response to stimulus such as ATM, RAB18, RHOA, IMPA1 and BSG and signaling namely ATM, RAB18, RHOA and IMPA1 were modulated by treatment of sodium butyrate in chicken macrophages (Table 1, Fig. 2)

Likewise, sodium butyrate treatment led to upregulation of proteins related with biological regulation like Proteasome activator complex subunit 3 (PSME3); cellular component

biogenesis includes Heat shock cognate 71 kDa protein (HSPA8), NSFL1 cofactor p47 (NSFL1C) and PSME3; cellular process such as HSPA8, Proteasome subunit alpha type (PSMA6), Aspartate aminotransferase (GOT1), Inosine-5'-monophosphate dehydrogenase (IMPDH2), Protein disulfide-isomerase (PDIA3), NSFL1C and PSME3. Similarly, proteins associated with localization such as HSPA8; metabolic process namely HSPA8, PSMA6, GOT1, IMPDH2, NSFL1C and PSME3; and response to stimulus such as HSPA8 and PDIA3 were also upregulated by treatment of sodium butyrate in chicken macrophages (Table 1, Fig. 2).

Signaling pathway analysis by STRING revealed that sodium butyrate treatment downregulated proteins involved in GTP binding, ATP binding, nucleotide binding, intracellular membrane bound organelle changes and metabolic activity inside the cell (Table 2, Fig. 3). Additionally, sodium butyrate upregulated proteins associated with regulation of cellular process, metabolic activity, innate immune system, IL-1 signaling, MAPK6/MAPK4 signaling pathway and activation of NF- κ B in the chicken macrophages (Table 3, Fig. 4).

3.4.2 Effect of SE on the proteome of chicken macrophages

Quantitative comparison showed downregulation of 22 proteins and upregulation of 9 proteins after SE infection in chicken macrophages compared to control ($P < 0.05$), however 358 proteins were not affected ($P > 0.05$). SE infection in chicken macrophages downregulated the protein expression correlated with various biological process. Proteins related with biological regulation such as ATM, Anaphase promoting complex subunit 1 (ANAPC1), Zinc finger protein 462 (E1C5J4), Actin-related protein 3 (ACTR3) and Zinc finger homeobox protein 4 (ZFHX4) were downregulated by SE infection in chicken macrophages. In addition, proteins related with cellular component biogenesis such as ATM, ANAPC1, CENPE, Hsc70-interacting protein (ST13), E1C5J4 and ACTR3; cellular process such as ATM, ANAPC1, CENPE, NKTR,

Ubiquitin-conjugating enzyme E2 (UBE2K), ST13, E1C5J4, ACTR3 and ZFHX4 were also downregulated after SE infection in chicken macrophages. Likewise, proteins involved in localization such as CENPE; metabolic process such as ATM, ANAPC1, UBE2K, E1C5J4 and ZFHX4; developmental process and multicellular organismal process such as NAV3; response to stimulus and signaling such as ATM were also modulated (Table 4, Fig. 5).

Furthermore, SE infected chicken macrophages upregulated the expression of proteins correlated with distinct biological processes. SE upregulated the protein expression related with biological regulation such as Cytochrome C (CYC); cellular component biogenesis and response to stimulus includes HSPA8. In addition, SE infection in chicken macrophages also upregulated proteins associated with cellular process such as HSPA8, ATP synthase subunit-d (ATP5PD), Peptidylprolyl isomerase (FKBP12), CYC, Bifunctional purine biosynthesis protein (ATIC) and Hydroxymethylbilane synthase (HMBS); localization such as CYC, HSPA8 and ATP5PD and metabolic process such as HSPA8, ATP5PD, FKBP12, CYC, ATIC and HMBS (Table 4, Fig. 5).

Signaling pathway analysis by STRING revealed that SE infection in chicken macrophages downregulated proteins involved in nucleotide binding, cytoplasmic and cytoskeletal changes and actin binding (Table 5, Fig. 6). Additionally, SE infected chicken macrophages upregulated proteins related with various metabolic pathways (Table 6, Fig. 7).

3.4.3 Effect of sodium butyrate on the proteome of chicken macrophages infected with SE

Quantitative profile determined downregulation of 14 proteins and upregulation of 6 proteins after treatment of SE infected chicken macrophages with 45 mM sodium butyrate ($P < 0.05$), whereas 369 proteins weren't affected ($P > 0.05$).

Sodium butyrate treatment in SE infected chicken macrophages downregulated proteins allied with different biological processes like biological regulation such as WD repeat-containing protein-1 (WDR1) and cellular component biogenesis such as ATP-dependent 6-phosphofructokinase (PFKP) and WDR1. Similarly, proteins associated with cellular process such as Protein disulfide-isomerase (P4HB), WDR1, PFKP and Rab GDP dissociation inhibitor (F1NCZ2); localization such as F1NCZ2; metabolic process such as PFKP and response to stimulus such as P4HB was modulated by sodium butyrate treatment in SE infected macrophages (Table 7, Fig. 8).

Moreover, sodium butyrate treatment in SE infected chicken macrophages upregulated proteins related with cellular component organization such as HSPB9, Ras-related protein Rab-11A (RAB11A), Vimentin (VIM) and Actin-related protein 2/3 complex (ARPC4); cellular process such as RAB11A, VIM, ATPSF1B and ARPC4; and metabolic process such as ENO1 and ATP5F1B (Table 7, Fig. 8).

Signaling pathway analysis by STRING revealed that sodium butyrate downregulated the protein expression of SE infected chicken macrophages plays role in actin filament binding, intracellular membrane bound changes, cellular homeostasis and cortical actin cytoskeletal changes (Table 8, Fig. 9). Additionally, sodium butyrate upregulated the proteins involved in membrane trafficking, endocytosis, metabolic pathways and cytoskeleton changes (Table 9, Fig. 10).

3.5 DISCUSSION

Salmonella is a food borne bacterial pathogen that colonizes the intestinal tract of chickens and induces inflammation for its transmission (Larock et al., 2015). We have recently found that sodium butyrate exerts its anti-inflammatory effect on SE-infected macrophages by

downregulation of inflammatory cytokine mediators. However, it remains elusive how sodium butyrate influence proteins expression in cells infected with SE. Therefore, the aim of our study was to determine the proteomic changes in chicken macrophages infected with *Salmonella* Enteritidis in response to treatment with sodium butyrate.

One of the notable findings was that sodium butyrate treatment downregulated proteins associated with various biological processes. For example, sodium butyrate reduced cellular metabolic process of glycolysis proteins PGK1, PGAM1, and TPI1. Since sodium butyrate is an energy source for cells, it is reasonable to expect that sodium butyrate reduce cellular energy generating activity. Besides its role on energy metabolism, glycolysis is essential in regulating immune responses. Elevated glycolysis increases inflammatory IL-17 signaling pathways and increases infiltration of inflammatory cells such as neutrophils (Transl et al., 2020).

Inflammation leads to increase rate of glycolysis and lactate production in primary mixed glia cells of brain (Giri et al., 2019). In addition, Berg Soren et al. (2003) reported that pro-inflammatory cytokines increase the rate of glycolysis in rat enterocytes. All these findings suggest that sodium butyrate will reduce inflammatory response. In consistence, sodium butyrate reduced inflammatory gene expression in uninfected macrophage cells.

Sodium butyrate reduced expression of protein PSMA7. This protein constitutes a proteasome complex that causes proteolytic degradation of proteins present inside the cells through ubiquitin –proteasome pathway and target proteins, which regulates cell cycle, cell proliferation, apoptosis, immune response and antigen processing (Du et al., 2009). Yang et al. (2013) revealed that PSMA7 interacts with NOD1, which is a cytoplasmic protein of Nod-like receptor family and modulates its function in colorectal cancer. NOD1 induces the secretion of pro-inflammatory cytokines by the activation of NF- κ B and MAPK pathways. The reduced

PSMA7 is in accordance with the reduced inflammatory response in uninfected chicken macrophages.

Sodium butyrate reduced expression of nucleotide binding proteins (CMPK, PAICS, CENPE) and proteins of intracellular protein transport and signal transduction (RHOA, RAB18). Nucleotide synthesis is a major biological process, which helps in cell proliferation and maintenance of physiological functions inside the cells (Xu et al., 2008). UMP-CMP kinase catalyzes the phosphorylation of CMP and UMP, which forms UDP and CDP and helps in nucleic acid synthesis (Sugino et al., 1966; Liou et al., 2002). Muller et al. (2017) reported that protein expression of nucleotide metabolic process such as CMPK1, NME2, PRPS2, ADSL altered after treatment with 2 mM butyrate. GNB1 are G-proteins attached to cell membrane of cytoplasm and plays role in many cellular processes such as signal transduction, protein transport. GNB1, RHOA and Rab18 are GTP binding proteins, which convert inactive GDP to active GTP form and plays an important role in signal transduction (Vincent and Settleman, 1997; Herroeder et al., 2009). RhOA proteins are small GTPases that helps in actin cytoskeleton arrangements (Bros et al., 2019). Rab18 are small GTPases that regulates ER-Golgi trafficking (Stenmark, 2009; Kiral et al., 2018). Our results suggest that sodium butyrate reduced nucleotide binding and protein transport and signal transduction in uninfected cells.

Notably, sodium butyrate increased expression of proteins regulating metabolism such as PSME3, IMPDH2, ATP5A1, ATP5A1W, MDH2, PSMA6. Protein catabolism pathways play crucial role in maintaining normal physiological functions, host defense and immune response (Jiang and Chen, 2012). PSME3 is a proteasome protein that activates NF- κ B signaling pathway and promotes cell survival (Nicole Jung-Eun Kim et al., 2017; Xie et al., 2019). IMPDH catalyzes conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP)

which is a first rate-limiting step in de novo synthesis of guanidine monophosphate (Calise et al., 2018) and plays role in cellular proliferation and immune response (Zimmermann et al., 1998). ATP5A1 catalyzed ATP synthesis in mitochondria during oxidative phosphorylation. Xiaoyun et al. (2017) reported that mitochondrial membrane ATP synthase (F₁F₀ ATP synthase) plays critical role in cell energy metabolism and cellular homeostasis through ATP synthesis. Wang et al. (2018) also showed that sulfhydrylation of ATP5A1 maintains ATPase activity and survival of adrenocortical cells. Sodium butyrate may increase these metabolism proteins to promote cell survival. Similarly, sodium butyrate treatment also upregulated proteins associated with cellular process such as PDIA3. This protein is an isomerase enzyme located in endoplasmic reticulum and regulates folding of glycoproteins by promoting disulfide bonds in the glycoprotein substrates to prevent protein aggregation (Zhuang et al., 2015). PDIA3 interacts with ERP27 to regulate cytokine dependent signal transduction mediated by STAT3 signaling. In addition, PDIA3 modulates cell growth in bone metastasis and interacts with cytoskeletal proteins such as vimentin for protein binding (Santana-Codina et al., 2013). Sodium butyrate may increase expression of PDIA3 to regulate STAT3 signaling in uninfected cells.

SE downregulated the cellular protein expression of SCIN, ACTR3 and ARPC4 as compared to uninfected control cells. The three molecules are actin-binding proteins and regulates actin cytoskeleton by capping F-actin and nucleating F actin filaments (Hassanpour et al., 2014). Actin related protein complex such as ARPC4 promotes actin cytoskeletal nucleation and results in cell migration (Su et al., 2018). Hanisch et al. (2011) reported that *Salmonella* invasion in host cells requires activation of RHOA/Myosin-II pathway but ARP 2/3 complex-independent pathway. Further work investigating the effect of SCIN, ACTR3 and ARPC4 on influencing SE is needed. In contrast, SE upregulated proteins related with ATP synthesis such

as ATP5A1 that maintains cellular homeostasis (Xiaoyun et al., 2017). In addition, CYC protein was also upregulated in present of SE challenge. CYC is a cytochrome protein and involved in bacterial respiration during pathogenesis of *Salmonella* in host cells (Thony-Meyer, 1997; Jones-Carson et al., 2016).

Interestingly, in SE-infected chicken macrophages sodium butyrate downregulated proteins associated with cellular homeostasis, cortical actin cytoskeleton and anatomical structural morphogenesis such as WDR1, ACTN1, VCL, P4HB and ATP6V1A. WDR1 is an actin interacting protein plays role in actin cytoskeleton modifications and WDR1 mutation causes lymphoid immune defect disease (Pfajfer et al., 2018). VCL is a cytoskeletal actin binding protein and maintains various physiological processes, such as adhesion and motility, by promoting actin polymerization and binding to specific phospholipids (Izard and Brown, 2016; Bays and DeMali, 2017). ACTN1 is a cytoskeleton actin binding protein and regulates cell-cell matrix adhesion. Hamill et al. (2015) reported that ACTN1 controls motility of keratinocytes by normalizing actin cytoskeleton and focal adhesion proteins, which results in cell migration. ATP6V1A is a vacuolar ATPase and acidifies intracellular compartments inside cells to increase permeability of endosomes, vesicular swelling and intracellular bacterial growth. Expression of ATPV1A significantly increases in macrophages during *Salmonella* infection for its intracellular replication (Huang et al., 2018). Sodium butyrate may downregulate the expression of ATPV1A in SE challenged macrophages to inhibit the intracellular growth of SE. In addition, sodium butyrate downregulated CTSD protein expression, a soluble lysosomal aspartic endopeptidase responsible for causing degradation of proteins. Dien et al. (2008) revealed that CTSD is a major lysosomal enzyme causes α -synuclein degradation, which plays a crucial role in the development of Parkinson's disease. Qiao et al. (2008) also reported that CTSD protects against α -synuclein, a

critical component of Lewy bodies that exists in many neurodegenerative diseases such as Parkinson's disease, dementia and atrophy.

