THE USE OF SELECTED RED MACROALGAE (SEAWEEDS) FOR THE REDUCTION OF *SALMONELLA* ENTERITIDIS IN POULTRY

by

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With love, I dedicate this work to my father
"My biggest critic"

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ABSTRACT

Red seaweeds are rich in bioactive compounds and secondary metabolites that are known to improve human and animal health. Seaweeds are commercially harvested along Pacific and Atlantic coasts of Canada for a wide range of applications in the pharmaceutical, nutritional, cosmetic, agrichemical and food industries. *Salmonella* Enteritidis is a zoonotic pathogen which causes infections in humans through contaminated poultry products. Worldwide, *Salmonella* outbreaks have become an important public health and economic concern. Moreover, the rising concerns for bacterial resistance to antibiotics and increasing consumer concern for drug residues in meat and animal products highlight an urgent need for alternative strategies to antibiotics. To evaluate red seaweeds as a natural alternative to antibiotics against *Salmonella* Enteritidis infection in poultry, six selected red seaweed species were tested. An *in vitro* study revealed that two red seaweed species namely *Sarcodiotheca gaudichaudii* (SG) and *Chondrus crispus* (CC) exhibited antimicrobial activity against *S.* Enteritidis. Extracts of both seaweeds reduced biofilm formation and motility of *S.* Entertitidis. Moreover, lower concentrations of seaweed extracts and compounds isolated from these seaweed species potentiated the activity of an antibiotic (tetracycline). Sub lethal concentrations of tetracycline (MIC_{25}; 4 μg mL^{-1}) in combination with seaweed extracts exhibited antimicrobial activity comparable to full strength tetracycline (25 μg mL^{-1}). Further investigation revealed that seaweed components suppressed the expression of multi-drug efflux pump related genes. The *in vivo* effect of seaweeds was tested using the *Caenorhabditis elegans – S.* Enteritidis infection model. Seaweeds reduced bacterial proliferation within the nematode gut and increased the survival of worms which correlated with an up regulation of innate immune response genes in the worm. Since the innate immune response pathways between *C. elegans* and higher animals are conserved, these results provide evidence that seaweed extract (SWE, CC and SG) may also impart beneficial effects on animal and human health. A poultry trial further revealed that dietary inclusion of the red seaweeds CC and SG in layer feed improved the performance of layers, shifted the gut microbiome towards beneficial bacteria, improved villi height, crypt depth, and elicited an increase in the concentration of Short chain fatty acids (SCFA). This suggests that the cultivated strains of *C. crispus* and *S. gaudichaudii* could be used as prebiotics for layer hens. Further, a challenge study showed that seaweed dietary inclusion reduced the negative effect on growth and egg production of *S.* Enteritidis challenged layer hens. Dietary inclusion of *Chondrus crispus* (CC) inhibited colonization of *S.* Enteritidis in the ceca and lowered fecal pathogen shedding. This could be by promoting the growth of *Lactobacillus* and increasing the concentration of SCFA. To conclude, the results suggested that innovative on-farm strategies such as red seaweed feed supplements can reduce *S.* Enteritidis colonization in birds. Hence, it can serve as an effective alternative to antibiotics to limit *S.* Enteritidis associated human infections. Additionally, producers (including organic farmers) would likely accept a natural feed additive like seaweed without concerns for toxicity.

LIST OF ABBREVIATIONS USED

ABC	ATP Binding Cassette
AGP	Antibiotic Growth Promoters
AHL	Acyl-homoserine Lactone
AM	Aspergillus Meal
ASM	American Society of Microbiology
AST	Aspartate transaminase
BW	Body Weight
CC	*Chondrus crispus*
CCAC	Canadian Council of Animal Care
CCWE	*Chondrus crispus* Water Extract
CD	Crypt Depth
CDC	Centers for Disease Control and Prevention
CF	Cystic Fibrosis
CFIA	Canadian Food Inspection Agency
CFU	Colony Forming Units
CM	Cytoplasmic Membrane
CM	Cytoplasmic Membrane
Csg	Curli Fimbriae
CTC	Chlortetracycline
DP	Degree of Polymerization

DPD	4,5-dihydroxy-2,3-pentanedione
ELISA	Enzyme-linked Immunosorbent Assay
EPS	Extracellular polymeric substances
FCR	Feed Conversion Ratio
FDA	Food and Drug Administration
FOS	Fructooligosaccharide
GC	Guanine-Cytosine
GD	*Gymnogongrus devoniensis*
GIT	Gastrointestinal Tract
GOS	Glucooligosaccharides
GUT	Gastrointestinal Tracts
HACCP	Hazard Analysis Critical Control Points
HPV	Human Papillomavirus
HS	Heparin Sulphate
IAV	Influenza A Virus
ICTEC	Tubular Epithelial Cells of the Isthmus
IDSA	Infectious Diseases Society of America
IN	Inulin
LD_{50}	Lethal Doses
LPS	Lipopolysaccharides
MAPK	p38 Mitogen-Activated Protein Kinase

MATE	Multidrug and Toxic Compound Exporters
MCTEC	Tubular Epithelial Cells of the Magnum
MDR	Multidrug Resistance
MFP	Membrane Fusion Proteins
MFS	Major Facilitator Superfamily
MIC	Minimum inhibitory concentration
MOI	Multiplicity of Infection
MOS	Mannan oligossacharides
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAOS	Neoagaro-oligosaccharides
NE	Norepinephrine
NESP	The National Enteric Disease Surveillance
NGM	Nematode Growth Medium
OM	Outer Membrane
OMP	Outer Membrane Channel Proteins
PCD	Programmed Cell Death
PO	Phenoloxidase
PPMS	*Palmaria Palmate*
QS	Quorum sensing
RB	Respiratory Burst
RBC	Red Blood Cells
RND	Resistance-Nodulation-Division Proteins

SAM	*S*-adenosylmethionine
SC	*Solieria chordalis*
SCFA	Short Chain Fatty Acids
SCV	*Salmonella* Containing Vacuole
SE	*Salmonella* Enteritidis
SG	*Sarcodiotheca gaudichaudii*
SMR	Small Multidrug Resistance
SOD	Superoxide Dismutase
SPI1	*Salmonella* Pathogenicity Island 1
SPI2	*Salmonella* Pathogenicity Island 2
SUK	*Sarcodiotheca spp*
SWE	Seaweed Extract
T3SS	Type Three secretion system
TBD	2,3,6-Tribromo4,5-dihydroxybenzyl methyl
TOS	Transgalactooligosaccharides
USDA	United States Department of Agriculture
VH	Villi Height
VRE	Vancomycin-resistant Enterococci
WBC	White Blood Cells
WHO	World Health Organization
XOS	Xylooligosaccharide

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CHAPTER 1. INTRODUCTION

Food-borne *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) is a major cause of egg-associated salmonellosis in humans in America and Europe (Govaris *et al.* 2010). In humans, *Salmonella* infection causes food poisoning and intestinal infections leading to high mortality (Yim *et al.* 2010). Worldwide, *Salmonella* outbreaks have become an important public health and economic problem (WHO, 2014). The survival capabilities of *S.* Enteritidis in host cells are favored by bacterial organelles (flagella, and fimbriae) and virulence factors that are primarily associated with biofilm formation, motility and quorum sensing (Brossard & Campagnari, 2012, De Kievit & Iglewski, 2000). The invasion of *Salmonella* into the intestinal epithelial cells is mediated by Type III secretion systems (T3SS). Type 3 secretion systems are complex needle like apparatus used by pathogens to deliver effector proteins into host cells. *S. enterica* encodes two large gene clusters: SPI-I and SPI-II (*Salmonella* pathogenicity island). SPI1 becomes active on contact with host epithelium and translocates virulence factors across the host cell plasma membrane, and *Salmonella* pathogenicity island II (SPI2) modulates the intracellular environment, which enables the survival and replication of *Salmonella* within the host cell (Malik-Kale *et al.* 2011). Further, biofilm formation and quorum sensing enhance *Salmonella* survival and pathogenesis in the host.

Recent reports have shown that the use of antibiotics in livestock has increased the prevalence of pathogenic bacterial strains that are resistant to a wide range of antibiotics (Laevens *et al.* 2003, Phillips *et al.* 2004). The emergence of drug resistant pathogens has become a major health concern in the current phase of diminishing antibiotic discovery (Lewis, 2013). The transfer of these pathogens to humans via direct contact with animals or through consumption of contaminated food or water is a significant public health problem. This has generated debate on the safety of antibiotics as growth promoters in young animals (Dibner & Richards, 2005). The factors that contribute to antibiotic resistance are exceedingly complex and dependent on the interactions between the infecting bacterium, its localization in the body, antibiotic concentration at the site of infection and the immune status of the patient (Wright, 2003). This necessitates a need to find alternative strategies to control the spread of *Salmonella*, to reduce the use of antibiotics.

Natural antimicrobial products such as prebiotics and probiotics have been widely investigated as feed additives to limit pathogen infection in poultry (Hinton & Mead, 1991). For example supplementation of probiotic *Lactobacillus* (1 g/kg) and prebiotic IMO (isomalto-oligosaccharides, 10 g/kg) significantly decreased the cecal *E. coli* in 21 day old broiler chickens (Mookiah *et al.* 2014). Additionally, natural compounds isolated from seaweeds such as halogenated and brominated metabolites, micosporine -like amino acids and sulphated polysaccharides have shown antimicrobial, antiproliferative, immune-modulating and anti-oxidant properties (Nishino *et al.* 1989, Vairappan *et al.* 2008, Stephanie *et al.* 2010). Red seaweeds (Rhodophyta) contain about 5,000–6,000 species (Guiry & Guiry, 2015) and are characterized by the presence of pigments such as phycoerythrin, phycocyanin and allophycocyanins (Muller *et al.* 2006). The bioactive compounds isolated from red seaweeds have shown efficacy against several infectious and parasitic diseases (Vairappan, 2003). While, these studies have shown *in vitro* antibacterial activity of seaweed components, their *in vivo* affect is not much explored. Model organisms including mice and rodents are commonly used to validate the bioactivity *in vivo*. However, due to the budget and space limitations of such animal trials, a relatively simple model system is required to test the efficacy and several concentrations of potential antimicrobials.

Caenorhabditis elegans (*C. elegans*) is an anatomically and genetically simple nematode and has been extensively used as a high throughput model to screen antimicrobial compounds (Aballay & Ausubel, 2001, Moy *et al.* 2009). *Salmonella* can infect the soil nematode *C. elegans* (Sifri *et al.* 2005). Bacterial virulence factors required for pathogenesis and innate immune responses are conserved between *C. elegans* and mammals (Sifri *et al.* 2005). In *C. elegans*, signal transduction pathways are activated in response to infection and effector molecules produced by pathogens. There are four main pathways identified in *C. elegans*; the p38 MAP kinase pathway, programmed cell death (PCD), the toll-like pathway and the DAF-2 insulin-signaling pathway. The *C. elegans* gonadal PCD is triggered in response to *Salmonella* virulence factors and PCD plays an important role to protect worms against *Salmonella* colonization (Aballay & Ausubel, 2001). Red seaweeds have been shown to enhance the immune response of *C. elegans* to *Pseudomonas aeruginosa* (PA-14) through the induction of PMK-1 and Daf-2/daf-16

insulin signalling pathways (Liu *et al.* 2013). Thus *C. elegans* can be used as an ideal model to screen antimicrobial compounds for testing in higher animals.

The use of natural products as anti-infective agents in commercial livestock production is a relatively new area of research, one that has arisen in response to rising concerns for bacterial resistance to antibiotics and an increasing consumer concern for animal drug residues in meat. Feed ingredients containing seaweeds for layer hens enhance beneficial bacteria in the gut, as seaweeds remain largely undigested in the lower GIT and therefore act as substrates in bacterial fermentation (MacArtain *et al.* 2007). While seaweeds have been previously determined to enhance the immune system, modulate growth, and microbial population in pigs and ruminants, their effects in poultry is not explored (Evans & Critchley, 2014). The aim of this study is to investigate the effect of red seaweeds on *Salmonella* Enteritidis in poultry using a *C. elegans* infection model. A study on red seaweeds as feed additives to control *Salmonella* infection in layer hens may offer an alternative to the use of antibiotics in poultry.

CHAPTER 2. LITERATURE REVIEW

2.1 Seaweeds

Marine environments represent a rich source of unique biological and chemical diversity. This diversity serves as a reserve of bioactive compounds; especially seaweeds which have been implicated to improve human and animal health. Seaweeds are primarily classified into three groups: green algae (Chlorophyta), brown algae (Phaeophyta) and red algae (Rhodophyta) (Garson, 1989). The green algae constitute approximately 4500 species. The characteristic green color is due to the presence of chlorophyll *a* and *b* which are similar to higher plants (Guiry, 2012). Brown algae contain about 1800 species and the brown color of these algae is due the abundance of xanthophyll pigment and fucoxanthin. Red algae (Rhodophyta) contain about 5,000–6,000 species (Guiry & Guiry, 2015) and are characterized by the presence of phycoerythrin, phycocyanin and allophycocyanins as accessory pigments, which give them their red color (Muller *et al.* 2006). Seaweeds are used as food in the Asian countries of Japan and China. Additionally seaweeds are also used in the treatment of intestinal disorders and many diseases such as goiter and hyperthyroidism (Aquaron *et al.* 2002).

2.1.1 Red seaweeds

Red algae are found from polar to tropical coastlines, most commonly in the intertidal and subtidal zones to a depth of 40-250 m (Muller *et al.* 2006). Main properties of red algae include the presence of *β*-carotene, zeaxanthane, chlorophyll *a* and floridean starch as reserve. Carrageenans and sulphated galactans represent the main matrix polysaccharides of red seaweeds. These polysaccharides contain alternately linked linear chains of β-d-galactopyranosyl residues (A units) and α-galactopyranosyl (or 3,6-anhydrogalactosyl) residues (B units) with sulfate esters, pyruvic acid ketals, and methoxyl group substitutions. These natural polysulfates have been reported as active metabolites and have been attributed diverse biological activities (Bouhlal *et al.* 2011).

2.1.2 Bioactive metabolites in red seaweeds

The bioactive compounds from red seaweeds have a wide range of applications in the pharmaceuticals, nutritional, cosmetics, agrichemicals and the food industry. Their

cell wall constitutes long chain polysaccharides including cellulose, agars, and carrageenans, which are of commercial value in the food industry (Guiry & Guiry, 2015). Red seaweeds including *Palmaria palmata*, and *Chondrus crispus* are edible. Nori, from the red algae *Porphyra*, is a most valuable marine crop in Japan with a value of more than $1billion (US). Red algae including *Kappaphycus* and *Betaphycus* are commercially used to extract carrageenans, a common food ingredient in yogurt, chocolate milk and ice creams. Red algae such as *Gracilaria, Gelidium, Pterocladia* are used as a source of agar, commonly used as a solidifying agent in microbiological media (Mabeau & Fleurence, 1993). Many compounds isolated from red seaweeds have shown efficacy against several infectious and parasitic diseases as described below.

2.1.2.1 Antiviral activity

Several seaweed polysaccharides, such as carrageenans, sulfated proteoglycans, and dextran sulphates have been reported to exhibit antiviral activities against human papillomavirus (HPV), influenza A virus (IAV) and human herpes virus HSV-1 and HSV-2 (Carlucci *et al.* 2004, Talarico & Damonte, 2007, Wang *et al.* 2012). Previously, a strong antiviral activity with EC_{50} between 23.0 and 101.1 µg/mL at a Multiplicity of Infection (MOI) of 0.001 ID50/cells was observed with *Solieria Chordalis* extract. Additionally, 500 µg/mL of carbohydrase (C3) hydrolysate extracts displayed complete protection to cells (Hardouin *et al.* 2014).

Sulphated polysaccharides exhibit high antiviral activity against enveloped viruses, such as HSV. Viral envelope glycoprotein C interacts with the heparin sulphate (HS) on the host cell surface, which leads to the attachment of the virus to the cells. The virus-cell complex is made of ionic interactions between the anionic sulphate groups in the polysaccharide and basic amino acids of the glycoprotein. The sulphated polysaccharides from seaweeds have been shown to mimic cell bound sulphates and are known to alter the polysaccharide complexes on the cells, thereby blocking the entry of viruses into the host cells (Damonte *et al.* 2004). Bourgougnon *et al.* (1993) reported that the aqueous *Schizymenia dubyi* seaweed extracts with higher sulfate content were effective in inhibiting HSV-1 replication at an EC_{50} = 2.5-80 µg/mL without cytotoxic effect (Bourgougnon *et al.* 1993). Methanol extract and 2,3,6-Tribromo4,5-

dihydroxybenzyl methyl (TDB) ether isolated from the red alga *Symphyocladia latiuscula* exhibited antiviral activities against wild type HSV-1 and acyclovir (ACV) resistant-HSV-1 (IC_{50} values of 5.48, and 4.81µg /mL, respectively). Daily oral administrations of the methanolic extract and TDB delayed the appearance of lesions in infected mice without toxicity (Park *et al.* 2005). Similarly, lambda-carrageenans from red seaweed *Gigartina skottsbergii* (Gigartinaceae, Rhodophyta) displayed antiviral activity against animal viruses belonging to the *Alphaherpesvirinae* subfamily, BoHV-1 (bovine herpesvirus type 1) strain Cooper and SuHV-1 (suid herpesvirus type 1) strain Bartha. Diogo *et al.* (2015) first reported the antiviral potential of lambda-carrageenan against animal viruses. These results indicate that red seaweed compounds could be used as potential antiviral agents to treat humans and animals (Diogo *et al.* 2015).

2.1.2.2 Antifungal activity

With an increase in the number of immunocompromised individuals, invasive fungal infections have emerged as a major concern. There has been a sharp increase (almost 50%) in the rate of mortality associated with invasive fungal infections despite the availability of several antifungal drugs (Brown *et al.* 2012). Therefore, new sources of antifungal compounds are required to combat this growing problem. Bioactives from seaweeds are viable sources of antifungal compounds that could be potentially used for drug development. Several compounds isolated from seaweeds exhibit antifungal activity against pathogenic fungi. The most promising component includes diterpene benzoates (bromophycolide) from *Callophycus serratus*, which displayed a wide range of activities against human tumor cell lines, malarial parasites and bacterial pathogens (Teasdale *et al.* 2012). A protein fraction of red seaweed *Hypnea musciformis* showed inhibitory affects against the growth of human pathogen i.e. yeasts *Candida albicans* and *C. guilliermondi* (Cordeiro *et al.* 2006). In another study, mahorones, a brominated cyclopentenone, from the red seaweed *Asparagopsis taxiformis* exhibited antifungal activity against *Aspergillus fumigatus and Candida albicans* (Greff *et al.* 2014).

2.1.2.3 Antibacterial activity

The current drift towards an increase in antibiotic resistant bacterial strains highlights the urgent need to find natural alternatives. Previously, several reports have

highlighted the identification of antimicrobial metabolites. For example, halogenated metabolites (elatol and iso-obtusol) isolated from red algae inhibited six species of bacteria including *Staphylococcus epidermidis*, *Klebsiella pneumonia* and *Salmonella* sp. As the subculture of the assay tubes showed growth on agar, it was concluded that the mode of action of elatol and iso-obtusol was bacteriostatic (Vairappan, 2003). Additionally, the isolated halogenated compounds (laurinterol and isolaurinterol) were shown to be effective against seven strains of antibiotic resistant bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, and vancomycin resistant *Enterococcus faecalis* and *E. faecium* (VRE) (Vairappan *et al.* 2004). Lutein, β- carotene, and chlorophyll *a* isolated from edible extracts of red seaweed *Porphyra tenera* have been shown to suppress mutagen-induced umu C gene (indicator of DNA damage) expression (measure of β- galactosidase activity) in *Salmonella* Typhimurium (TA 1535/ pSK 1002) (Okai *et al.* 1996). Similarly, hot water extracts of red seaweed *Gracilaria tenuistipitata* and *Gelidium amansii* showed higher survival of white shrimp (*Litopenaeus vannamei*) infected with the bacterium *Vibrio alginolyticus*. Further, the water extract significantly increased the immune response associated parameters such as the haemocyte count, phenoloxidase (PO) activity, respiratory burst (RB), and superoxide dismutase (SOD) activity (Yeh & Chen, 2009). Aqueous extract from five red seaweeds *Gelidium latifolium, Hypnea musciformis, Jania rubens, Jania spp.* and *Laurencia obtuse* have shown significant antimicrobial activity against pathogenic Gram-negative bacteria including *Escherichia coli, Klebsiella spp,* and *Pseudomonas aeruginosa* (Alghazeer *et al.* 2013). Moreover, sulfated galactans and polysaccharides (carrageenans) from the aqueous extract of red algae, *Corallina,* has shown bactericidal activity against Gram-positive pathogenic bacteria including *Enterococcus faecalis* and *Staphylococcus epidermidis*. This indicates that, in addition to organic extracts, the polysaccharide fractions in red seaweeds, which are water-soluble, can be effective against Gram-negative and positive bacterial pathogens. Furthermore, it will be interesting to identify antimicrobial activity of extracts from cultivated edible red seaweeds such as *Chondrus crispus* and *Sarcodiotheca* spp.