In contrast, sodium butyrate treatment upregulated protein associated with cytoskeletal changes such as VIM in SE infected chicken macrophages. VIM is an intermediate filament protein present in cytoskeleton, maintains cell integrity and promotes many cellular processes such as cell adhesion, immune response and autophagy (Mak and Bruggemann, 2016). Morvaknin et al. (2003) reported that VIM released by activated macrophages helps in bacterial killing and production of oxidative metabolites, in response to pro-inflammatory signaling pathways. *Salmonella* infection in host intestinal epithelium induces actin cytoskeletal rearrangements and promotes pro-inflammatory cytokine immune response mediated by MAP kinase for its invasion and survival. However, VIM protein inhibits pro-inflammatory immune response and involved in bacterial killing (Mak and Brüggemann, 2016). In addition, VIM modulates apoptosis of immune cells and inflammatory response in patients with sepsis (Su et al., 2019). Also, sodium butyrate treatment upregulated ENO1, a glycolytic enzyme expresses in cytosol and involved in autoimmune and inflammatory diseases (Rottenberg et al., 2016). Bae et al. (2012) showed that cell surface expression of ENO1 was enhanced in hematopoietic cells during inflammatory process and antibody against ENO1 stimulates these cells to produce pro-inflammatory mediators such as IL-1 β , TNF- α through MAPK and NF- κ B pathway.

3.6 CONCLUSIONS

In this study, we have found that sodium butyrate treatment in chicken macrophages reduced expression of proteins involved in glycolysis, which would be increased during pro-inflammatory signaling pathways. In addition, sodium butyrate treatment downregulated proteins which secrete pro-inflammatory cytokines by activating MAPK and NF- κ B pathway. GTP

binding proteins were also downregulated by sodium butyrate treatment in chicken macrophages, which cause actin cytoskeletal modifications inside host cells. We have also observed that sodium butyrate treatment in SE infected chicken macrophages significantly downregulated actin binding proteins, essential for host cytoskeleton rearrangements inside host cells after SE infection. Interestingly, sodium butyrate treatment in SE infected chicken macrophages also downregulated protein responsible for intracellular growth of SE in the intestinal tract of chickens. Collectively, these results suggest that sodium butyrate modulates protein expression essential for SE invasion and replication in chicken intestine.

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Table 3.1 Differentially regulated proteins in chicken macrophages by sodium butyrate (SB) treatment

Proteins downregulated by SB	Alternate id	Accession number	M.Wt (kDa)	Fold change	P<0.05
Phosphoglycerate kinase	PGK1	F1NU17	45	0.7	0.03
Phosphoglycerate mutase	PGAM1	F1NHM9	29	0.5	0.0058
Triosephosphate isomerase	TPI1	TPIS	27	0.8	0.015
Uncharacterized protein	PAICS	R9PXP8	48	0.4	0.036
Proteasome subunit alpha type-7	PSMA7	PSA7	28	0.7	0.037
Uncharacterized protein	DNAH9	F1NVK1	482	0.4	0.0095
Basigin	BSG	BASI	42	0.7	0.0072
Ryanodine receptor type 3	RyR3	Q90985	553	0.1	0.014
Uncharacterized protein	NAV3	F1NAH8	250	0	0.0082
GTP-binding protein	RHOA	O93467	22	0.04	0.019
Non-specific serine/threonine protein kinase	ATM	E1C0Q6	348	0.3	0.038
Ras-related protein Rab-18	RAB18	RAB18	23	0.2	0.047
Receptor expression-enhancing protein	REEP5	E1BZN6	21	0.1	0.047
UMP-CMP kinase	CMPK	KCY	22	0.1	0.0042
Uncharacterized protein	CENPE	E1BQJ6	258	0.1	0.01
Uncharacterized protein	SSB	F6R1X6	46	0	0.0027
Uncharacterized protein	HSPA4L	F1NC26	95	0.3	0.035
Prostaglandin E synthase 3	PTGES3	A0A1D5PZE1	19	0.08	0.036
Uncharacterized protein	GNB1	A0A1L1RM11	44	0	0.046
PRA1 family protein 3	ARL6IP5	PRAF3	22	0	0.039
Uncharacterized protein	GMFB	A0A1D5PTE8	17	0.3	0.041
Uncharacterized protein	AKR1B10L1	F1NT57	37	0	0.032
Natural killer cell triggering receptor	NKTR	A0A1D5PRM6	161	0.2	
Uncharacterized protein	SCCPDH	R4GHA5	47	0	0.0044
Adseverin	SCIN	A0A1D5PBC3	79	0	0.0022
Ribonuclease/angiogenin inhibitor 1	RNH1	Q5ZIY8	50	0	0.0038
Inositol-1-monophosphatase	IMPA1	A0A1D5PHE2	30	0	0.0095

Table 3.1 (Cont.)

Proteins upregulated by SB	Alternate id	Accession number	M.Wt	Fold change	P<0.05
Heat shock cognate 71 kDa protein	HSPA8	F1NWP3	71	1.6	0.0095
Malate dehydrogenase	MDH2	E1BVT3	37	1.3	0.045
ATP synthase subunit alpha	ATP5A1	A0A175KE65	60	1.6	0.0064
Protein disulfide-isomerase A3	PDIA3	PDIA3	56	1.8	0.00023
Stress-70 protein, mitochondrial	HSPA9	F1NZ86	73	1.8	0.037
Inosine-5'-monophosphate dehydrogenase	IMPDH2	Q5F4A4	56	2	0.02
Annexin A5	ANXA5	ANXA5	36	1.7	0.025
Proteasome subunit alpha type	PSMA6	F1NEQ6	27	1.3	0.035
Proteasome activator complex subunit 3	PSME3	PSME3	29	4.1	0.0087
Na(+)/H(+) exchange regulatory cofactor NHERF	SLC9A3R1	F1NK29	36	2.8	0.019
Uncharacterized protein	WDR77	A0A1D5PD41	38	4.8	0.0027
Aspartate aminotransferase, cytoplasmic	GOT1	AATC	46	3.6	0.023
NSFL1 cofactor p47	NSFL1C	NSF1C	41	6.9	0.045
ATP synthase subunit alpha	ATP5A1W	A0A182C637	60	1.5	0.0098

Table 3.2 Pathways downregulated by SB treatment in chicken macrophages

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GO0005525	GTP binding	2	0.0226
2	GO0044237	Cellular metabolic process	7	0.0118
3	GGA392451	G beta: gamma signaling through PI3Kgamma	2	0.0154
4	GO0005488	Binding	7	0.0435
5	GGA109582	Hemostasis	5	0.0166
6	KW0547	Nucleotide binding	9	0.0017
7	GO0043231	Intracellular membrane-bounded organelle	7	0.0338
8	GGA1660598	Metabolism	4	0.00035
9	KW0067	ATP-binding	6	0.0427

Table 3.3 Pathways upregulated by SB treatment in chicken macrophages

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GO0050794	Regulation of cellular process	5	0.0375
2	GGA9020702	Interleukin-1 signaling	2	0.0131
3	GGA5687128	MAPK6/MAPK4 signaling	2	0.0131
4	GGA5668541	TNFR2 non-canonical NF-kB pathway	2	0.0131
5	GGA5658442	Regulation of RAS by GAPs	2	0.0131
6	GGA168256	Immune System	4	0.0131
7	GGA1430728	Metabolism	6	0.0131
8	GGA1169091	Activation of NF-kB in B cells	2	0.0131
9	GGA168249	Innate Immune System	3	0.0162
10	GGA597592	Post-translational protein modification	3	0.042

Table 3.4 Differentially regulated proteins in chicken macrophages after *Salmonella* Enteritidis (SE) infection

Proteins downregulated by SE	Alternate id	Accession number	M.Wt	Fold change	P<0.05
Uncharacterized protein	DNAH9	F1NVK1	482	0.2	0.032
Ryanodine receptor 2		F1NLZ9	563	0.2	0.035
Uncharacterized protein	NAV3	F1NAH8	250	0	0.0082
Biorientation of chromosomes in cell division 1 like 1	BOD1L1	R4GKR8	329	0	0.032
Actin-related protein 3	ACTR3	ARP3	47	0.4	0.0074
Zinc finger homeobox protein 4	ZFHX4	ZFHX4	395	0	0.0049
Non-specific serine/threonine protein kinase	ATM	E1C0Q6	348	0.2	0.022
Spectrin beta chain	SPTBN1	A0A1D5PJY1	274	0	0.013
Collagen type V alpha 2 chain	COL5A2	A0A1D5P6W1	145	0.1	0.029
Elongation factor 1-alpha	EEF1A1	A0A1L1RRR1	49	0.2	0.02
Zinc finger protein 462		E1C5J4	278	0	0.014
Actin-related protein 2/3 complex subunit 4	ARPC4	F1P010	20	0.3	0.049
Anaphase promoting complex subunit 1	ANAPC1	E1C2U7	216	0	0.024
Uncharacterized protein	CENPE	E1BQJ6	258	0	0.001
Uncharacterized protein	GMFB	A0A1D5PTE8	17	0.09	0.015
Uncharacterized protein	A0A1D5P0W7	A0A1D5P7P7	25	0.2	0.02
Natural killer cell triggering receptor	NKTR	A0A1D5PRM6	161	0	0.0069
Hsc70-interacting protein	ST13	A0A1L1RVN1	30	0	0.029
Terpene cyclase/mutase family member	LSS	A0A1D5PDR0	85	0	0.032
Adseverin OS=Gallus gallus	SCIN	A0A1D5PBC3	79	0.1	0.011
Uncharacterized protein	UBE2K	A0A1L1RJI2	22	0.1	0.0087
Histidine triad nucleotide binding protein 2	HINT2Z	R4GGS3	17	0	0.0076

Table 3.4 (Cont.)

Proteins upregulated by SE	Alternate id	Accession number	M.W t	Fold change	P<0.05
Heat shock cognate 71 kDa protein	HSPA8	F1NWP3	71	1.2	0.0017
Bifunctional purine biosynthesis protein	ATIC	F7AXZ3	69	1.3	0.025
Peptidylprolyl isomerase	FKBP12	Q90ZG0	12	2	0.043
Hydroxymethylbilane synthase	HMBS	A0A1D5NYN8	37	2.6	0.04
EF-hand domain family member D2	EFHD2	A0A1D5PD25	25	3.5	0.017
Uncharacterized protein		A0A1D5P4K6	20	2.5	0.02
ATP synthase subunit d, mitochondrial	ATP5PD	E1C658	18	2	0.042
ATP synthase subunit alpha	ATP5A1	A0A182C637	60	1.3	0.039
Cytochrome c	CYC	CYC	12	12	0.03

Table 3.5 Pathways downregulated by SE infected chicken macrophages

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GO0051303	Establishment of chromosome localization	2	0.0337
2	GO0030833	Regulation of actin filament polymerization	2	0.0337
3	GO0007049	Cell cycle	3	0.0337
4	GO0043232	Intracellular non-membrane-bounded organelle	5	0.009
5	KW0206	Cytoskeleton	5	0.00028
6	KW0009	Cytoplasm	7	0.0018
7	KW0547	Nucleotide binding	6	0.0167
8	KW0009	Actin-binding	4	0.00047

Table 3.6 Pathways upregulated by SE infected chicken macrophages

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GGA1592230	Mitochondrial biogenesis	2	0.0016
2	GGA163200	Respiratory electron transport, ATP synthesis	2	0.007
3	GGA01100	Metabolic pathways	4	0.0084
4	GO0009167	Purine ribonucleoside monophosphate metabolic process	2	0.0197

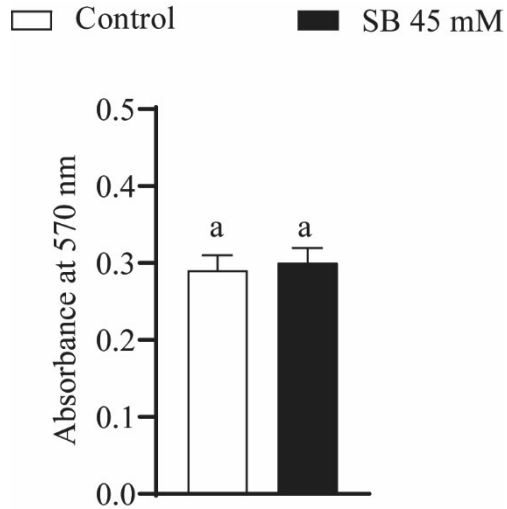


Figure 3.1 Effect of SIC of sodium butyrate (SB) 45 mM on viability of chicken macrophages using MTT assay. Chicken macrophages were treated with SB at 45 mM for 4 h. Data presented as mean absorbance and error bar represents SEM (n=6). Bar with different letters represents a statistical difference at $P < 0.05$.