2.1.3 Mechanism of antimicrobial activity of seaweeds

Seaweeds are constantly exposed to a range of environmental stresses such as desiccation, osmotic stress, light, and extreme temperatures as well as pathogenic microbes. In response, the seaweeds have evolved protective mechanisms to survive in such stressful conditions (Sampath-Wiley *et al.* 2008). The beneficial effect of seaweeds are attributed to the complex mixture of bioactives which posses antioxidant, antimicrobial, anticancer and antiviral properties. Amongst other compounds responsible for these activities sulphated polysaccharides, organic acids, and phenolic compounds are well characterized (Bedoux *et al.* 2014). For example, the phenolic compounds permeate the cell wall or cell membrane of the pathogen and exhibit antimicrobial action by releasing the intracellular content (O'Connor & Rubino, 1991). Phenolic compounds also interfere and impair nutrient uptake, nucleic acid, protein synthesis, enzyme activity and disrupt electron transport (O'Connor & Rubino, 1991). Previously, seaweed-associated polysaccharides have been shown to elicit defense responses similar to pathogen recognition (PAMP triggered immunity) in host and activate signalling pathways downstream to activate defense responses (Potin *et al.* 1999). To my knowledge, there are no reports of the direct antimicrobial mechanism of seaweed polysaccharides against Gram-negative enteric pathogens. Interestingly, polysaccharides (mannans) derived from the yeast cell wall have been shown to inhibit Gram-negative pathogens (*E.coli* and *Salmonella*) by binding to their fimbria (Ballou, 1970). Fimbriae are surface appendages of bacteria, which bind to the mannan specific receptors in host intestinal epithelial cells. Mannanoligosaccharides have high affinity ligands and can competitively bind to pathogenic bacteria. As a result, pathogens get adsorbed to the mannans; thereby reducing the attachment of pathogenic bacteria to the intestinal epithelial cells and get removed from the intestine without successful colonization (Ofek *et al.* 1977). Similarly, the antimicrobial activity of the polysaccharides in red seaweeds could be attributed to their affinity to the pathogen surface appendages such as pilli, fimbriae and flagella. Polysaccharides from seaweeds with ionic properties have been shown to exhibit antimicrobial activity against Gram-negative pathogens. As demonstrated by Karbassi *et al.* (2014), alginic acid (carbohydrate polymer) from brown seaweeds showed bacteriostatic effects against *E.coli*. The mechanism of bacteriostatic action was not

reported; however, the polyanionic nature of alginic acid was assumed to be pivotal for antimicrobial activity against *E. coli* (Karbassi *et al.* 2014).

2.1.4 Red seaweeds as prebiotics

Seaweeds are rich in dietary fiber (25–75% dry weight), and have been shown to improve intestinal microflora (Evans & Critchley, 2014). Among others, red seaweed polysaccharides can be considered prebiotic. They are resistant to acid and enzymatic digestion in the upper gastrointestinal tract and selectively stimulate the growth of beneficial gut bacteria in the host. Red seaweeds are rich in unique carbohydrate including floridean starch, sulfated galactans, agar, carrageenans and uronic acid. Agars are known as gel forming polyssacharides and are commercially extracted from Gracilariaceae and Gelidiaceae families. They are linear polymers characterized by alternate 3-linked β-D-galactopyranosl and 4-linked 3,6-anhydro-α-L-galactopyranosyl units. In addition to the use of agar in microbiology and biotechnology applications, agar -derived polysaccharides have also been shown to act as prebiotics. Ramnani *et al.* (2012) reported that the low molecular weight polysaccharides derived from agar and alginates significantly increased the population of beneficial bacteria, *Bifidobacterium* (from $\log_{10}$ 8.06 at 0 h to $\log_{10}$ 8.55 at 24 h) in anaerobic batch cultures inoculated with human feces. Additionally, the polysaccharide-supplemented cultures also showed an increase in short chain fatty acids (SCFA, acetic and propionic acid) indicating their optimum utilization by fermentation (Ramnani *et al.* 2012). Neoagaro-oligosaccharides (NAOS) obtained from the enzymatic hydrolysis of agarose showed an increase in the colonic bacteria of mice fed with 2.5% (w/v) NAOS (Hu *et al.* 2006).

Carrageenans are a class of sulphated polysaccharides made of alternately linked β-D-galactose with α-D-galactose. Based on their physical properties carrageenans are categorized as kappa, iota and lambda carrageenans. Red seaweed rich in sulfated galactans and carrageenans has been evaluated for its prebiotic property in rats. Though seaweed intake significantly increased cecal moisture and the proportion of acetic and propionic acid (SCFA), no direct prebiotic activity was observed (Gomez-Ordonez *et al.* 2012). Hayisama *et al.* (2014) studied a potential synbiotic effect of fermented red seaweed (*Gracilaria fisheri*) using *Lactobacillus plantarum*. The cell free extract was

shown to inhibit the growth of food borne pathogens including *Staphylococcus aureus, Escherichia coli, Salmonella* Typhi and *Vibrio parahaemolyticus*. Moreover, the cell free extract also increased the abundance of lactic acid bacteria (Hayisama-ae *et al.* 2014). In another study, *Bifidobacterium* readily fermented nori sheets from red seaweed *Porphyra yezoensis*. The rate of fermentation was dependent on the amount of floridoside (glycerol galactoside), which was determined as the major substrate for the fermentation by intestinal bacteria. Floridoside was neither broken down by digestive enzymes nor absorbed by the small intestine. Muraoka *et al.* (2008) concluded that floridoside can be used as a prebiotic substrate for *Bifidobacterium* (Muraoka *et al.* 2008). However, these were *in vitro* studies, which examined the prebiotic potential of red seaweed polysaccharide, and to date there has been no report on the prebiotic effect of red seaweeds in poultry.

2.1.5 Seaweeds in livestock production

The algal biomass and algal products are widely used as animal feed in a number of countries. It is estimated that about 30% of the algal biomass produced is used as animal feed (Richmond, 2004). Health-promoting oligosaccharides derived from seaweeds are currently investigated for their use in poultry (Dibaji *et al.* 2014). Recent studies with broilers suggested that seaweed in the diet enhanced the health and productivity largely by increasing the growth of beneficial gut-microbiota in the lower gastrointestinal tract (GIT) (Abudabos *et al.* 2013).

The use of natural products as anti-infective agents in commercial livestock production is a relatively new area of research, one that has arisen in response to rising concerns for bacterial resistance to antibiotics and an increasing consumer concern for animal drug residues in meat. Seaweeds are a rich source of dietary fiber, minerals, vitamins, proteins, phlorotannins, and carotenoids (Ventura *et al.* 1994). Ingredients containing seaweeds fed to commercial laying hens enhance gut micro biota, as seaweeds remain largely undigested in the lower GIT and therefore act as substrates in bacterial fermentation (MacArtain *et al.* 2007).

Gudiel and Goni (2002), reported that red and brown seaweeds have prebiotic like properties that can alter metabolic activity of beneficial microflora and reduce the

prevalence of pathogenic bacteria (Gudiel-Urbano & Goñi, 2002). Seaweeds such as *Porphyra yezoensis, Undaria pinnatifida, Laminaria japonica,* and *Hizikia fusiformis* have also been reported to alter nutrient digestibility by binding to bile salts in the gut and inhibiting uptake of fats resulting in lower levels of blood cholesterol (Wang *et al.* 2001). Additionally, supplementation of layer diets with seaweed, *M. pyrifera,* resulted in elevated levels of omega -3 fatty acids in eggs (Carrillo *et al.* 2008). Moreover, feed supplements of seaweeds have resulted in increased growth and nutrient availability in chickens and ducks (El-Deek & Brikaa, 2009).

2.2 *Salmonella*

2.2.1 History and nomenclature of the genus *Salmonella*

Salmonella are Gram-negative facultative anaerobes that are capable of causing gastroenteritis and systemic infection in a wide variety of hosts (Doyle *et al.* 1997). *Salmonella* is named after an American veterinary pathologist, Daniel Elmer Salmon. In 1884, D.E. Salmon and his colleague bacteriologist Theobald Smith first isolated "hog cholera bacillus" from pig intestines and named it *Bacillus choleraesuis* (Smith, 1894). In 1900, Lignieres coined the generic name *Salmonella*, and the newly discovered *Bacillus choleraesuis* was subsequently given the name *Salmonella choleraesuis*. Bacilli with similar phenotype were added to the genus *Salmonella*. These included clinically important organisms that were named either describing the specific disease they cause or the animal from which they were isolated (*S. typhi and S. typhimurium*). However, this nomenclature became inaccurate with time and the organisms were named by the geographic location where they were first isolated (*S. london* and *S. panama*). In 1966, Kauffmann proposed a new antigen-based system (Kauffmann, 1966). According to this system, *Salmonella* serovars were identified by the interaction between antibody and surface antigens of the organisms as well as the biochemical characteristics. Each serovar was considered a separate species and this complexity of multiple species lead to the division of the genus *Salmonella* into three sub-species namely, *S. choleraesuis*, *S. typhosa*, and *S. kauffmannii* (Ewing, 1972). Later, Le Minor *et al.* (1982) proposed a one species concept, where *S. choleraesuis* was named the single *Salmonella* species with six subspecies. Under the one species concept, *choleraesuis* was named as both a species and

a serovar (Leminor *et al.* 1982). In 1987 the World Health Organization (WHO) proposed to replace *S. choleraesuis* with *S. enterica* as the type species (Le Minor, 1987). They suggested the division of the genus *Salmonella* into seven subgenera called as subspecies (I, II, IIIa, IIIb, IV, V, and VI) (Euzeby, 1999). The subspecies were grouped together on the basis of DNA similarity and phenotype. In 2005, a Judicial Commission approved the recommendation by the WHO Collaborating Centre for Reference and Research and replaced *S. choleraesuis* with *S. enterica* as the type species of the genus *Salmonella* (Judicial Commission of International Committee on Systematics of Prokaryotes, 2005). This nomenclature system is currently used by the WHO, the Centers for Disease Control and Prevention (CDC) and the American Society for Microbiology (ASM). Every year newly recognized serovars are reported in the document called the Kauffmann-White scheme, Journal of Research in Microbiology (Tindall *et al.* 2005).

According to the new system, the genus *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica*. *S. enterica* is further divided into six subspecies I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa *S. enterica* subsp. *arizonae*; IIIb *S. enterica* subsp. diarizonae; IV *S. enterica* subsp. *houtenae,* and VI *S. enterica* subsp. *indica*. Under supspecies I, serovars are named after geographical location, habitat or the associated disease. For other subspecies, the antigen formula by Kauffmann-White is used to designate the serovars (Popoff *et al.* 2004). According to the latest report published in 2014, there are 2659 serovars in the genus *Salmonella*, which includes 63 new *Salmonella* serovars identified from 2008-2010 (Issenhuth-Jeanjean *et al.* 2014). To differentiate serovars from species, serovar names were written in Roman font (not italicised) and started with a capital letter, for example, *Salmonella enterica* serovar Typhimurium.

In this thesis I will use the approved nomenclature *Salmonella enterica* subsp. *enterica* serovar Enteritidis and the short version *Salmonella* Enteritidis or *S.* Enteritidis (SE).

2.2.2 General characteristics of the genus *Salmonella*

The genus *Salmonella* belongs to the family Enterobacteriaceae and is characterized by the presence of peritrichous flagella, is rod shaped, Gram-negative, non-

spore forming, and is facultatively anaerobic (Doyle *et al.* 1997). *Salmonella* are oxidase negative and catalase positive. These characteristics enable identification of *Salmonella* at the subspecies level. Some species are ubiquitous while other species are specifically adapted to a particular host. Most of the strains of *Salmonella* are capable of producing a biofilm, a complex matrix of polysaccharides which acts as a barrier for antimicrobial agents (Galan, 2001)

2.2.3 *Salmonella* pathogenicity

Salmonella are zoonotic bacteria capable of causing disease in wide range of host species including humans, animals, birds, reptiles and rodents. They are ubiquitous in nature and can be isolated from fruits, vegetables and environmental sources such as rivers, sewage and soil. The primary mode of transmission is the orofecal route, moreover airborne transmission can also occur. Upon consumption of contaminated water or food, the bacteria survive the acidic condition of the gastric fluid to reach the small intestine and colon. Flagellar motility enables bacteria to recognize and adhere to the host epithelium (Pontier-Bres *et al.* 2012). The invasion of *Salmonella* into the intestinal epithelial cells is mediated by Type three secretion systems (T3SS). T3SS operon contains *Salmonella* pathogenicity island 1 (SPI1), which encodes for proteins to form a needle initiation complex. This enables bacteria to penetrate into the intestinal epithelium and inject effector proteins into the host cytosol. Delivery of effector proteins leads to host cytoskeleton rearrangement and aids in replication of bacteria in the Payer's patch (Groisman & Mouslim, 2000). After replication, bacteria migrate towards bone marrow, liver and spleen to initiate systemic infection. In systemic infection, macrophages are the primary site for bacterial replication. The survival of *Salmonella* in macrophages is critical for the establishment of an invasive infection. Virulence factors encoded by *Salmonella* pathogenicity island II (SPI2) enables the survival and replication of bacteria within the macrophages (Brown *et al.* 2005). Consequently, bacteria multiply and spread throughout the body, resulting in septicemia and death of the host. The survival capabilities of *S.* Enteritidis are favored by cell-associated organelle and virulence factors that are primarily associated with biofilm formation, motility and quorum sensing (Brossard & Campagnari, 2012, De Kievit & Iglewski, 2000).

2.2.4 Virulence factors of *Salmonella*

The invasion and intracellular survival of *Salmonella* in the host epithelium is mediated by virulence factors including Type 3 secretion systems (T3SS), flagella, and fimbriae adhesins.

2.2.4.1 Fimbriae

Fimbriae are hair like surface appendages composed of helically arranged repeated subunits of fimbrin monomers. The length of fimbria ranges from 0.5-10 μm from the surface of the cell and are usually 2-8 nm thick (Collinson *et al.* 1996). There are 13 fimbriae loci, which play a vital role in *Salmonella* pathogenesis by aiding biofilm formation and persistence on both biotic and abiotic surfaces. Most fimbriae have a conserved mechanism for assembly and translocation to the surface of the bacteria. In the periplasm, fimbrins are bound by chaperones to prevent premature aggregation and are translocated across the outer membrane by usher proteins for assembly. Hybridization studies suggest that many fimbrial gene sequences are conserved between *Salmonella* serovars while some are specific to known receptors.

2.2.4.2 Flagella

Bacterial flagella are complex molecular machines required for motility in Gram-positive and Gram-negative bacteria. Flagella are morphologically similar in both eukaryotes and archaea but share no functional homology with prokaryotes. The evolutionary studies of T3SS and flagella export proteins suggest that they have evolved from a common ancestor. This indicates that during evolution, the versatile export apparatus in bacteria would have secreted various proteins for diverse functions such as assembly of surface appendages and intrabacterial interaction (Gophna *et al.* 2003). Most *Salmonella* serovars contain 5-10 randomly arranged flagella on the cell surface that contribute to the virulence of the pathogen. Cogan *et al.* (2004) identified flagella as one of the virulence markers for poultry. Flagella and fimbriae were shown to be important for the growth of *Salmonella* serovars in chicken eggs (Cogan *et al.* 2004). However, there is conflicting evidence for the role of flagella in the virulence of *S.* Enteritidis. Parker & Guard-Petter (2001) identified a mutation in flagella that may aid in oral infection in chickens. They orally challenged 20 day old chicks with wild type, non-

flagellated *S.* Enteritidis mutants (*fliC* and *flhD*) and invasive protein insertion mutants (*sipD* and *iacP*). Surprisingly, the absence of flagella increased oral invasion by *S.* Enteritidis. This implies that *S.* Enteritidis did not utilize flagellation and motility to colonize the birds. However, invasive proteins were shown to be essential for the colonization of *S.* Enteritidis in birds as was observed in mammals. Thus, avian adapted non-motile *S.* Enteritidis might have evolved to optimize oral colonization in birds at the expense of a narrower host range (birds), while flagellation might still aid in the colonization of *Salmonella* in a wider host range (mammals) (Parker & Guard-Petter, 2001).

2.2.4.2.1 Structural composition of flagella

The structure of flagella resembles T3SS in *Salmonella* and contains three regulatory proteins, four structural chaperones, six cytoplasmic proteins and 22 structural proteins. The structure is composed of a L ring (FlgH) in the plane of lipopolysaccharide in the outer membrane, a P ring (FlgI) in the peptidoglycan, and a MS ring (membrane and supramembranous, FlgF and FliF) within the cytoplasmic membrane (Brutinel & Yahr, 2008). In Gram-positive bacteria L and P rings are absent. This difference in structure could be due to the variation in the cell wall of Gram-negative and -positive bacteria. The C ring (FliG, FliM and FliN) embedded into the cytoplasm controls the rotation as well as direction of rotation of flagella. An ATPase (FliI) extends into the cytoplasm of the rotating machinery. Basal body protein FliF assembles between two bacterial membranes connects inner and outer membrane rings to form the flagellar rod. The rod works as an axle and transmits basal body rotation to the hook and filament. Other basal body proteins (FlgG and FlgF) regulate the positioning of flagella. The flagellar motor, the driving force for motion, contains five proteins: MotA, Mot B, FliG, FliM, and FliN. MotB is linked to the peptidoglycan layer and extends into the periplasm, while MotA interacts with FliG in the cytoplasm. The Type 3 secretion system (T3SS) is essential for the assembly of flagellar components, namely the rod, hook and filament located in the extracellular space outside the cytoplasmic membrane. The hook is present outside the cytoplasmic membrane and FliK acts as molecular marker to determine its length. The hook extends into a hook filament junction (FlgK and FlgL), followed by a filament made of flagellin protein (FliC and FljB) (Jarrell & McBride, 2008). The

filament cap protein mediates the polymerization of flagellin at the tip of the growing flagella. These structural proteins have homologs in Gram-positive bacteria, however their regulatory mechanisms are different due to the presence of the thick peptidoglycan layer and absence of an outer membrane (Mukherjee & Kearns, 2014).

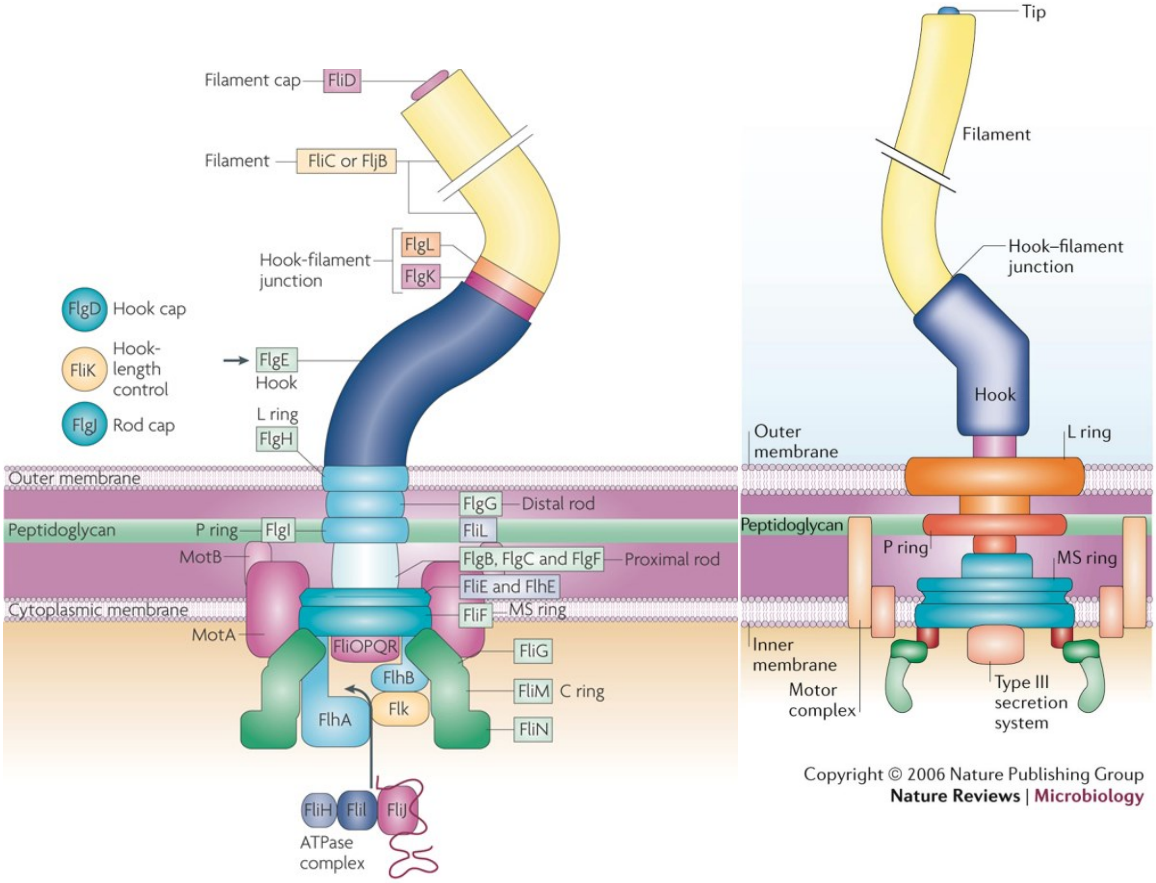

Figure 2.1 Schematic representations of components of flagellar system in *Salmonella.* (adapted from Fabienne *et al.* 2008)

2.2.4.2.2 Assembly of flagella

Bacterial flagellar assembly proceeds in three stages, the proximate structure (basal body) is assembled first, followed by hook and filament (cell distal structure). There are nearly 50 genes involved in the assembly and regulation of flagella. These genes are clustered into different regions on the chromosome and are divided into three classes: early (I), middle (II) and late (III) (Macnab, 1992). The early gene *flhDC* encodes

the master regulators FlhD and FlhC, which regulate the expression of the middle genes involved in the assembly of the T3SS apparatus, rod and the hook. In the basal body, the MS ring is formed first, followed by the assembly of motor, rod, L and P ring mediated by the Type 3 secretion system (Minamino & Namba, 2004). A class II gene *fliA* (flagella-specific sigma factor, σ^{28}) is required for the activation of late gene transcription. In addition, FlgN serves as chaperone for the hook-filament junction protein and FliT serves as chaperone for the filament cap protein (FliD). Three hook-associated proteins FlgK, FlgL and FliD are involved in the polymerization of flagellin on the tip of the hook. After formation of the hook- basal body complex, FlgK and FlgL present at the junction of hook-filament layer serve as a polymerization nucleus for the flagellin subunits. FliD is secreted on the tip of the growing filament and acts as capping protein for the polymerization of flagellin subunits (Yokoseki *et al.* 1995). The depletion of cap proteins in the cytoplasm signals the completion of flagellum structure. This initiates the binding of FliT to FlhC, and inhibits the transcription of middle genes indicating that rod hook substrates are no longer required for the assembly process (Jarrell & McBride, 2008).