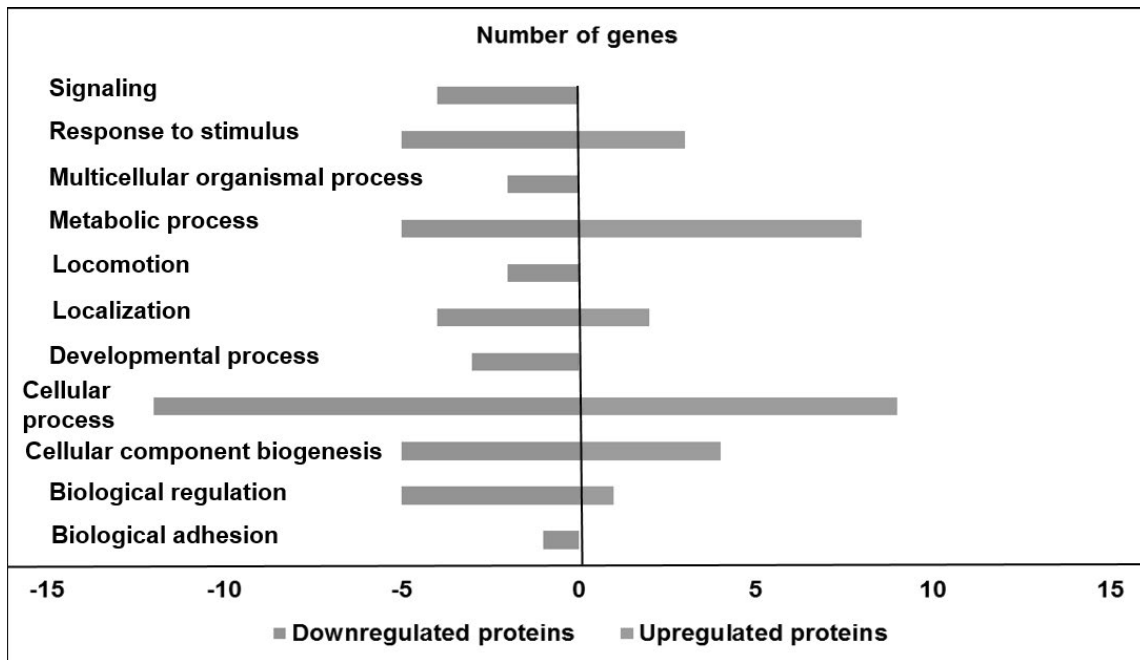


Figure 3.2 SB treatment in chicken macrophages induced down and upregulated proteins in different biological processes

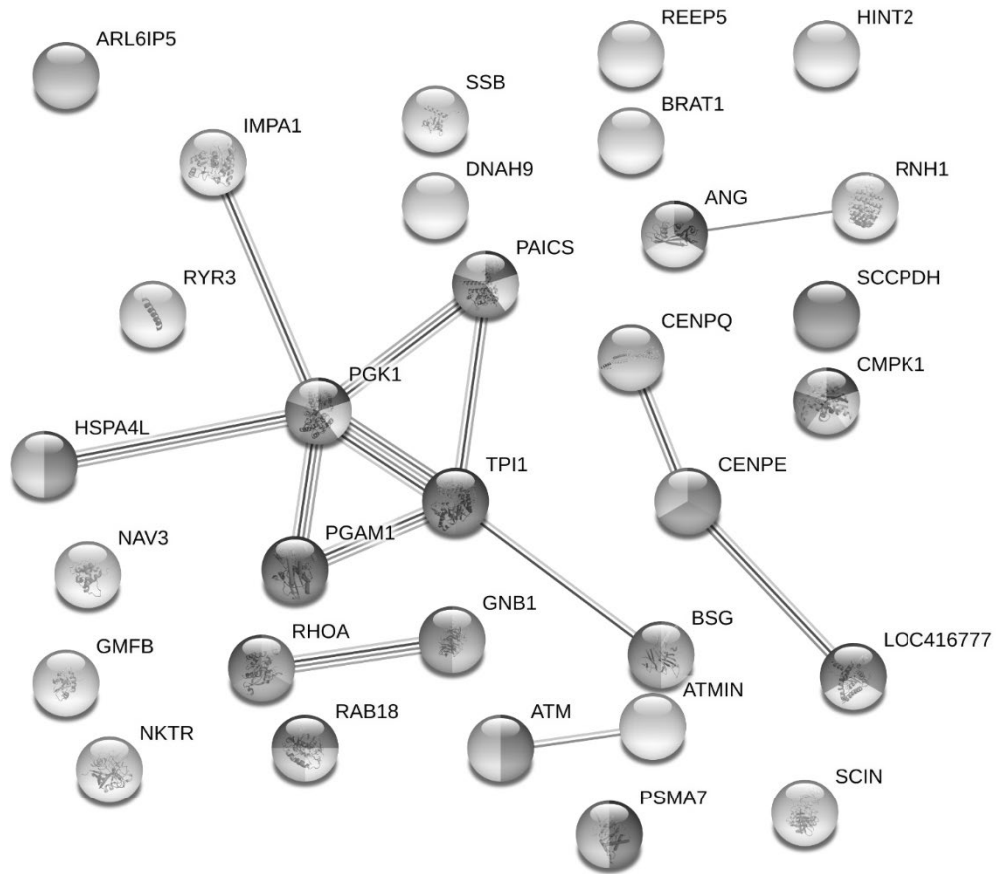


Figure 3.3 Signaling pathway analysis by STRING showed pathways downregulated by treatment of SB in chicken macrophages.

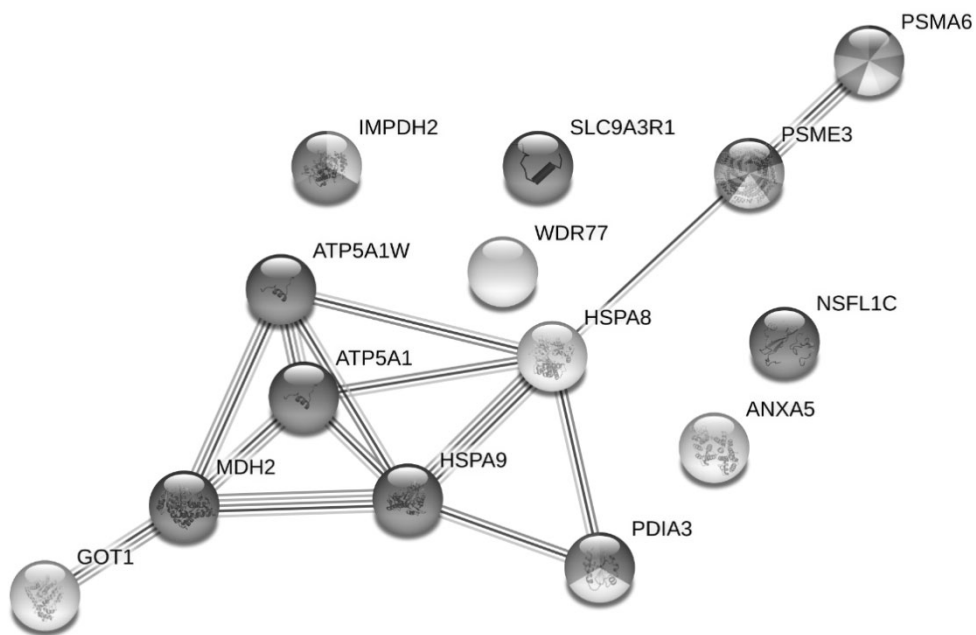


Figure 3.4 Signaling pathway analysis by STRING showed pathways upregulated by treatment of SB in chicken macrophages

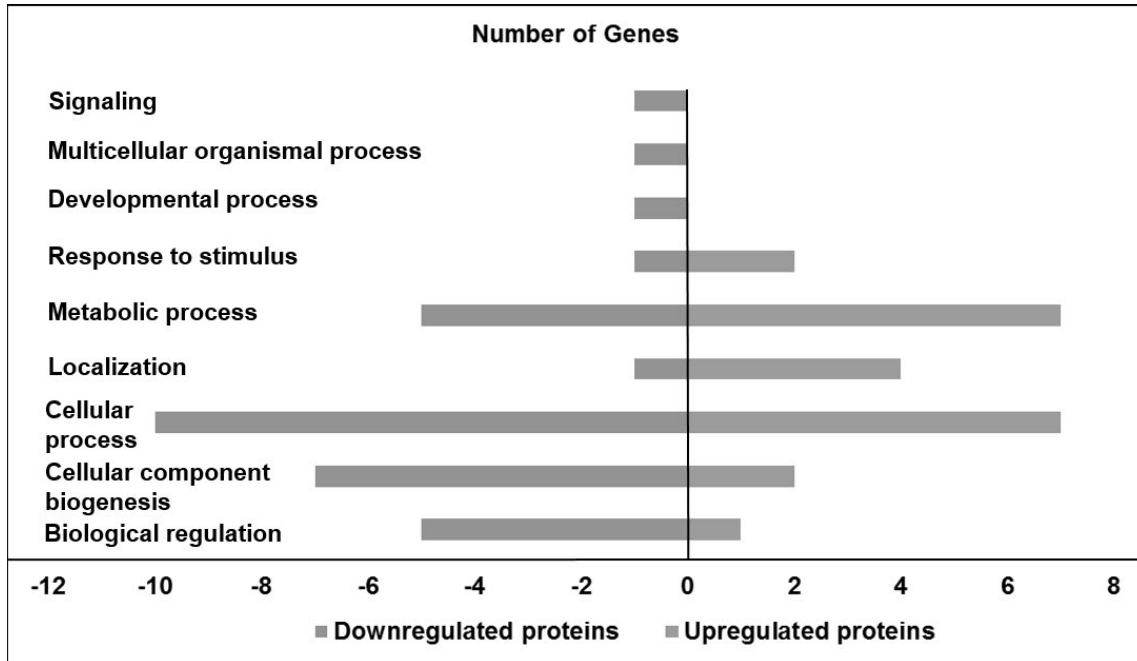


Figure 3.5 *Salmonella* Enteritidis (SE) infection in chicken macrophages induced down and upregulated proteins in different biological processes

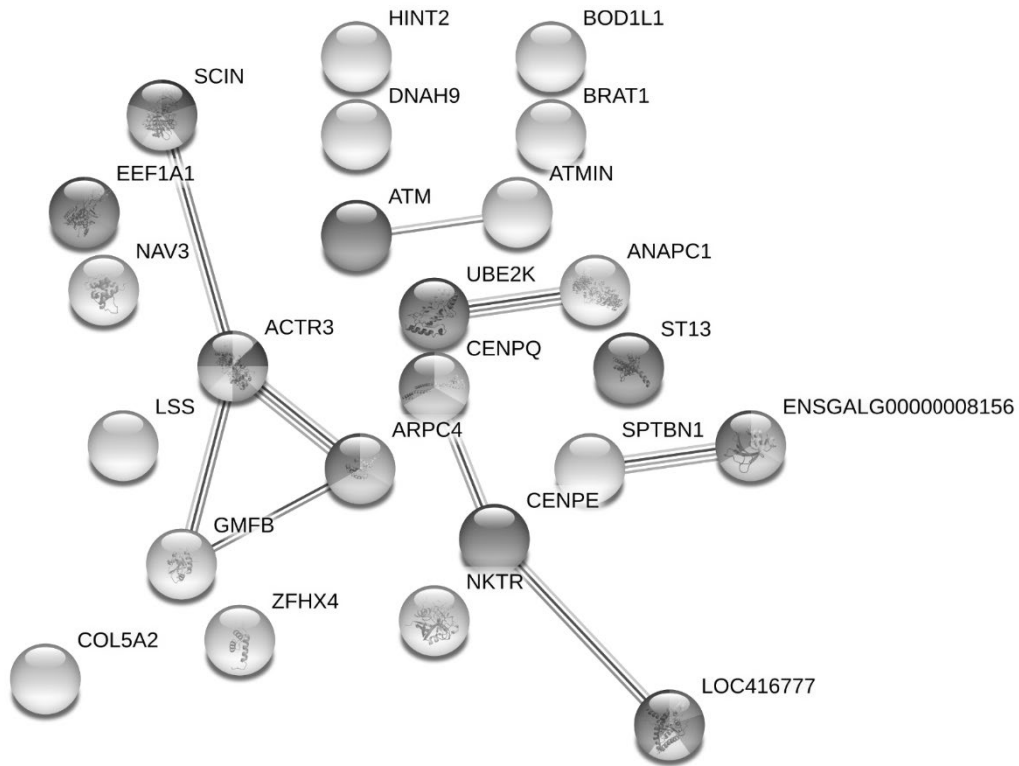


Figure 3.6 Signaling pathway analysis by STRING showed pathways downregulated by SE infection in chicken macrophages