2.2.4.3 Type 3 secretion system (T3SS)

The Type 3 secretion systems is a complex needle-like apparatus used by pathogenic bacteria to deliver effector proteins directly into host cells. This mechanism was visualized in the1980s for a cluster of hypersensitive response and pathogenicity (*hrp*) genes in *Pseudomonas syringae.* It was demonstrated that the *hrp* gene cluster in bacteria is required to trigger hypersensitive response in resistant plants and disease in susceptible plants (Lindgren *et al.* 1986). Later in 1998, the first three-component structure of T3SS was isolated from *S.* Typhimurium (Kubori *et al.* 1998). Initially, T3SS were thought to be intermediate complexes of flagellar assembly, however, later it was found that T3SS genes evolved by multiple horizontal gene transfer (Gophna *et al.* 2003). Two large gene clusters encode T3SS: SPI-I and SPI-II (*Salmonella* pathogenicity island) pathogenicity islands are located within a chromosome or on a plasmid. They have different guanine-cytosine (GC) content than the rest of the genome and are bordered by transposon insertion sequences or bacteriophage genes. There are 21 SPIs identified in different serovars of *Salmonella* of which SPI-I and SPI-II are most characterized.

2.2.4.3.1 *Salmonella* pathogenicity island 1 (SPI-I) T3SS

The 40 kb SPI-I is located at centrosome 63 on the chromosome. The GC content of SPI-I is 47% and it contains overall 41 genes encoding for various components of T3SS (regulatory, effectors and metal transport). The virulence of SPI-I is determined by its ability to deliver effector proteins into the host cell. Previously, Galan and Curtiss (1989) showed that an SPI-I mutant (invC) of *S.* Typhimurium was defective in penetrating into cells, however there was no observed effect on the attachment of bacteria to the cultured cells. Also, the mutant strain had 50% higher lethal doses (LD_{50}) than their wild-type parent strains when administered orally to Balb/c mice. Surprisingly, no differences were observed in LD_{50} when strains were given intraperitoneally. This indicates that SPI-I is essential for the effective penetration of *Salmonella* into host intestinal epithelial cells (Galan & Curtiss, 1989). In another study, Hung *et al.* (2013) demonstrated that intestinal fatty acid propionate reduced *Salmonella* penetration of cultured intestinal epithelial cells. As the expression of SPI-1 regulators was significantly reduced in the SPI-1 mutants, the mechanism of control by propionate was through reduced expression of SPI-1 transcriptional regulators (*hilD*) (Hung *et al.* 2013). Moreover, SPI-I is involved in the invasion during the initial phase of infection and not during systemic phase. The subcellular localization and expression of T3SS-I and T3SS-II were differentially regulated during *Salmonella* Typhimurium infection. It was observed that the majority of bacteria at 2 h post infection were associated with the epithelium and expressed T3SS-I but not T3SS-II. After 8 h post infection, bacteria expressing T3SS-II were identified in the lamina propria and mucosa, whereas T3SS-1-expressing bacteria were in the lumen (Laughlin *et al.* 2014).

2.2.4.3.2 Structural component of *Salmonella* pathogenicity island 1 (SPI-1)

The assembly of SPI-1 is initiated by the formation of inner membrane and outer membrane rings, followed by association of rings and formation of basal body complex. Invasive protein InvJ acts as a molecular marker and controls the formation of the needle complex. M-cells are the preferred target site for the infection of the small intestine by *Salmonella*. Several environmental factors such as pH, osmolarity, bile acid, ion concentration (Mg^{2+}), and short chain fatty acids can regulate the expression of SPI-1. The bile acid in the upper intestinal tract or short chain fatty acids produced by intestinal

microbiota repress the expression of SPI-1 (Hung *et al.* 2013). Increase in osmolality and neutral pH have been shown to induce the expression of SPI-I. HilA is homologous to transcriptional activators involved in regulation of genes in response to physiological signals. It belongs to a two-component global regulatory systems OmpR/ToxR family. HilA is characterized by the presence of a conserved DNA binding/transcriptional activation domain at its amino-terminus and a carboxyl-terminal domain. HilA regulates the transcription of invasion genes in response to the growth conditions of *Salmonella* during infection. The cellular activity of HilA can be regulated by change in the expression of *hilA* (Bajaj *et al.* 1995). HilA can activate transcription of the inv/spa operons. The inv locus genes (*invF, invA* and *invG*) also regulate the entry of *Salmonella* into the epithelial cells. The role of *invF* is to maintain the proper expression of every component of the organelle involved in entry of *Salmonella* and *invG* regulates the supramolecular structure required for the entry (Kaniga *et al.* 1994). InvF activates transcription of the sic/sip operon of SPI-1, and genes within SPI-4 and SPI-5 (sopB). *Salmonella* invasion protein (SipA) plays a critical role in rearrangement of the host actin cytoskeleton. SipA binds to actin and inhibits the depolymerization of actin filaments. This facilitates the spatial localization and outward extension of membrane ruffles, which results in uptake of *Salmonella* (Zhou *et al.* 1999).

2.2.4.3.3 *Salmonella* pathogenicity island 2 (SPI2) T3SS

SPI-II is 40 kb insertion located on centrosome 37. The GC content of SPI-II is 43%. SPI-II is important for the survival in the *Salmonella* containing vacuole (SCV) and is required for systemic infection in mice. It contains more than 40 genes, including regulatory genes, T3SS effectors, and tetrathionate reductase genes. Jones *et al.* (2001) demonstrated the role of pathogenicity islands in interactions between *Salmonella* and avian cells *in vitro* and in chickens. They concluded that SPI-II functions mainly by facilitating survival within macrophages and enabling multiplication within the reticuloendothelial system (Jones *et al.* 2001).

It has been shown that SPI-1 and SPI-2 are inversely regulated; however, the gene regulation of both pathogenicity islands is complex. Bustamante *et al.* (2008) reported transcriptional cross-talk between SPI-1 and SPI-2. They showed that HilD, a SPI-I encoded regulator, is essential for the expression of both SPI-1 and SPI-2 under

stationary growth phase in LB medium. This reflects the notion that the bacterial system is constantly evolving to maximize their survival by co-ordinating the expression of virulence factors in the infection process (Bustamante *et al.* 2008).

2.2.5 Structure and function of *Salmonella* biofilms

A biofilm is defined as a sessile microbial population attached irreversibly to a surface and each other by a bacterium-initiated matrix (extracellular polymeric substance). They exhibit altered phenotype with respect to regulation of genes and growth (Donlan, 2002). Biofilms are mainly composed of proteinaceous fractions (curli, fimbrie, flagella), and exopolysaccharides (cellulose, colanic acid, O antigen). Major biofilm components required for the attachment on epithelial cells are fimbriae (type 1 fimbriae, plasmid encoded fimbriae) (Pef), curli fimbriae (Csg), colanic acid and cellulose (Ledeboer & Jones, 2005). Essential biofilm components on gall stone surfaces are flagella and O-A- antigen capsules (Crawford *et al.* 2010). Moreover, cellulose, lipopolysaccharide, functional T3SS and flagella based motility play an important role in the formation of biofilm on abiotic surfaces (Prouty & Gunn, 2003).

Curli (*csg)* are aggregates of amyloid like cell surface proteins which exist in complex with cellulose and the O-Ag-capsule. In biofilm formation, curli plays an important role in initiating cell-surface and cell-cell interactions. Fimbrial operons, most importantly Type 1 fimbriae, are required for adherence of biofilms on the epithelial cell layer. As observed in HEp-2 cells and chicken intestinal tissue, where *Salmonella* biofilms were formed in a type 1 fimbria-dependent manner. The global gene expression of *Salmonella* was compared during planktonic and biofilm growth using a microarray. The results indicated that five fimbrial gene clusters (*pef, csg, lpf, bcf and sth*) were altered in biofilm formation. Additionally, *pef* and *csg* mutants showed defective biofilm formation on the tested surfaces (Ledeboer *et al.* 2006). This indicates that curli and fimbria are essential for the genesis and maturation of biofilm on cell surfaces. In addition to curli, fimbriae and cellulose, biofilm formation protein (BapA) is required for the formation of the matrix. Multidomain protein BapA is large proline-threonine-rich 386 kDa *S.* Enteritidis protein encoded by the *bapABCD* operon. BapA is required for the aggregation and subsequent pellicle formation of the bacterial cells on air liquid surfaces.

Latasa *et al.* (2005), demonstrated that deletion of *bapA* in *Salmonella* Enteritidis resulted in the loss of the capacity of bacteria to form biofilms, whereas the overexpression of *bapA* resulted in increased biomass of biofilm. However, the overproduction of curli fimbriae can compensate for *bapA* deletion (Latasa *et al.* 2005). This indicates that although BapA facilitates the biomass of biofilm, it does not have a major impact on biofilm formation as its absence can be overcome by other protein components such as curli and fimbriae.

Exopolysaccaharide such as cellulose is required for the matrix structuralization during biofilm formation. Cellulose, encoded by *bcsABZC– bcsEFG* operons are a β-1-4-D-glucose polymer responsible for the sticky texture in biofilms and support long range cell-cell interactions (Solano *et al.* 2002). Another expopolyssacaride sugar called colanic acid is required for the formation of complex three-dimensional structures of *Salmonella* biofilms on both biotic and abiotic surfaces. Colanic acid forms a tight pellicle under most environmental conditions. This indicates that matrix production is triggered in response to environmental conditions (Solano *et al.* 2002). The extrapolymeric substance (EPS) fraction also contains the O-Ag capsule, which is made of repeat units of tetrasaccharide. The O-Ag capsule encoded by *yihU-yshA* and *yihVW* operons are covalently attached to lipids. They are important in the environmental persistance of the biofilm attached to the abiotic surface. Gibson *et al.* (2006) showed that the O-antigen capsule was co-regulated with fimbria- and cellulose associated matrix expression. As compared to the wild type, the *yih* mutant strains that lacked the capsule assembly were not able to survive lyophilisation (represented as long term stress). However, the mutant had no effect on pellicle formation, the rdar morphotype and biofilm formation. This indicated that the O-Ag capsule was crucial for survival during desiccation stress but not for multicellular aggregation (Gibson *et al.* 2006). Thus, the formation of these surface structures signifies a conserved survival strategy of *Salmonella* against environmental stress.