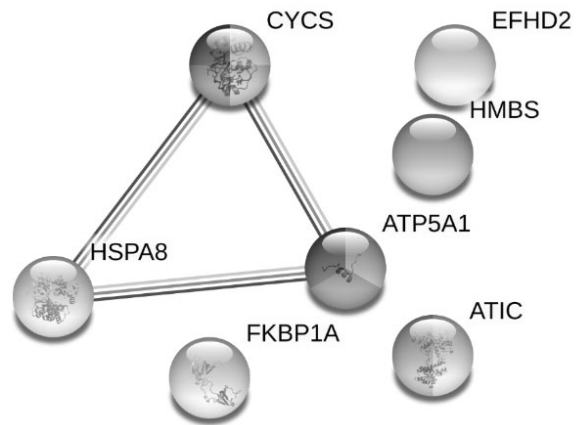


Figure 3.7 Signaling pathway analysis by STRING showed pathways upregulated by SE infection in chicken macrophages

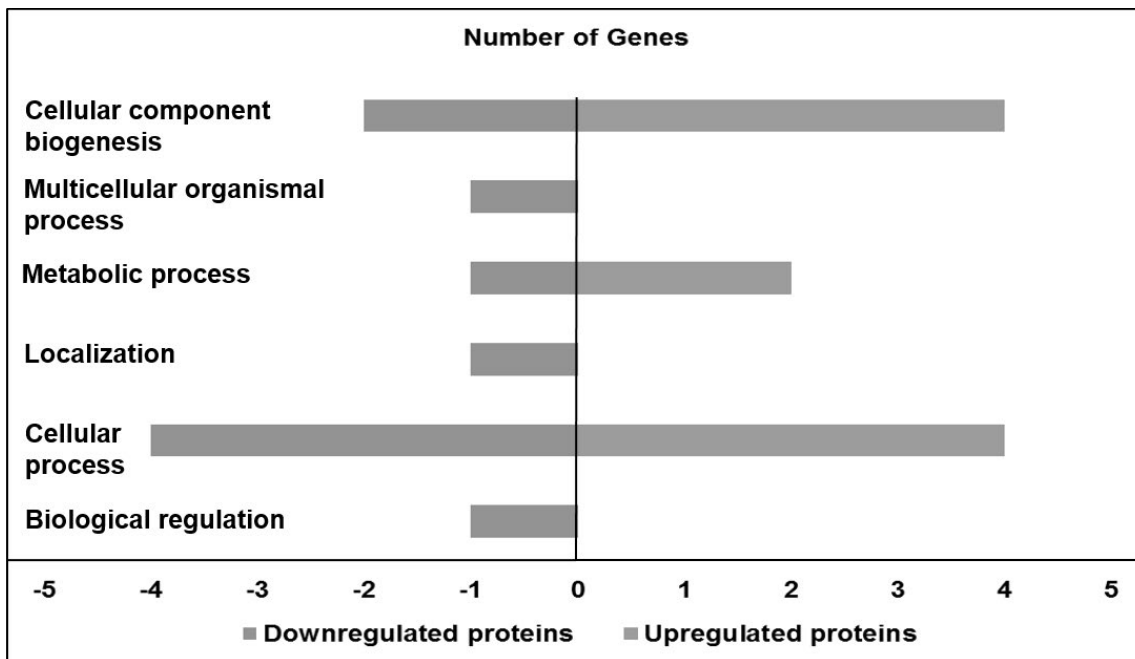


Figure 3.8 SB treatment in SE infected chicken macrophages induced down and upregulated proteins in different biological processes

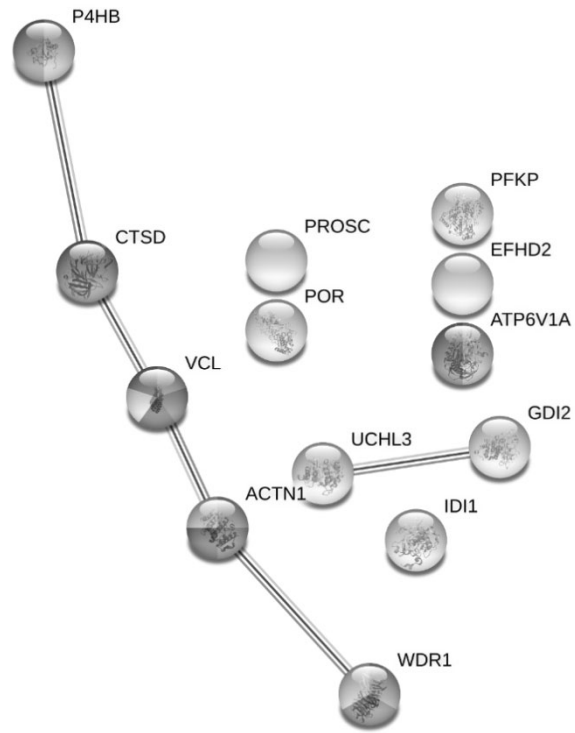


Figure 3.9 Signaling pathway analysis by STRING showed pathways downregulated by SB treatment in SE infected chicken macrophages

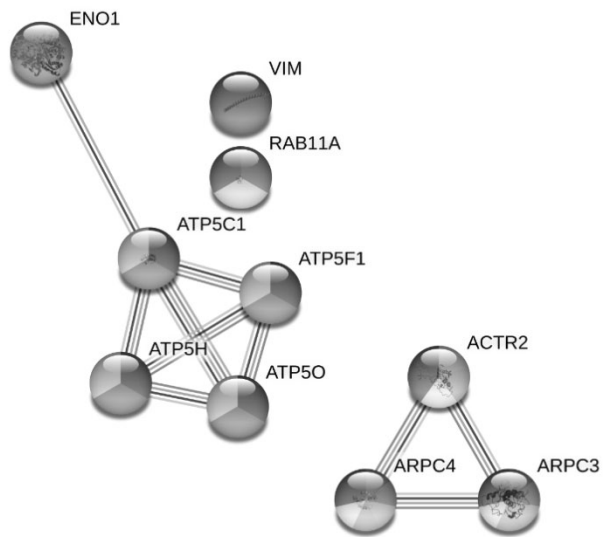


Figure 3.10 Signaling pathway analysis by STRING showed pathways upregulated by SB treatment in SE infected chicken macrophages

CHAPTER IV

Sodium butyrate modulates chicken intestinal epithelial proteins responsible for *Salmonella* Enteritidis colonization and replication

4.1 ABSTRACT

Salmonella Enteritidis (SE) is an intracellular poultry associated food borne pathogen that has developed multiple mechanisms to alter chicken intestinal physiology to effectively infect the gut. Butyrate, a short chain fatty acid from microbiota metabolism, maintains gut homeostasis and plays a protective role against enteric pathogens. However, there is limited information on the mechanism of interaction among SE, butyrate and intestinal host response. To elucidate the effect of sodium butyrate on SE-infected chicken intestinal cellular pathways, proteomic analysis was performed on primary chicken enterocytes infected with SE and treated with sodium butyrate. The effect of sub-inhibitory concentration (SIC, concentration not inhibiting bacterial growth) of sodium butyrate at 45 mM was studied on the viability and proliferation of primary chicken enterocytes. The primary chicken enterocytes were cultured (10^5 cells per well) and the effect of sodium butyrate was determined on the proteome of primary chicken enterocytes infected with SE. The proteins were extracted, and the differentially regulated proteins were identified by tandem mass spectrometry (LC-MS/MS). The results showed that the treatment of primary chicken enterocytes with sodium butyrate downregulated ($P < 0.05$) proteins involved in glycolysis such as Triose-phosphate isomerase (TPI1) and Aldolase A; regulation of actin cytoskeleton such as GTP binding protein (RHOA) and Serine/threonine-protein phosphatase (PPP1CC); membrane trafficking such as 14-3-3 protein (YWHAQ, YWHAZ) and Cathepsin Z (CTSZ), compared to control cells. In addition, sodium butyrate in SE infected primary chicken enterocytes downregulated ($P < 0.05$) proteins involved in pro-inflammatory immune response and phagocytosis such as Annexin A1 (ANXA1); intracellular trafficking of SE such as Ras related protein (RAB1A, RAB11B); host actin cytoskeleton rearrangements such as, Alpha-actinin-4 (ACTN4), Tropomyosin alpha-3 chain (TPM3), Actin related protein 2/3 complex subunit 5 (ARPC5), Actin related protein 2 (ACTR2), WD repeat-containing protein 1 (WDR1), Capping

actin protein, gelsolin (CAPG). In contrast, sodium butyrate treatment in SE infected cells upregulated ($P<0.05$) protein involved in bacterial killing during pro-inflammatory signaling pathway such as Vimentin (VIM). Collectively, sodium butyrate reduced proteins involved in glycolysis, pro-inflammatory immune response, host actin cytoskeleton changes and intracellular trafficking of SE. The results suggest that sodium butyrate can be used as an anti-inflammatory compound to reduce SE infection in chicken intestinal tract.

Keywords: *Salmonella*, primary chicken enterocytes, sodium butyrate, proteomics, anti-inflammatory

4.2 INTRODUCTION

In the United States, *Salmonella* Enteritidis (SE) is one of the major food borne pathogens causing enteric illness in humans and increases public health concerns (Heredia and García, 2018). Humans usually acquired SE infection through contaminated poultry and poultry-derived products, which are vital food products associated with human salmonellosis (Foley et al., 2011). SE colonizes in the intestinal tract of chickens and spreads to the environment through feces, which further increases contamination of chicken carcass during slaughter and poultry processing (Kollanoor-Johny et al., 2012). Despite using various pre-harvest and post-harvest strategies to reduce Salmonellosis, SE infection is still an extensive problem in poultry industry with increased incidences since SE has developed strategies to adapt in the chicken intestinal tract mainly ceca (Betancor et al., 2010; Galis et al., 2013). Hence, reducing SE in the intestine of chickens would reduce chances of contamination of poultry derived foods and minimize risk of human infection.

SE interacts with host intestine through different bacterial proteins, which manipulates host intestinal immune responses and leads to induction of inflammatory response for its pathogenesis and systemic dissemination from the chicken's intestinal tract (Foley et al., 2013; Upadhyaya et al., 2013). By using the type three secretion system (T3SS) encoded on *Salmonella* Pathogenicity island 1 (SPI1) and 2 (SPI2), SE injects bacterial effector proteins into the cytoplasm of host intestinal epithelium. SE induces T3SS encoded on SPI1 and invades the intestinal epithelium through intestinal epithelial cells (Larock et al., 2015; Lhocine et al., 2015). Invasion of SE through intestinal epithelial cells leads to alteration of host intestinal cells physiology and cytoskeletal changes resulting in bacterial uptake inside cells (Ly and Casanova, 2007; Haraga et al., 2008). Internalization of SE results in the formation of phagosome membrane *Salmonella* containing vacuole (SCV). After coming inside SCV, SE induces second

T3SS encoded on SPI2, replicates, and survives inside the cytoplasm of intestinal epithelial cells and macrophages (Gart et al., 2016). Therefore, reducing the SE colonization and virulence mechanisms in chicken intestine could potentially be a feasible approach of reducing SE infection in chickens.

Different approaches have been taken to reduce the SE colonization and persistence in chicken's intestinal tract but to a limited success. Butyric acid, a short chain fatty acid and a microbiota metabolite maintains gastrointestinal health, since it serves as main energy source for colonocytes, enhances epithelial barrier integrity, and inhibits inflammation (Hamer et al., 2008; Kasubuchi et al., 2015). Butyric acid has been Generally Recognised as Safe (GRAS) for its use in foods (Butyric acid- 21CFR182.60, Food and Drug Administration (FDA), 2017). We recently found that sodium butyrate was effective in reducing SE attachment and invasion *in-vitro* at its SIC 45 mM in primary chicken enterocytes as compared to control. Furthermore, we observed that SIC of sodium butyrate reduced invasion of SE in chicken macrophages. In addition, sodium butyrate 45 mM significantly downregulated the expression of inflammatory genes (*Il1 β* , *Il8* and *Mmp9*) in chicken macrophages infected with SE. Despite these findings from our previous research, it remained elusive on protein expression response to sodium butyrate on SE infected primary chicken enterocytes.