2.2.6 Quorum sensing mechanism in *Salmonella* Enteritidis

Bacteria use quorum sensing to control gene expression in response to a change in environmental conditions. Quorum sensing is cell-to-cell communication in which

bacteria sense signalling molecules called auto inducers in response to the change in population density. In the process of quorum sensing (regulated by LuxI/LuxR), bacteria produce and secrete auto inducers in the environment (Surette *et al.* 1999). The concentration of auto inducers increases with the bacterial cell density, until it reaches a critical threshold, at which time auto inducers diffuse back into the cell and regulate downstream processes such as virulence and biofilm formation. In *Salmonella*, three types of quorum sensing signalling systems have been described: LuxR/acyl-homoserine lactone (AHL) in which *Salmonella* detect auto inducers produced by other species of bacteria, LuxS/ autoinducer-2 (AI-2) in which *Salmonella* recognizes inter/intra species signals and autoinducer-3 (AI-3) signaling in which *Salmonella* recognizes signals as well as molecules produced by a mammalian hosts (Surette *et al.* 1999).

Salmonella is deficient in LuxI, and thus do not produce AHL. However, it encodes a transcriptional factor for protein SdiA (LuxR homologue), which recognizes and binds to AHL produced by other bacterial species. SdiA activates the expression of two *srg* (SdiA-regulated gene) loci: the *rck* operon and *srgE* gene (Ahmer *et al.* 1998). The *rck* operon present on the *Salmonella* virulence plasmid (pSLT) encodes for six genes: *pefI, srgD, srgA, srgB, rck,* and *srgC*. Two *rck* operon genes, *pef1* and *srgA*, regulate the expression and folding of the *pef* operon (Plasmid Encoded Fimbriae) (Nicholson & Low, 2000). Previously it has been shown that the *pef* operon encodes for fimbriae of *Salmonella* Typhimurium, required for adhesion to the mouse small intestine (Baumler *et al.* 1996). Barrel protein Rck is localized in the outer membrane of *Salmonella* and is required for adhesion to epithelial cells (Crago & Koronakis, 1999). SdiA does not have a direct function in biofilm formation by *Salmonella,* however SdiA regulates the assembly of the genes required for the expression of plasmid-encoded fimbriae and thus indirectly influences biofilm formation by *Salmonella*.

The second quorum sensing system is LuxS mediated AI-2 synthesis. LuxS synthesizes AI-2 by catalyzing the recycling of *S*-adenosylmethionine (SAM) to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) in bacteria. SAM is a methyl donor in bacteria while DPD cyclizes to form different furanones, which act as precursor of AI-2 (Wang *et al.* 2005). *Salmonella* AI-2 is a tetrahydroxyte- tetrahydrofuran, which

is produced in response to growth condition and environment factors such as pH, nutrient availability and osmolarity (DeLisa *et al.* 2001). The Lsr (LuxS-regulated) transport system detects and transports AI-2 into the cell. Alternatively, the rbs transporter has also been shown to be involved in the uptake of AI-2. Mutation in *luxS* has been shown to affect biofilm formation in *Salmonella* Typhimurium. The global gene expression of the RNA from wild-type (WT) *Salmonella* Typhimurium and a *luxS* mutant was used to analyze the genes controlled by luxS/AI-2. The microarray data revealed that, compared to WT, the motility and biofilm-related genes were down-regulated in the *luxS* deletion mutant. The results indicated that luxS and AI-2 are important in motility and biofilm formation (Jesudhasan *et al.* 2010).

The third signaling system involves signaling between bacteria and mammalian cells, where the autoinducer-3 (AI-3) produced by the GIT microbiota, and molecular signals epinephrine (epi) and norepinephrine (NE) produced by the host utilizes a two component regulatory system to activate the transcription of genes related to pathogenesis (Sperandio *et al.* 2003). Additionally, in *S.*Typhimurium this signaling system has also been shown to regulate motility in response to norepinephrine. As shown by Bearson and Bearson (2008), the mutation in the *qseC* gene encoding for the two-component (QseBC) quorum-sensing system reduced motility of *S.* Typhimurium in the presence of the mammalian hormone norepinephrine. Additionally, the *qseC* mutants also showed reduced colonization in the gastrointestinal tract of swine compared to the wild type (WT). This explains the role of quorum sensing by *Salmonella* in colonizing the epithelium in response to host hormones (Bearson & Bearson, 2008).

2.2.7 Cross talk and regulation of motility, virulence and biofilm formation

In *Salmonella*, expression of multiple processes simultaneously would be energetically expensive; therefore coordinated expression of all the processes under desirable conditions is carried out when they are required. Major bacterial processes (motility, virulence and biofilm) are triggered in response to environmental conditions such as osmolarity, pH and ion concentration. For example SPI-I T3SS is activated in the small intestine in response to low oxygen, high osmolarity, neutral pH and SPI-II T3SS is expressed in SCV in response to low osmolarity, low cationic concentration, and acidic

pH (Vescovi *et al.* 1996).

SirA/BarA is a two-component regulatory system which regulates SPI-I genes, but is a negative regulator of flagella encoding genes *(CsrB)*. As a result, the environmental system in which BarA/SirA encoded SPI-I T3SS is expressed would repress the flagellar regulon (Teplitski *et al.* 2003). In another study, regulatory proteins RtsA and RtsB have been shown to coordinate the invasion and flagellar genes in *Salmonella* Typhimurium. RtsA induces the expression of SPI-I T3SS effector hilA. RtsB binds to the *flhDC* promoter region and represses the expression of the flagellar genes. Thus in the small intestine, these proteins act together to induce virulence and repress motility (Ellermeier & Slauch, 2003). Similarly, the concentration of Ca^{2+} and Mg^{2+} regulates the PhoP/PhoQ mediated expression of SPI-I and SPI-II T3SS. At high ionic concentration PhoP remains unphosphorylated and allows the expression of SPI-I proteins. However, in SCV due to low ionic concentration PhoP becomes phosphorylated. This results in activation of SPI-II genes and down regulation of SPI-I. Thus, when the SPI-I T3SS is no longer required for invasion of non-phagocytic cells, PhoP activates SPI-II in SCV (Vescovi *et al.* 1996). Biofilm-associated protein, BapA is required for aggregation of cells in *Salmonella* Enteritidis and has been shown to contribute to invasive *Salmonella* infection, suggesting a link between biofilm formation and virulence. Latasa *et al.* (2005) showed that orally inoculated BALB/c mice with a *bapA* mutant strain survived longer than those inoculated with the WT. Additionally, colonization of *bapA* mutants at the intestinal cell barrier was significantly lower compared to the wild type strain. This indicates that BapA mediates both biofilm formation and invasion of *Salmonella* in host epithelium (Latasa *et al.* 2005). Thus, the regulatory pathways including virulence gene expression, motility and biofilm formation are interlinked and decision making to coordinate these processes is crucial for the survival of bacterial cells.

2.3 Antibiotic classification and mode of action

Since their introduction in 1937, antibiotics have been one of the greatest discoveries in modern medicine and have revolutionized the treatment of infectious diseases. Antimicrobial therapy on the basis of mode of action can be grouped into three

classes: inhibition of DNA replication and repair, inhibition of protein synthesis, and inhibition of cell-wall turnover (Walsh, 2000). Drugs targeting ribosomal function of the 30S subunit (tetracycline and aminoglycoside) and 50S subunit (macrolide family and chloramphenicol) are bacteriostatic (growth inhibitors). Inhibitors of cell wall synthesis (β lactam and lipopeptides) and nucleic acid synthesis (fluoroquinolones, sulfonamides and rifamycins) are bactericidal drugs, which lead to cell death. Fluoroquinolones are broad-spectrum antimicrobials effective for treatment of invasive gastrointestinal infections. Antimicrobial therapy (ciprofloxacin) for *Salmonella* gastroenteritis is recommended only in immunocompromised patients suffering with enteric fever.

2.3.1 Resistance against antibiotics

Antibiotics have been widely used for treatment of infectious diseases, however, their excessive and indiscriminate use has resulted in the development of drug resistant strains of human and animal pathogenic bacteria. In recent years drug resistant microbes have gained much attention and pose a major threat to public health (Davies & Davies, 2010). Bacteria have developed numerous biochemical mechanisms to overcome the toxic effects of antibiotics. These mechanisms are specific to a type of drug or an antibiotic. However, there are generalized mechanisms of bacterial resistance such as restricting the entry of a drug into the cell and also actively exporting the drug out of the cell (drug efflux) (Piddock, 2006a). Bacteria restrict the influx of a drug into the cell by surrounding itself with a low permeability barrier and also by the extruding drug out of the cell through an energy-dependent mechanism (Depardieu *et al.* 2007). However, after entry into the cell, the barrier cannot prevent the toxic action of the drugs. Hence, alteration of membrane permeability and efflux of the drug are essential in bacterial drug resistance (Levy, 1992, Nikaido, 1994).

2.3.2 Bacterial resistance mechanisms to antimicrobial agents

Worldwide, bacterial resistance to antimicrobial agents continues to increase. This challenges the treatment of diseases caused by microorganisms (Levy, 1995, Nikaido, 2009). The factors that contribute to antibiotic resistance are exceedingly complex and dependent on the interactions between the infecting bacterium, its localization in the body, antibiotic concentration at the site of infection and the immune status of the patient

(Wright, 2003). Bacteria acquire antibiotic resistance by several mechanisms including i) drug inactivation/modification ii) alteration of the target site iii) bypass pathways and iv) decreased membrane permeability (Nikaido, 1994, Nikaido, 2009). In *E. coli*, enzymatic deactivation of penicillin (drug inactivation) was the first identified mechanism of antibiotic resistance. Gram-positive and negative bacteria have been shown to produce enzymes (penicillinases, *β*- lactamase, aminoglycoside phosphoryltransferase (APH), aminoglycoside acetyltransferase (AAC), aminoglycoside adenyltransferase or nucleotidyltransferase), capable of deactivating *β*- lactams and aminoglycosides (Datta & Kontomic, 1965, Davies & Wright, 1997, Wright, 1999).

Another mechanism of bacterial resistance is the modification of drug target, where a structural change in the target site disables antibiotic activity (Spratt, 1994). This mechanism leads to drug resistance even after its entry into the cell. In a typical example, bacteria can acquire fluoroquinolone resistance by mutation in the target enzymes, DNA gyrase and topoisomerase IV, involved in DNA replication. Hooper, (2000) showed that a single mutational event in the target enzyme of the bacteria (*S. aureus*) can lead to an increase in the minimum inhibitory concentration (MIC) of drugs (ciprofloxacin and gatifloxacin) (Hooper, 2000). Moreover, some bacterial cells acquire resistance by "bypassing" the effect of antibiotics. Such cells can replace (bypass) a metabolic step that is usually inhibited by the antibiotic with an alternate drug resistant metabolic enzyme. Thus, despite the presence of an antibiotic-sensitive target in the cell; the bacteria continue to flourish due to an altered binding site (McManus, 1997). An example of this mechanism was observed in vancomycin resistant enterococci, where the end of the pentapeptide, $_{D}$-Ala-$_{D}$-Ala (the binding site of vancomycin) was replaced by an ester structure, $_{D}$-Ala-$_{D}$-lactic acid. This prevented the binding of vancomycin to the target site in the resistant strain (Courvalin, 2006).

An efficient antibiotic must reach its target and accumulate at an optimal concentration to prevent bacterial replication. For example, the inhibitors of bacterial protein synthesis must pass through the cell membrane and accumulate in the cytoplasm at a concentration sufficient to block bacterial protein machinery. Hence, by preventing the entry of antibiotics into the bacterial cell or by pumping the antibiotics out constantly

at the same rate as it enter the cell, the sensitive antibacterial targets in the bacterial cell can be shielded from antibiotic action (Walsh, 2000). For instance, in Gram-negative bacteria, *β*- lactams enters the cell via porins (water-filled hollow membrane protein) and loss of porins may lead to resistance. Previously, the imipenem resistance in *Pseudomonas aeruginosa* was acquired due to the mutational losses of D2 porin (Livermore, 1992). These resistance mechanisms could be explored as potential targets to modulate bacterial drug resistance and to restore the efficacy of antimicrobial agents.

2.3.3 Efflux pumps in bacteria

Multidrug efflux pumps are membrane transport proteins, which use cellular energy to extrude toxic substances (antibiotics) from the cells into the external environment (Littlejohn *et al.* 1992, Paulsen *et al.* 1996). These proteins are attributed to enhance resistance against the toxic compounds in diverse life forms including Gram-positive, Gram-negative bacteria and eukaryotic organisms (Webber & Piddock, 2003, Wu *et al.* 2011a). In bacteria, several genes have been identified that encode such multidrug efflux proteins. Multiple drug efflux system can be classified into five families based on the number of pump components (single/multiple), the substrate exported by pump, the number of transmembrane-spanning regions, and the source of energy used by the pump (Piddock, 2006a). These include the ATP binding cassette (ABC) family, multidrug and toxic compound exporters (MATE), the small multidrug resistance (SMR) family, resistance-nodulation-division proteins (RND), and the major facilitator superfamily (MFS). Four of these systems require proton motive force as an energy source, but not the ABC family that utilizes ATP (hydrolysis) to mediate substrate extrusion (Van Bambeke *et al.* 2000, Webber & Piddock, 2003). The efflux pump activity is affected when i) efflux pumps are inactivated ii) their expression is down regulated or iii) the antibiotic compound is modified to escape efflux activity (Kourtesi *et al.* 2013).

Bacterial efflux pumps are integral membrane transporters responsible for the efflux of inhibitory substances such as antibiotics and drugs. (Li & Nikaido, 2004). These efflux pumps are present either as single-component transporters mediating the efflux of toxic compounds across the cytoplasmic membrane (CM), or as multiple component transporters: catalyzing efflux across the outer membrane (OM) and the periplasmic

membrane (Johnson & Church, 1999). Proteins involved in the efflux include outer membrane channel proteins (OMP) and periplasmic membrane fusion proteins (MFP) (Dinh *et al.* 1994, Paulsen *et al.* 1997). Efflux pumps capable of transporting a range of structurally dissimilar compounds confer multidrug resistance (MDR) and are known as multidrug efflux pumps (Piddock, 2006a).

2.3.4 Drug efflux pumps in *Salmonella*

Salmonella enterica is a gastrointestinal pathogen that causes diseases such as gastroenteritis, inflammation, diarrhoea and life threatening systemic infections (Nishino *et al.* 2009, Piddock, 2006a). The commonly used antibiotics for treatment of such diseases are fluoroquinolones (ciprofloxacin), cephalosporin and ceftriaxone (Baucheron *et al.* 2004). The emergence of drug resistant *Salmonella* in the human food chain is a major concern in both veterinary and human medicine. For instance, *S. enterica* serovar Typhimurium has been shown to acquire fluoroquinolone resistance due to multiple target gene mutations and active efflux by the AcrAB-TolC system (Baucheron *et al.* 2004). *S. enterica* serovar Typhimurium contains nine drug efflux pumps (AcrAB, AcrD, AcrEF, MdtABC, MdsAB, EmrAB, MdfA, MdtK and MacAB) (Nishino *et al.* 2006). These pumps are classified into four efflux pump families as follows:

a) The major facilitator family - EmrAB and MdfA

b) The Resistance Nodulation D family - AcrAB, AcrD, AcrEF, MdtABC and MdsAB

c) The multidrug and toxic compound extrusion family - MdtK

d) The ATP-binding cassette family - MacAB

Seven drug efflux pumps (AcrAB, AcrD, AcrEF, MdsAB, MdtABC, EmrAB and MacAB) require TolC as an outer membrane protein to expel drug out of the cells. MdsABC have been reported to be TolC dependent, however, it could function with both TolC and outer membrane component MdsC. Moreover, MdfA or MdtK efflux pumps have been identified as single components independent of TolC (Horiyama *et al.* 2010). In the plasma membrane, single component efflux system function as the transporter of antibiotics (away from their targets) from the cytoplasm to the periplasmic space. AcrAB-TolC removes the antibiotics from the periplasm to the outside of bacterial cell (Tal & Schuldiner, 2009). *S. enterica* serovar Typhimurium efflux pump AcrAB-TolC

has been found to have close homology to *E. coli* AcrAB-TolC (AcrA (94%) and AcrB (97%) respectively) (Piddock, 2006b). Its substrates include antibiotics such as chloramphenicol, quinolones and tetracycline, acriflavin, ethidium bromide, bile salts, SDS, Triton X-100, centrimide and triclosan. In *Salmonella*, a major regulator, RamA controls the *acrAB* expression in response to environmental signals (indole and bile) and AcrR works as a repressor (Nishino *et al.* 2009).

2.4 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans (*C. elegans*) is an anatomically simple, genetically tractable nematode with advantages of small body length (1.5 mm adults), rapid generation time (3 days) and large brood size (approximately 300 progeny per gravid adult) (Sifri *et al.* 2005). It is used as a model organism to investigate many biological processes including host pathogen interactions and for antimicrobial drug discovery (Aballay & Ausubel, 2002, Moy *et al.* 2006). Furthermore, transparency of *C. elegans* enables direct real time monitoring of pathogenesis and virulence mechanisms. Several studies have shown that bacterial pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio sp*, *Salmonella* Typhimurium, *E. coli* O157:H7, and *Enterococcus faecalis* have similar modes of pathogenesis in nematodes and higher animals, with a similar sub-set of genes involved in the infection process (Tan *et al.* 1999, Breger *et al.* 2007).

2.4.1 Anatomy and life cycle of *C. elegans*

C. elegans lifecycle is anatomically simple with about 1000 somatic cells. The entire genome of *C. elegans* has been sequenced and is used as a model to study more complex organisms such as humans as about 35-40% of *C. elegans* genes are homologous to humans. Further, the hermaphroditic nature and fast generation time of *C. elegans* makes it a suitable model organism for isolation and characterization of genetic mutants (Brenner, 1973, Byerly *et al.* 1976)

There are two sexes in *C. elegans*, a self-fertilizing hermaphrodite (XX) and a male (XO). The most common sexual form of *C. elegans* is the hermaphrodite. The hermaphrodite is a female in which sperms are produced and stored in gonads before the

production of eggs (Brenner, 1973). Self-fertilization of the hermaphrodite allows homozygous worms to generate genetically identical progeny and male mating facilitates the isolation and maintenance of mutant strains. Mutant animals are readily obtained by genetic screens, and elaborate screens are used to identify mutation in biological processes (Jorgensen & Mango, 2002).

The life cycle of *C. elegans* is comprised of the embryonic stage, four larval stages (L1-L4) and adulthood. *C. elegans* goes through a reproductive life cycle (egg to egg-laying parent) in 5.5 days at 15°C, 3.5 days at 20°C, and in 2.5 days at 25°C. When worms reach adulthood, they produce about 300 progeny each with a life span of two weeks. *C. elegans* can adopt an alternative life form, called the dauer larval stage. Dauer larvae can remain viable for about three months, they are thin with a plugged mouth. The dauer larvae stage is non-ageing and can re-enter the L4 stage in the presence of a food source (Golden & Riddle, 1984).

2.4.2 *Salmonella* proliferates in *C. elegans*

Since the 1960s, *C. elegans* has been an economical and facile model host for the study of evolutionarily conserved mechanisms of microbial pathogenesis, innate immunity, neurobiology and aging (Riddle *et al.* 1997). Previous studies have shown that bacterial pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio* sp, *Salmonella* Typhimurium, *E. coli* O157:H7, and *Enterococcus feacalis* have similar pathogenic mechanisms in nematodes and higher animals, with a similar sub-set of genes involved in the infection process (Sifri *et al.* 2005). Ausubel's laboratory at the Harvard Medical School first showed that *Salmonella* Typhimurium and other *Salmonella* serovars including *S.* Dublin could kill *C. elegans.* Interestingly, most of the *Salmonella* virulence factors identified to affect this killing were also important for mammalian pathogenesis. For example, the *S.* Typhimurium PhoP/PhoQ signal transduction system regulates virulence-related genes in mammals (Vescovi *et al.* 1994). *S.* Typhimurium mutants with a *phoP /phoQ / purB* deletion and *phoQ* point mutation resulted in less killing of *C. elegans*. It was shown that *Salmonella* attaches to intestinal epithelial cells and establishes persistent infection in *C. elegans* (Aballay *et al.* 2000). Thus, the *C. elegans*- *S.* Enteritidis model system could be used for identifying

Salmonella virulence factors that cause disease in humans.

In the same year, another study by Labrousse *et al.* (2000) showed *C. elegans* as a host for the enteric pathogen *S.* Typhimurium. They concluded that feeding *C. elegans* with *S.* Typhimurium resulted in a significantly shorter life span (9-14 days) compared to the *E. coli* OP50 (20 days). They also concluded that for pathogenesis, *S.* Typhimurium should be alive, as *C. elegans* worms fed on heat-killed *Salmonella* showed no symptoms of infection. This indicates that unlike *Pseudomonas, Salmonella* does not kill worms by secreting toxins (Labrousse *et al.* 2000). Tenor *et al.* (2004) identified *Salmonella* effector proteins required for the infection in *C. elegans.* A library of transposon mutants (*hilD, hilC, hilA, invH)* corresponding to the virulence factors encoded by SPI-I T3SS was evaluated for the ability to kill *C. elegans*. Compared to the wild type, mutants showed a significant reduction in epithelial invasion. This indicates that the *C. elegans* model can be used to determine *Salmonella* virulence factors required for pathogenicity in the host (Tenor *et al.* 2004).

Khoo *et al.* (2010) used *C. elegans* to evaluate the pathogenesis of *Salmonella* serovars recovered from vegetables and poultry meat. All the isolates were capable of colonizing the intestine of *C. elegans* and significantly reduced the survival of worms (9-18 days) compared to the controls at 28 days, fed *E. coli*. This indicates that the mechanism of *Salmonella* colonization in vegetables and poultry products can be correlated to the interaction of *Salmonella* with *C. elegans* (Khoo *et al.* 2010). This infers that the *C. elegans* model can be used to provide preliminary insight to study *Salmonella* pathogenesis in poultry.