In light of our previous findings and current state of knowledge, the objective of this study was to investigate the effect of sodium butyrate on molecular and functional pathways involved in SE infected primary chicken enterocytes using mass spectrometry.

4.3 MATERIALS AND METHODS

4.3.1 Primary chicken enterocyte cell culture

Male broiler day old chicks (Cobb 500) obtained from Cobb-Vantress, Fayetteville, AR were kept under a brooding temperature of 90°F overnight as approved by Institutional Animal Care and Use Committee, University of Arkansas. Chicks were sacrificed by cervical dislocation and small intestines were collected to harvest villi in a petri-dish containing Dulbecco's modified minimum essential medium (DMEM F-12; HiMedia Laboratories Pvt. Ltd, Mumbai, India) as described earlier (Rath et al., 2018, 2019). Intestinal villi were subjected to 0.1% Streptomyces hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) incubation at 37°C in a humidified 5% CO₂ incubator for 1 h to separate mucus present inside the villi. The villi were centrifuged at 300 g for 10 min and further digested with 0.025% Trypsin: Cell dissociation solution (Sigma-Aldrich) in the ratio of 1:9 for 15 min at 37°C in a humidified 5% CO₂ incubator to dissociate the cells. Dissociated cells were laid over Histopaque®-1119 (Sigma-Aldrich) at 400 g for 30 min to separate the cell debris and dead cells from live cells and cell clusters by density gradient centrifugation. Subsequently, cells present at the interface of two liquids were collected gently and cultured in DMEM F-12 culture medium containing 10% heat inactivated fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA), and growth factors such as 1X Insulin Transferrin Selenium (Sigma-Aldrich), 1X Epithelial cell growth supplement (EpiCGS, Sigma-Aldrich), 20 ng/mL epidermal growth factor (EGF, Thermo Fisher Scientific) for 48 h in a humidified 5% CO₂ incubator at 37°C till they reached confluency. After 24 h, supernatant containing dead cells were discarded and cells were replaced with fresh DMEM F-12 culture medium to grow them at semi-confluent stage. Cells were dissociated with Accutase (Sigma-Aldrich) after 3-4 passages to perform cell culture assays and stored in liquid nitrogen up to 6 passages for further *in-vitro* assays.

4.3.2 Effect of SIC of sodium butyrate on viability of primary chicken enterocytes

Primary chicken enterocytes (10^4 cells/well) were seeded in 96 well plate for 24 h at 37°C in a humidified, incubator containing 5% CO₂ to form a monolayer. The primary chicken enterocytes were incubated with 45 mM sodium butyrate for 4 h at 37°C. The cellular viability and metabolic activity of primary chicken enterocytes in response to SIC of sodium butyrate was determined by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Jung et al., 2005; Sakurazawa and Ohkusa, 2005). For testing the cell viability of primary chicken enterocytes against SIC of sodium butyrate, the MTT reagent (10 µL) was added to the chicken enterocytes and the plate was incubated at 37°C for 2 h. Subsequently, 100 µL of isopropanol (Sigma-Aldrich) was added to each well to dissolve the formazan crystals and the plate was further incubated at room temperature in dark for 1 h. The absorbance was measured at 570 nm by using microplate reader (Benchmark; Bio-Rad Laboratories, Hercules, CA, USA).

4.3.3 Bacterial strains and culture conditions

Salmonella Enteritidis (SE) GFP 338 was cultured in 10 mL of tryptic soy broth (TSB, Hardy Diagnostics CRITERION™, Santa Maria, CA, USA) at 37°C for 18 h. Following subculture in 10 mL TSB for another 10 h, the culture was centrifuged at 2504 g for 10 min. The pellet was suspended in sterilized phosphate buffer saline (PBS, pH 7) and used as the inoculum. The enumeration of SE counts in the inoculum was made by plating serial 5-fold dilutions on brilliant green agar (BGA; Difco Laboratories, Detroit, Michigan, USA) and the plates were incubated at 37°C for 24 h for bacterial enumeration.

4.3.4 Proteomic sample collection, preparation and In-gel protein digestion

Primary chicken enterocytes (10^5 cells/well) were seeded into 6-well plate (Costar) containing DMEM F-12 with 10% FBS for 48 h at 37°C in a humidified, 5% CO₂ incubator to

form a monolayer. A mid-log phase (10 h) culture of SE was inoculated on the primary chicken enterocytes (~6 Log CFU/mL; multiplicity of infection 10:1) in the presence or absence of SIC of sodium butyrate (45 mM). Infected monolayer was incubated for 4 h followed by rinsing with DMEM F-12 serum free media two times. The primary chicken enterocytes were then lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) as described earlier (Rath et al., 2018, 2019).

Cell lysate were subjected to 4-20% SDS Page gel electrophoresis and each sample was run in triplicate. Gel segments were excised and triturate into small pieces followed by washing with 50 mM ammonium bicarbonate (NH₄HCO₃, Thermo Fisher Scientific). Destaining of gel segments was performed by adding 50% Acetonitrile (ACN, Bio-Rad Laboratories, Hercules, CA, USA) in 50 mM NH₄HCO₃ and evaporated completely with 100% ACN to get white color crystals. Reduction of gel particles were determined by using 10 mM dithiothreitol (DTT, Bio-Rad) in 25 mM NH₄HCO₃ (1.5 mg/mL) at 60°C for 1 h and alkylation was performed with 55 mM iodoacetamide (Bio-Rad) with 25 mM NH₄HCO₃ (10 mg/mL) at room temperature for 1 h. The iodoacetamide was removed completely by rinsing the gel particles with 50 mM NH₄HCO₃ followed by digestion with MS Grade trypsin (20 ng/mL in 25 mM NH₄HCO₃) and incubated overnight at 37°C. The peptides extracted were dried completely and resuspended in 0.1% formic acid for analyses by liquid chromatography tandem mass spectrometry (LC-MS/MS). Three samples were used for each group and data was analyzed individually for each sample.

4.3.4.1 Mass spectrometry analysis

LC-MS/MS was performed by using an Agilent 1200 series micro-flow high-performance liquid chromatography (HPLC) coupled to a Bruker AmaZon SL quadrupole ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA, United States) with a captive spray ionization

source as described earlier (Rath et al., 2019, 2018; Karash et al., 2017). Tryptic peptides were separated by using C₁₈ capillary column (150 mm × 0.1 mm, 3.5 μm particle size, 300 Å pore size; ZORBAX SB) with 5-40% gradients of 0.1% formic acid (solvent A) and ACN in 0.1% formic acid (solvent B). Solvent flows at a rate of 4 μL/min over a duration of 300 min each.

LC-MS/MS data were acquired in positive ion mode. Bruker captive electro spray source was operated with a dry gas temperature of 150°C and a dry nitrogen flow rate of 3 L/min with captive spray voltage of 1500 volts. The data acquisition was in the Auto MS (n) mode optimized the trapping condition for the ions at m/z 1000. MS scans were performed in enhanced scanning mode (8100 m/z/second), MS/MS fragmentation scans performed automatically for top 10 precursor ions. The samples were run three times for each group as technical replicates and experiment was repeated two times for analyzing results.

By using Bruker Data Analysis 4.0 software, peaks were picked from LC-MS/MS chromatogram using default peak picking method recommended and to created Protein Analysis Results.xml file. This was used for searching Mascot database. In Mascot search, parent ion and fragment ion mass tolerances were set at 0.6 Da with cysteine carbamidomethylation as fixed modification and methionine oxidation as variable modifications. For the identification of proteins in cell extracts, Mascot search was performed against Gallus UniProt database. Identification of proteins is with 95% confidence limit and with less than 5% false discovery rate (FDR). FDR was calculated in during the Mascot search by simultaneously searching the reverse sequence database. Uncharacterized Gallus proteins were identified based on gene sequence similarities tentatively. For evaluation of differentially expressed proteins, Mascot.dat files _were exported to Scaffold Proteome Software version 4.8 and quantitative differences were determined based on 95% confidence limit. To determine the signaling pathway of proteins, the differentially regulated

proteins were analyzed using software such as Protein Analysis through Evolutionary Relationships software (PANTHER) and STRING protein association network.

4.3.5 Statistical Analysis

The CFU counts of SE were logarithmically transformed (Log CFU) to maintain homogeneity of variance (Byrd et al., 2001). In the present study, we used triplicate samples and the experiment was repeated two times. Scaffold Proteome Software version 4.8 (Proteome Software Inc, Portland, OR) was used to analyze Mascot files for the proteomic analysis. Differentially expressed proteins were determined using Student's t-test and probability of $P < 0.05$ was required for statistically significant differences.

4.4 RESULTS

4.4.1 Effect of sodium butyrate on the proteome of primary chicken enterocytes

Fig. 1 showed the effect of SIC of sodium butyrate 45 mM on cell viability of primary chicken enterocytes. 45 mM concentration of sodium butyrate did not affect the cell viability as compared to control ($P > 0.05$). Therefore, we selected the highest SIC 45 mM of sodium butyrate for proteomic analysis. A total of 408 proteins were identified when primary chicken enterocytes were treated with 45 mM sodium butyrate. Quantitative comparison showed downregulation of 13 proteins and upregulation of 7 proteins in sodium butyrate treated cells compared to control ($P < 0.05$), whereas 388 proteins were not affected ($P > 0.05$).

Sodium butyrate treatment in primary chicken enterocytes downregulated expression of proteins associated with different biological processes, for example biological regulation, cellular component biogenesis, developmental process, localization, locomotion, response to stimulus and signaling involves GTP binding protein (RhOA); cellular process such as Triosephosphate

isomerase (TPI1), Malate dehydrogenase (MDH1), Cathepsin Z (CTSZ) and RhOA; metabolic process includes TPI1, MDH1 and CTSZ (Table 1, Fig. 2).

Additionally, sodium butyrate treatment upregulated proteins associated with cellular process and localization such as Coatamer subunit gamma (COPG1) and Clathrin heavy chain (CLTC); cellular component biogenesis includes CLTC (Table 1, Fig. 2).

Signaling pathway analysis by STRING showed that sodium butyrate treatment in primary chicken enterocytes downregulated proteins involved in Glycolysis/Gluconeogenesis, membrane trafficking, Rho-GTPase effectors, and regulation of actin cytoskeleton (Table 2. Fig. 3). Fig. 4 showed the signaling pathway analysis of proteins upregulated by sodium butyrate 45 mM treatment in primary chicken enterocytes.

4.4.2 Effect of SE on the proteome of primary chicken enterocytes

Quantitative comparison determined downregulation of 4 proteins and upregulation of 9 proteins after SE infection in primary chicken enterocytes compared to control ($P < 0.05$), while 395 proteins were not affected ($P > 0.05$). SE infection in primary chicken enterocytes downregulated proteins related with cellular process and cellular component biogenesis such as Tubulin beta-7 chain (TBB7) and Tubulin alpha-5 chain (TUBA1C) (Table 3, Fig. 5).

In addition, SE infection in primary chicken enterocytes upregulated proteins associated with biological adhesion, cell population proliferation, developmental process, growth, immune system, metabolic process and signaling such as Annexin A1 (ANXA1); biological regulation such as Actin related protein 2/3 complex subunit 5 (ARPC5), Actin related protein 2 (ACTR2), WD repeat-containing protein 1 (WDR1) and ANXA1; cellular component biogenesis and cellular process includes Fascin (FSCN1), Ras related protein RAB-11B (RAB11B), ARPC5,

ACTR2, WDR1 and ANXA1; localization such as ANXA1, RAB11B, FSCN1 and ARPC5; locomotion includes ANXA1, FSCN1 and ARPC5 (Table 3, Fig. 5).

Signaling pathway analysis by STRING revealed that SE infection in primary chicken enterocytes downregulated proteins involved in phagosome activity and COPI independent Golgi to ER retrograde trafficking (Table 4, Fig. 6). Moreover, SE infected cells upregulated proteins correlated with innate immune response, membrane trafficking, actin cytoskeleton organization, endocytosis and Rho-GTPase pathway (Table 5, Fig. 7).

4.4.3 Effect of sodium butyrate on the proteome of primary chicken enterocytes infected with SE

Quantitative profile resulted in downregulation of 15 proteins and upregulation of 14 proteins after treatment of SE infected primary chicken enterocytes with 45 mM sodium butyrate ($P < 0.05$), however 379 proteins were not affected ($P > 0.05$). Sodium butyrate downregulated protein expression of SE infected primary chicken enterocytes associated with various biological processes such as biological adhesion, cell population proliferation, developmental process, growth, immune system process, locomotion and reproductive process, which includes ANXA1; biological regulation comprises of Endoplasmic reticulum chaperone BiP (HSPA5), Rho GDP dissociation inhibitor beta (ARHGDIB) and ANXA1; cellular component biogenesis includes Tropomyosin alpha-3 chain (TPM3), ANXA1 and RAB11B; cellular process contains Elongation factor 1-beta (EEF1B2), HSPA5, ANXA1, TPM3, RAB11B and ARHGDIB; localization consists of ANXA1 and RAB11B; metabolic process such as EEF1B2, HSPA5 and ANXA1; multicellular organismal process contains ANXA1 and TPM3; response to stimulus and signaling involves HSPA5, ANXA1 and ARHGDIB (Table 6, Fig. 8).

Likewise, sodium butyrate treatment upregulated protein expression of SE infected primary chicken enterocytes related with various biological processes such as cellular component biogenesis and cellular process [Tubulin beta chain (TUBB4B), Fibronectin (FN1), Tubulin beta chain (TUBB2A), TBB7 and 60S ribosomal protein L6 (RPL6)]; developmental process and multicellular organismal process includes TUBB2A and FN1; growth such as FN1; localization consists of Exportin-2 (CSE1L) and TUBB2A; locomotion such as TUBB2A; metabolic process incorporates Prolyl 3-hydroxylase 1 (P3H1) and RPL6 (Table 6, Fig. 8).

Signaling pathway analysis by STRING indicated that sodium butyrate downregulated the protein expression of SE infected cells critical for membrane trafficking, actin binding, Rab-regulation of trafficking, negative regulation of response to stimulus, and binding of proteins (Table 7, Fig. 9). Additionally, sodium butyrate upregulated the protein expression of SE infected cells important for nucleotide binding, GTPase and phagosome activity, and cytoskeleton changes (Table 8, Fig. 9).

4.5 DISCUSSION

SE is a facultative intracellular food borne pathogen that colonize intestine of chickens and invades intestinal epithelial cells, which promotes transmission of bacterium in chicken's intestinal tract (Larock et al., 2015; Wemyss and Pearson, 2019; Ly and Casanova, 2007; Lhocine et al., 2015). Much is unknown on the molecular and functional pathways when, SE infects chicken intestine and causes host cytoskeleton modifications, the objective of our study to determine the effect of sodium butyrate on proteomic changes in primary chicken enterocytes infected with SE.

A diverse repertoire of proteins was downregulated in response to treatment of primary chicken enterocytes with sodium butyrate, including, proteins involved in glycolysis (TPI1 and

Aldolase C); oxidation reduction process (MDH1 and P4HA1); membrane trafficking (YWHAQ, YWHAZ and CTSZ); Rho GTPase effectors and regulation of actin cytoskeleton (RHOA and PPP1CC). Glycolysis is a metabolic pathway which upregulates inflammation pathways IL-17, with increased infiltration of Th2 cell and macrophages in breast cancer (Transl et al., 2020). In addition, Giri et al. (2019) had recently reported that stimulation of primary mouse brain glia cells with LPS induces the expression of monocarboxylate transporter (MCT4), that generates inflammatory signals and results in increased glycolysis and lactate production. Likewise, Berg Soren et al. (2003) reported that pro-inflammatory cytokines increased the rate of glycolysis in cultured rat enterocytes. Our results showed that sodium butyrate downregulated glycolytic proteins, which would decrease pro-inflammatory immune response. In addition, sodium butyrate also downregulated Cathepsin Z protein (also known as cathepsin X), a member of cysteine cathepsin proteases. Cathepsin Z is an exopeptidase, which possess carboxy-monopeptidase activity (Akkari et al., 2014) and stimulates maturation of dendritic cells, adhesion of macrophages and migration of T cells (Nanut et al., 2014; Jakoš et al., 2019). Allan et al. (2017) had reported that Cathepsin Z promotes IL-1 β associated neuro-inflammation in mice. Treatment of sodium butyrate in primary chicken enterocytes downregulated Cathepsin Z protein, which would reduce inflammation.

In addition, infection of primary chicken enterocytes with SE upregulated a number of proteins, including proteins correlated with actin cytoskeleton [Beta-tropomyosin (BRT-2/TPM2), WDR1, CAPZ, ACTR2, TPM3, ARPC5, ACTN4], Rab trafficking (RAB1A, RAB11B) and innate immune response (ANXA1, ACTR2). Notably, these proteins were significantly downregulated after treatment of infected cells with sodium butyrate.

ANXA1 is a glucocorticoid-regulated protein, and modulates inflammatory response (Oliveira et al., 2017). Recent reports showed that ANXA1 mediates pro-inflammatory immune response in neutrophils trans-endothelial migration (Williams et al., 2010) and is secreted from rheumatoid arthritis synovial fibroblasts (Tagoe et al., 2008; E Gavins et al., 2012; Zhao et al., 2016). In addition, Patel et al. (2011) showed that ANXA1 plays a crucial role in phagocytosis by facilitating interaction of actin filaments with phagosomes. Sodium butyrate treatment in SE infected cells downregulated expression of ANXA1, which would reduce pro-inflammatory immune response and modulate phagocytosis process during SE infection.

After internalization of SE in chicken intestinal epithelial cells, it remains enclosed in a phagosome complex called as *Salmonella* containing vacuole (SCV) for its survival and intracellular replication (Haraga et al., 2008; Wemyss and Pearson, 2019). SE modulates endocytic and exocytic Rab GTPases for its growth and survival in intestinal epithelial cells (Stein et al., 2012). Rab GTPases are small GTPases that plays a pivotal role in intracellular vesicular trafficking (Bhuin and Roy, 2015). After SE infection, Rab GTPases mediates intracellular membrane trafficking and helps in intracellular transport of bacterium to lysosomes for degradation (Chen et al., 1998). However, SE infection in primary chicken enterocytes impairs intestinal epithelial barrier integrity by transcytosis and promotes phagocytosis by macrophages and other dendritic cells for its survival and systemic dissemination within the chickens (Stein et al., 2012). Different Rab GTPases are located in various membrane compartments and converts inactive GDP to active GTP form (Kiral et al., 2018). Rab GTPases are also acts as markers of different organelles and vesicles in the endocytic system (Barr and Lambright, 2010; Zhen and Stenmark, 2015). In our study infection of primary chicken enterocytes with SE, led to upregulation of Ras related protein RAB-1A (RAB1A) and RAB11B.

Rab-1 regulates endoplasmic reticulum (ER)-Golgi network trafficking, present at ER and the pre-Golgi intermediate compartment and helps exocytosis. Rab-11 mediates trafficking from Golgi and recycling endosomes, early endosomes and basolateral plasma membrane and helps in endocytosis (Stenmark, 2009; Stein et al., 2012; Kiral et al., 2018). Stein et al. (2012) had also reported that human bacterial pathogens modulate Rab GTPases in order to gain entry inside host epithelial cells. Likewise, Smith et al. (2007) described that *Salmonella* Typhimurium modulates Rab GTPases for its intracellular growth inside host cells. Our results showed that sodium butyrate reduced expression of RAB11B and RAB1A, suggesting reduced intracellular trafficking of SE in chicken intestine.

Sodium butyrate treatment in SE infected primary chicken enterocytes also downregulated proteins involved in host actin cytoskeleton modifications such as TPM2, WDR1, CAPZ, TPM3, ARPC5, ACTR2, ACTN4. WDR1 plays a necessary role in reorganization of actin cytoskeleton by promoting disassembly of actin filaments by actin depolymerizing factor/cofilin (Ono, 2018; Pfajfer et al., 2018; Yuan et al., 2018). Verbrugghe et al. (2016) had reported proteomic analyses of porcine alveolar macrophages and suggested that cortisol increases proliferation of *Salmonella* in macrophages and results in increased intracellular bacterial replication. Actin related protein 2/3 complex involves ARPC5 and ACTR2 and causes actin polymerization and induces trafficking of cellular surface, and further promotes invasion of *Salmonella* inside host cells (Goley and Welch, 2006; Misselwitz et al., 2011; Su et al., 2018). Our results revealed that sodium butyrate significantly reduced proteins responsible for actin cytoskeletal rearrangements in primary chicken enterocytes, which would reduce SE uptake and intracellular bacterial replication.

In contrast, sodium butyrate treatment in SE infected primary chicken enterocytes upregulated protein involved in structural molecular activity such as VIM. VIM is critical protein, which helps in cytoskeleton changes and maintaining cell integrity, cell adhesion and regulates bacterial infection (Mak and Brüggemann, 2016). Su et al. (2019) had recently reported that VIM regulates apoptosis of immune cells such as lymphocytes and inflammatory response in patients suffering from sepsis. Moreover, Mor-Vaknin et al. (2003) showed that VIM is secreted from activated macrophages and extracellular VIM is involved in bacterial killing during pro-inflammatory signaling pathways. Our results showed that sodium butyrate treatment in SE infected primary chicken enterocytes significantly upregulated VIM protein, which would potentially reduce bacterial infection and immune response.

4.6 CONCLUSIONS

In this study, we have found that sodium butyrate treatment in primary chicken enterocytes reduced expression of proteins involved in glycolysis, which would be increased during pro-inflammatory signaling pathways. We have also observed that sodium butyrate treatment in SE infected primary chicken enterocytes downregulated protein involved in pro-inflammatory immune response and the phagocytosis process during bacterial infection. Likewise, we have identified that sodium butyrate treatment in SE infected primary chicken enterocytes downregulated proteins involved in host cytoskeleton rearrangements, which would be essential for invasion and intracellular replication and survival of SE in intestinal epithelial cells and macrophages. In addition, sodium butyrate treatment in SE infected primary chicken enterocytes also downregulated proteins involved in membrane trafficking inside the intestinal epithelial cells of chickens, which would be important for the growth and survival of SE in the

intestinal tract of chickens. Altogether, these results suggest that butyrate modulates chicken intestinal epithelial proteins responsible for SE colonization and replication.

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Table 4.1 Differentially regulated proteins in primary chicken enterocytes by sodium butyrate (SB) treatment

Proteins downregulated by SB	Alternate id	Accession number	M.Wt (kDa)	Fold change	P<0.05
14-3-3 protein theta	YWHAQ	Q5ZMD1	28	0.4	0.046
14-3-3 protein zeta	YWHAZ	Q5ZKC9	28	0.4	0.025
Triosephosphate isomerase	TPI1	P00940	27	0.5	0.043
Glutathione S-transferase		A0A1D5PDF1	25	0.4	0.026
Calreticulin (Fragment)		Q6EE32	47	0	0.016
Prolyl 4-hydroxylase subunit alpha-1	P4HA1	A0A1D5P4R2	62	0	0.02
Proteasome subunit beta		A0A1L1RUE7	23	0.1	0.014
Transgelin	TAGLN	P19966	22	0.1	0.0056
Serine/threonine-protein phosphatase	PPP1CC	Q5ZL39	37	0	0.0037
Malate dehydrogenase	MDH1	A0A1D5PE17	37	0	0.047
Uncharacterized protein	RHOA	A0A1L1RK40	23	0	0.021
Aldolase A (Fragment)	Aldolase C	Q92007	4	0	<0.00010
Cathepsin Z	CTSZ	E1C4M3	34	0	0.006
Proteins upregulated by SB					
Vimentin	VIM	F1NJ08	53	2	0.035
Filamin		Q90WF1	273	4.6	0.035
Uncharacterized protein	FLNB	A0A1D5NYG3	284	5.1	0.025
CgABP260		Q90WF0	280	8.1	0.013
Clathrin heavy chain	CLTC	F1NW23	192	5.9	0.012
CHICK-DECOY		A0A1D5PP37		0	0.067
Coatomer subunit gamma	COPG1	F1NB52	98	INF	0.00012

Table 4.2 Pathways downregulated by SB treatment in primary chicken enterocytes

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GO:0055114	Oxidation-reduction process	2	0.0041
2	GGA00010	Glycolysis / Gluconeogenesis	2	0.0038
3	GGA04110	Cell cycle	2	0.0092
4	GGA04810	Regulation of actin cytoskeleton	2	0.0169
5	GGA562889 7	TP53 Regulates Metabolic Genes	2	0.0034
6	GGA199991	Membrane Trafficking	3	0.0258
7	GGA195258	RHO GTPase Effectors	2	0.039

Table 4.3 Differentially regulated proteins in primary chicken enterocytes after *Salmonella* Enteritidis (SE) infection

Proteins downregulated by SE	Alternate id	Accession number	M.Wt	Fold change	P<0.05
Tubulin beta-7 chain	TBB7	P09244	50	0.03	0.035
Tubulin alpha-5 chain	TUBA1C	P09644	50	0.2	0.036
ARP1 actin related protein 1	ACTR1A	Q5ZM58	43	0.4	0.036
Aldolase A	Aldolase C	Q92007	4	0	<0.00010
Proteins upregulated by SE					
Actin-related protein 2/3 complex	ARPC5	Q5ZMV5	16	INF	0.27
Beta-tropomyosin	BRT-2	Q05705	29	3.6	0.029
Actin-related protein 2	ACTR2	F1NRM5	45	INF	0.0047
Radixin	RDX	Q9PU45	69	INF	0.034
Uncharacterized protein	FSCN1	A0A1D6UPS 2	64	INF	<0.00010
Uncharacterized protein	RAB11B	F7AU58	24	6.1	0.044
Aldo-keto reductase family 7	AKR7A2	A0A1L1RZG 2	19	INF	0.0047
Annexin A1	ANXA1	F1N9S7	39	1.5	0.05
WD repeat-containing protein 1	WDR1	F1NRI3	67	INF	0.0047

Table 4.4 Pathways downregulated by SE infected primary chicken enterocytes

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GGA04145	Phagosome	2	0.0181
2	GGA6811436	COPI-independent Golgi-to-ER retrograde traffic	6	3.30E-13
3	GGA2565942	Regulation of PLK1 Activity at G2/M Transition	4	6.22E-08
4	GGA5620912	Anchoring of the basal body to the plasma membrane	4	1.04E-07

Table 4.5 Pathways upregulated by SE infected primary chicken enterocytes

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GO0030036	Actin cytoskeleton organization	4	8.89E-06
2	GO0045087	Innate immune response	2	0.0045
3	GGA04810	Regulation of actin cytoskeleton	2	0.0138
4	GGA04144	Endocytosis	2	0.0138
5	GGA5663213	RHO GTPases Activate WASPs and WAVES	2	0.0035
6	GGA3928662	EPHB-mediated forward signaling	2	0.0035
7	GGA2029482	Regulation of actin dynamics for phagocytic cup formation	2	0.0035
8	GGA199991	Membrane Trafficking	3	0.0062
9	GGA6798695	Neutrophil degranulation	2	0.0251

Table 4.6 Differentially regulated proteins by SB treatment in SE infected primary chicken enterocytes

Proteins downregulated by SB treatment in SE infected primary chicken enterocytes	Alternate id	Accession number	M.Wt	Fold change	P<0.05
Endoplasmic reticulum chaperone	HSPA5	Q90593	72	0.5	0.019
Alpha-actinin-4	ACTN4	A0A1D5NXS9	98	0.7	0.0055
Annexin A1	ANXA1	F1N9S7	39	0.6	0.041
14-3-3 protein theta	YWHAQ	Q5ZMD1	28	0.4	0.024
Transgelin 2	TAGLN2	A0A1D5PDK5	27	0.5	0.015
ATP synthase subunit beta	ATP5F1B	Q5ZLC5	57	0.6	0.013
Uncharacterized protein	RAB1A	A0A1D5PE33	23	0.5	0.029
Capping actin protein	CAPG	A0A1D5NXJ9	50	0.6	0.031
14-3-3 protein gamma	YWHAG	F1NMY1	28	0.4	0.0082
Uncharacterized protein	TPM3	A0A1D5P9B0	34	0.4	0.022
14-3-3 protein beta/alpha	YWHAB	Q5ZLQ6	28	0.2	0.022
Beta-tropomyosin	BRT-2/TPM2	Q05705	29	0.1	0.0088
Rho GDP dissociation inhibitor beta	ARHGDIB	F1NLT8	23	0	0.0047
Uncharacterized protein	RAB11B	F7AU58	24	0.1	0.021
Uncharacterized protein	EEF1B2	F1NYA9	25	0	0.0047

Table 4.6 (Cont.)

Proteins upregulated by SB treatment in SE infected primary chicken enterocytes	Alternate id	Accession number	M.Wt	Fold change	P<0.05
Vimentin	VIM	F1NJ08	53	4.7	0.016
Tubulin beta-7 chain	TBB7	P09244	50	35	0.015
Tubulin beta chain	TUBB4B	F1NYB1	50	8.7	0.014
Tubulin beta-1 chain	TUBB2A	P09203	50	INF	0.018
Gamma-enolase	ENO2	F1NG74	47	INF	0.0056
Tubulin beta-3 chain	TUBB3	P09206	50	INF	0.042
Fibronectin	FN1	F1NJT4	272	10	0.029
W-linked valosin containing protein		A0A182C5X0	89	INF	0.017
Septin-2	2-Sep	Q5ZMH1	40	INF	0.00044
60S ribosomal protein L6	RPL6	Q8UWG7	34	INF	0.00044
Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3	Q5F3D2	37	INF	0.033
Uncharacterized protein	HSPA4L	F1NC26	95	INF	0.00044
Chromosome segregation 1	CSE1L	E1BV44	110	INF	0.00044
Prolyl 3-hydroxylase 1	P3H1	Q6JHU8	82	INF	0.00044

Table 4.7 Pathways downregulated by SB treatment in SE infected primary chicken enterocytes

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GGA9007101	Rab regulation of trafficking	2	0.024
2	GGA199991	Membrane Trafficking	4	0.0087
3	GO0048585	Negative regulation of response to stimulus	3	0.0486
4	GGA04110	Cell cycle	3	0.001
5	GGA5628897	TP53 Regulates Metabolic Genes	2	0.0087
6	GGA1445148	Translocation of SLC2A4 (GLUT4) to the plasma membrane	2	0.0023
7	GO0003779	Actin binding	2	0.022
8	GO0005515	Protein binding	5	0.0046

Table 4.8 Pathways upregulated by sodium butyrate treatment in SE infected primary chicken enterocytes

S. No	Pathway ID	Pathway description	Count in gene set	False discovery rate
1	GO0005198	Structural molecule activity	3	0.0027
2	GO0005200	Structural constituent of cytoskeleton	2	0.0032
3	GO0003924	GTPase activity	2	0.0062
4	GGA04145	Phagosome	2	0.0131
5	KW0342	GTP-binding	3	0.0055
6	KW0547	Nucleotide-binding	4	0.0132

□ Control ■ SB 45 mM

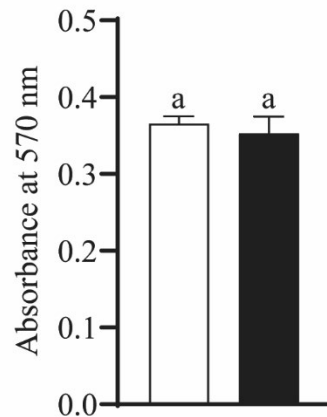


Figure 4.1 The effect of SIC of sodium butyrate (SB) 45 mM on cell viability of primary chicken enterocytes using MTT assay. Primary chicken enterocytes were treated with SB at 45 mM for 4 h. Data presented as mean absorbance and error bar represents SEM (n=6). Bar with different letters represents a statistical difference at $P < 0.05$.

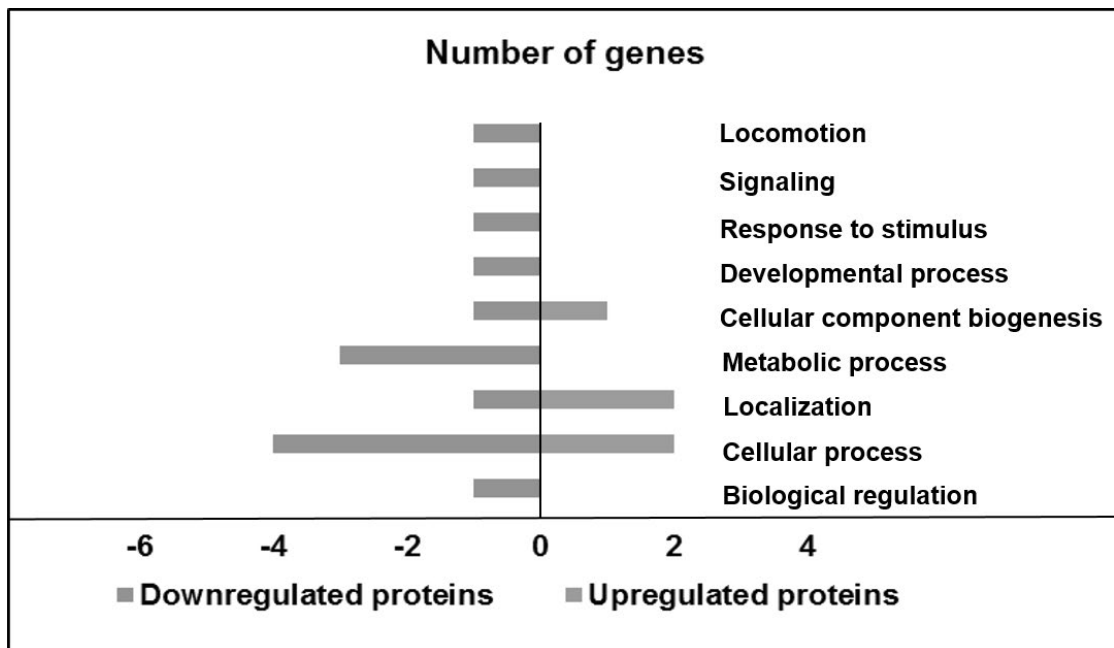


Figure 4.2 SB treatment in primary chicken enterocytes induced down and upregulated proteins in different biological processes

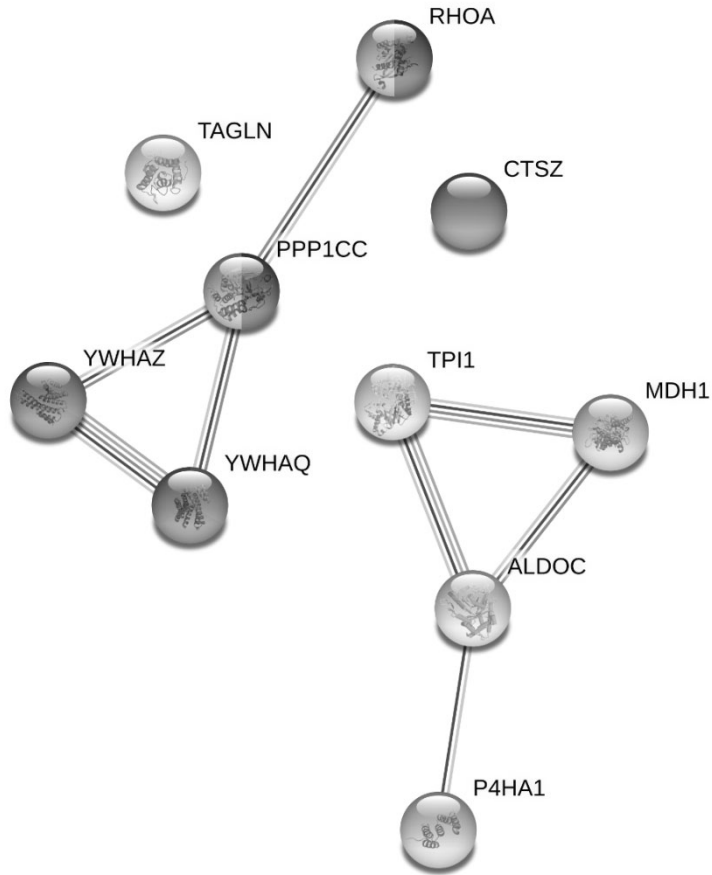


Figure 4.3 Signaling pathway analysis by STRING showed pathways downregulated by treatment of SB in primary chicken enterocytes

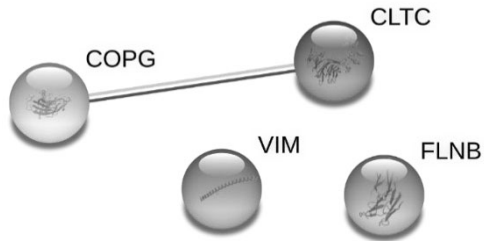


Figure 4.4 Signaling pathway analysis by STRING showed pathways upregulated by treatment of SB in primary chicken enterocytes

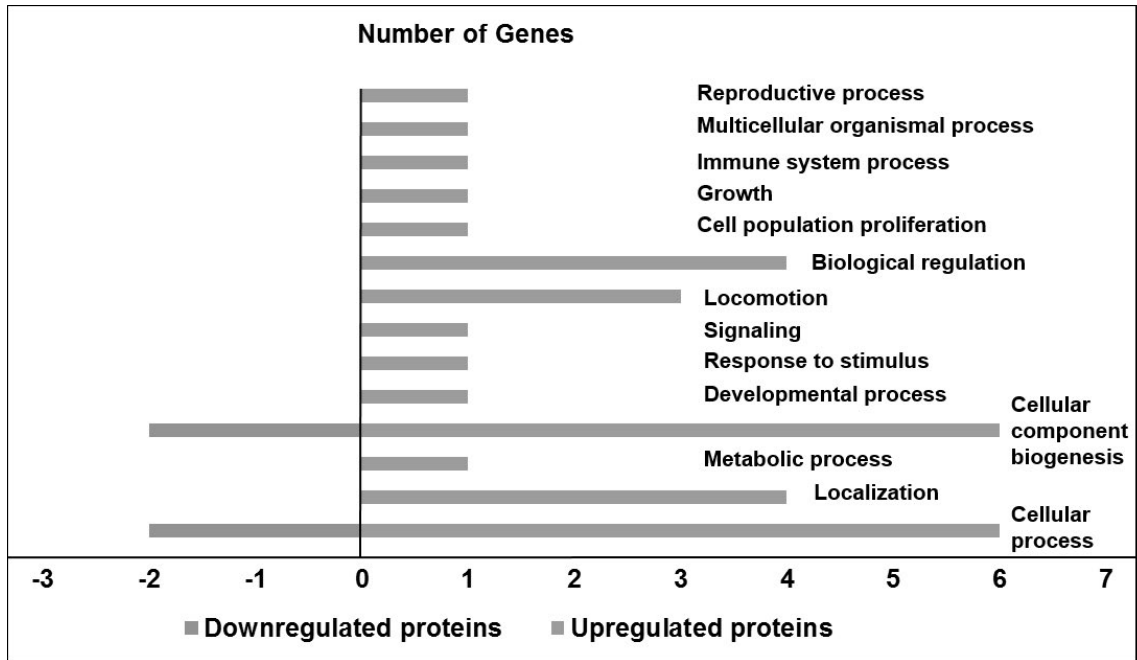


Figure 4.5 *Salmonella* Enteritidis (SE) infection in primary chicken enterocytes induced down and upregulated proteins in different biological processes

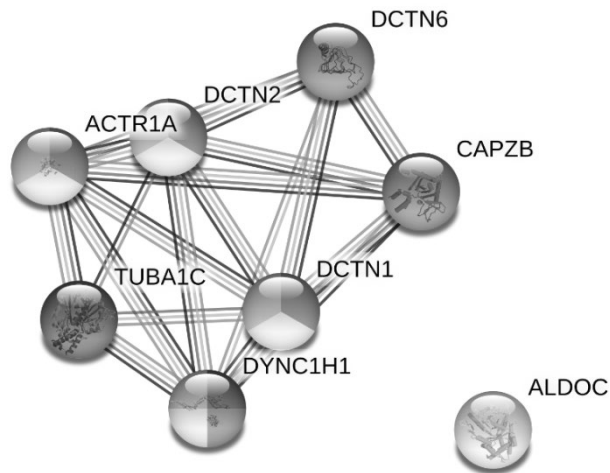


Figure 4.6 Signaling pathway analysis by STRING showed pathways downregulated by SE infection in primary chicken enterocytes

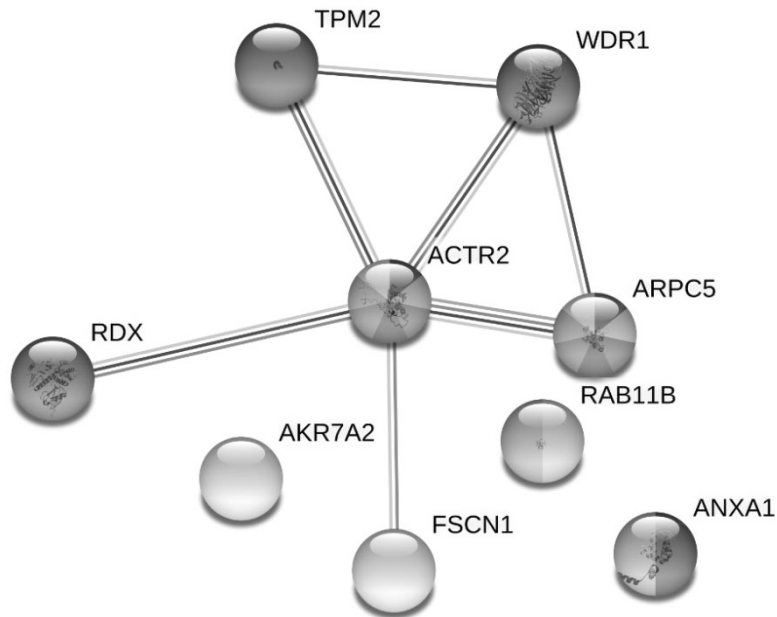


Figure 4.7 Signaling pathway analysis by STRING showed pathways upregulated by SE infection in primary chicken enterocytes

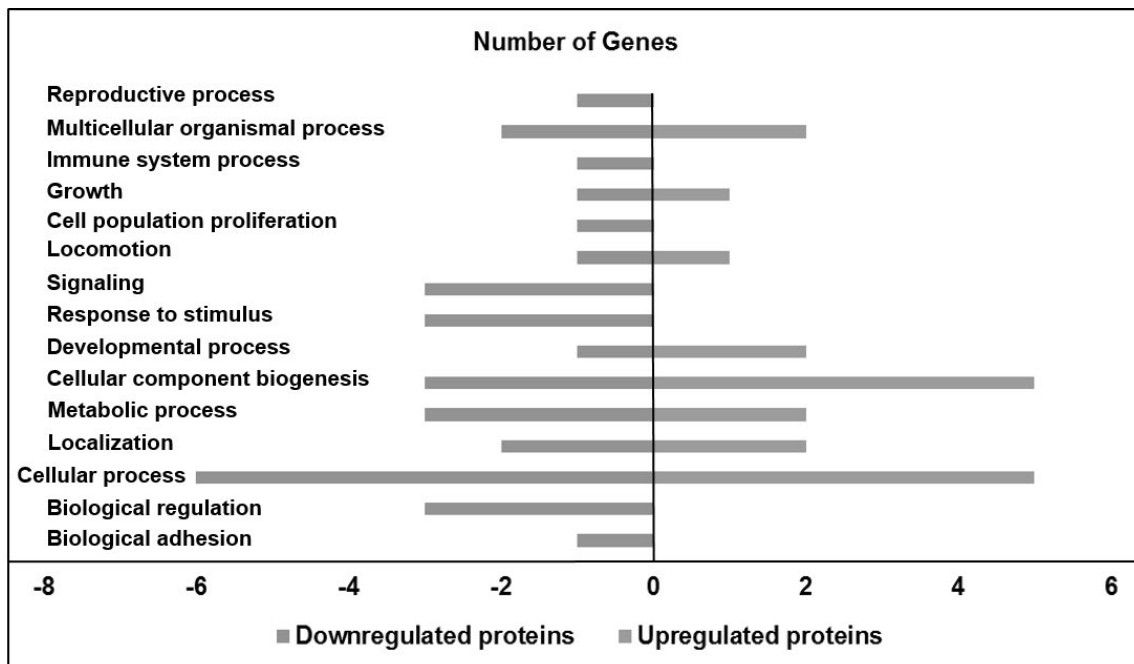


Figure 4.8 SB treatment in SE infected primary chicken enterocytes induced down and upregulated proteins in different biological processes

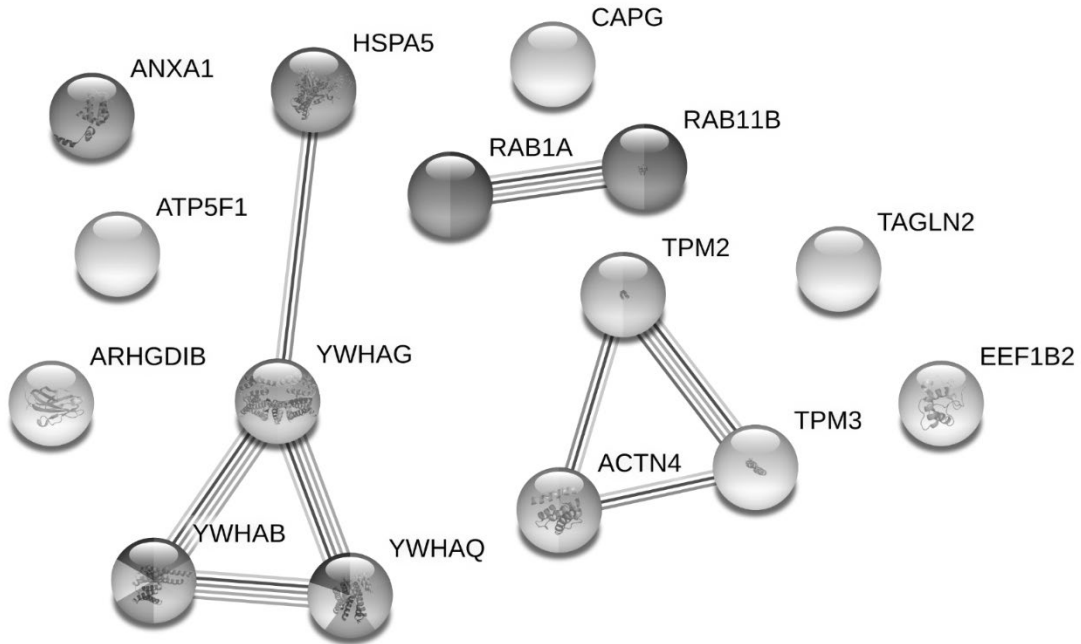


Figure 4.9 Signaling pathway analysis by STRING showed pathways downregulated by SB treatment in SE infected primary chicken enterocytes

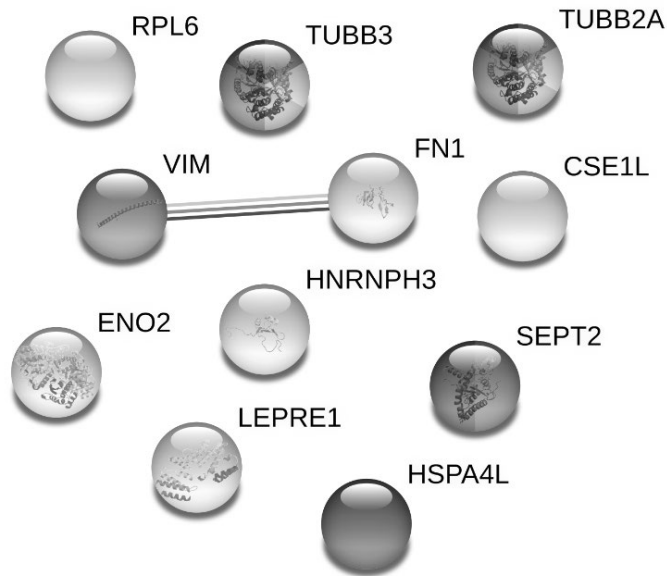


Figure 4.10 Signaling pathway analysis by STRING showed pathways upregulated by SB treatment in SE infected primary chicken enterocytes

CHAPTER V

Conclusions

Salmonella Enteritidis (SE) is a major food borne pathogen responsible for causing food borne illnesses in humans worldwide including United States. The primary source of SE in humans is primarily through contaminated poultry meat, eggs and poultry byproducts. The SE predominantly colonizes in the intestinal tract of chickens and acts as an asymptomatic carrier of infection without showing any clinical signs. However, limited success has been reported in the interventions targeting the colonization of SE in birds. The results highlight the need for novel preharvest inventions to reduce contamination of SE in chicken's intestinal tract.

In this project, we evaluated the anti-inflammatory effect of sodium butyrate during SE colonization in chickens (Study 1). We have determined the effect of sodium butyrate on the proteome of chicken macrophages in the presence and absence of SE (Study 2). We have also investigated the effect of sodium butyrate on the molecular and functional aspect of primary chicken enterocytes infected with SE using mass spectrometry (Study 3).

In the first study, effect of sodium butyrate on SE adhesion and invasion of primary chicken enterocytes and chicken macrophages was studied. In addition, effect of sodium butyrate on the expression of virulence genes of SE and inflammatory genes in chicken macrophages infected with SE was studied. We have identified that sodium butyrate at its sub-inhibitory concentration significantly reduced SE colonization by reducing attachment and invasion capacity. Moreover, sodium butyrate exerts its anti-inflammatory effect by downregulating inflammatory cytokines in SE infected chicken macrophages.

In the second study, effect of sodium butyrate on protein expression analysis of chicken macrophages infected with SE was studied using tandem mass spectrometry. We found that sodium butyrate treatment in uninfected chicken macrophages significantly downregulated proteins involved in glycolysis, GTP binding proteins and the proteins associated with secretion

of pro-inflammatory cytokine response. In addition, sodium butyrate treatment in SE infected chicken macrophages downregulated proteins involved in host cytoskeletal rearrangements and intracellular growth of bacteria in the intestinal tract of chickens.

In the third study, effect of sodium butyrate on protein expression analysis of primary chicken enterocytes infected with SE was studied using tandem mass spectrometry. We found that sodium butyrate treatment in uninfected primary chicken enterocytes significantly downregulated proteins involved in glycolysis. In addition, sodium butyrate treatment in SE infected primary chicken enterocytes significantly downregulated proteins involved in host cytoskeleton modifications and membrane trafficking for the invasion, intracellular replication and survival of SE in the intestinal tract of chickens.

In conclusion, sodium butyrate acts as an anti-inflammatory compound to reduce SE colonization in chickens. In addition, sodium butyrate significantly reduced proteins responsible for host cytoskeletal rearrangements, intracellular survival and growth of SE in primary chicken enterocytes and chicken macrophages.