2.4.3 Immune signalling pathways in response to *Salmonella* infection in *C. elegans*

In *C. elegans*, signal transduction pathways are activated in response to infection and effector molecules produced by pathogens. There are four main pathways identified in *C. elegans*; the p38 MAP kinase pathway, the programmed cell death (PCD), the toll like pathway and the DAF-2 insulin-signaling pathway. Programmed cell death of somatic and immune cells plays an important role in mammal-pathogen interaction. In mammals, programmed cell death is observed during disease conditions such as cancer,

autoimmune and neurodegenerative diseases. In *C. elegans* PCD is a part of normal developmental process, which occurs in 131 somatic cells during the development of *C. elegans*. In the adult hermaphrodites, more than half of the cells are eliminated by PCD, which is mediated by caspase CED-3, CED-4 and CED-9 (Gumienny *et al.* 1999). However, Aballay and Ausubel (2001) observed that colonization of *Salmonella* Typhimurium in the intestine of *C. elegans* stimulates PCD in the germline cells. The *C. elegans* PCD mutants (*ced-3* and *ced-4*) did not exhibit *S.* Typhimurium mediated PCD and were more susceptible to killing by *Salmonella* Typhimurium. Moreover, the *phoP Salmonella* mutant lacking the ability to synthesize virulence factors did not show germ line mediated cell death in *C. elegans.* This indicates that *C. elegans* gonadal PCD is triggered in response to *Salmonella* virulence factors and PCD plays an important role to protect worms against *Salmonella* colonization (Aballay & Ausubel, 2001). Aballay *et al.* (2003) further examined *Salmonella* mediated PCD in *C. elegans* and identified the *pmk-1* gene of *C. elegans* and bacterial lipopolysaccharides (LPS) as major factors for pathogenesis. They concluded that *Salmonella* mediated PCD in *C. elegans* requires a mammalian homologue of p38 mitogen-activated protein kinase (MAPK) which is encoded by the *pmk-1* gene. An enhanced level of gonadal PCD was not observed in *C. elegans*, when *pmk-1* was inactivated by RNAi and RNAi-*pmk-1* mutants were more susceptible to *Salmonella* mediated killing. Additionally, they also observed that the TOLL receptor pathway was not essential for the PMK-1 dependent gonadal PCD by *Salmonella*. Overall, *C. elegans* innate immune response to *Salmonella* is mediated through PMK-1-dependant PCD pathway (Aballay *et al.* 2003). Moreover, intestinal autophagy also plays an important role in the *C. elegans* defence against *Salmonella* infection. Elimination of the autophagy gene *bec-1* (RNAi-*bec-1*) in the intestine of *C. elegans* increased the susceptibility of *Salmonella* infection in the host. Similarly, in a mouse model, autophagy was necessary for the clearance of *Salmonella* from intestinal cells. This shows that autophagy in the host intestinal epithelial cells is required for resistance against *Salmonella* (Curt *et al.* 2014). Thus *C. elegans* can be used as a model to identify novel bioactive compounds that regulate host immune response to defend against *Salmonella* infection.

2.4.4 *Caenorhabditis elegans* as a tool for antimicrobial drug discovery

The *C. elegans* -*Salmonella* pathosystem has been used as high throughput model to screen compounds with potential anti-infective and anti-microbial properties, applicable to cure infections in higher animals and humans (Moy *et al.* 2009). *In vitro* antimicrobial assays including growth inhibition and cell culture assay, cannot replicate the *in vivo* conditions. Therefore, a whole organism model such as *C. elegans* can be more accurate to screen novel antimicrobial compounds. In addition, *C. elegans* can also be used to detect compounds that enhance the immune response. The first pioneering work of high throughput screening was a liquid culture assay, which was used to screen 6,000 synthetic compounds and 1,136 natural products using *C. elegans* infected with the opportunistic human pathogen *Enterococcus faecalis* (Moy *et al.* 2006). Antibiotic treatment used as a control significantly reduced bacterial colonization in the intestine of the nematodes as well as increased the survival of infected worms in a dose-dependent manner. This screening identified 16 compounds and 9 extracts that promoted the survival of *C. elegans.* However, in contrast to the antibiotics tested, the dose response of the identified compounds (*in vivo*) was significantly lower than the minimum inhibitory concentration (MIC) values required for inhibiting the growth of *E. faecalis* (*in vitro*). From the study, it was concluded that *C. elegans* infection model was effective not only for antibiotic screening, but also for compounds that target bacterial virulence and host immune response (Moy *et al.* 2006).

Previously, *C. elegans* has been used for pre-screening of antimicrobial agents and probiotics against enteric pathogens. For example, a strains of *Lactobacillus* isolated from chicken and pig intestines were tested to control *Salmonella* colonization in *C. elegans*. Two out of 17 *Lactobacillus* isolates were able to protect *C. elegans* from death caused by *Salmonella* colonization. Thus, authors concluded that a correlation exists between the amount of protection afforded the infected *C. elegans* and the performance of piglets, upon treatment with the similar *Lactobacillus* strain. Taken together, this suggests that *C. elegans* can be used as a laboratory model organism for preliminary screening of probiotics to control *Salmonella* infection. The results from the screening can be applied to higher animals including pigs (Wang *et al.* 2011). Similarly, *C. elegans*

can be used as a model for preselecting prebiotics such as seaweeds for control of *Salmonella* in poultry.

2.5 Overview of the gastrointestinal tract in chickens

2.5.1 Major parts and functions of gastrointestinal tract

Gastrointestinal (GIT) tract is a diverse organ system, which serves as primary surface for the entry of nutrients and macromolecules into a host's body. The avian digestive tract can be subdivided into anatomical sections including beak, esophagus, crop, proventriculus, gizzard, small and large intestines, ceca, and cloaca. The digestion of food through these organs is carried out in sequence of events including grinding, acidifying, hydrolyzing, emulsifying, and transporting of the end products (Klasing, 1999).

2.5.1.1 Function of anterior digestive organs

Digestion begins with grasping and break down of food by the beak followed by lubrication and passage of food to the esophagus by the tongue and oral cavity. The esophagus extends through the neck into thoracic cavity and ends in the proventriculus. Peristaltic contraction of inner and longitudinal muscles enables the movement of food in the esophagus. Just before entering the thoracic cavity, a region of the esophagus widens and is called the crop. The crop functions as a temporary storage of feed and enables the bird to eat a significant amount in food in short span of time. At the distal end, the crop narrows down and reforms into the esophagus, which is cranially connected to the proventriculus and gizzard. The proventriculus represents the first part of the stomach where digestive enzymes are produced to initiate digestion (Klasing, 1999). As in mammals, the secretory cells of the stomach secrete hydrochloric acid. The gastric juice maintains an acidic pH (2 to 4.5), which prevents the growth of pathogenic microorganisms. The feed remains in the proventriculus, depending on the filling state of the gizzard. The function of the gizzard is to grind the feed to reduce its size and increase the surface area. The gizzard is made of two pair of smooth muscles arranged asymmetrically to generate muscular strength during contractions. This aids in effective grinding and optimal mixing of the feed. The inner lining of the gizzard contains a hard cuticle composed of rod like projections secreted by tubular glands. The cuticle protects

the lumen from gastric juices (HCL and pepsin). From the gizzard, the ground feed is transferred into the small intestine (Svihus, 2014).

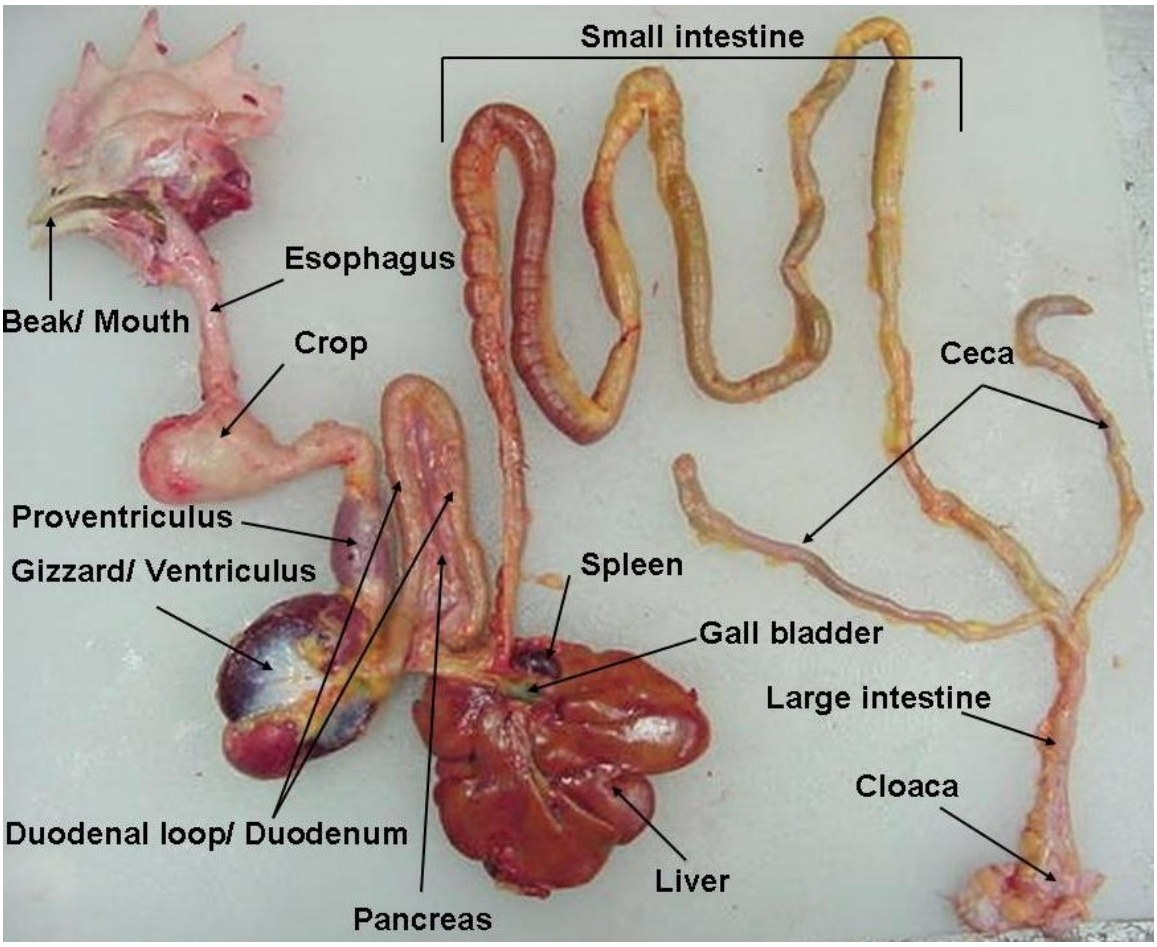

Figure 2.2 Diagramatic represtation of different parts of digestive organs of chicken. (adapted from Jacob and Pescatore, 2013)

2.5.1.2 Function of small and large intestine

The small intestine serves as the primary site for absorption of nutrients and enzymatic digestion. It is divided into three parts: the section from the junction of the gizzard to the pancreatic and bile ducts is known as the duodenum, the region from the end of the duodenum to the Meckel's diverticulum is called the jejunum and the ileocaecal junction works as the ileum. The duodenal loop encircles the pancreas and hepatic and pancreatic ducts enter the intestine in the duodenum near the beginning of the jejunum (Svihus, 2014). Bile mediates digestion of lipids and absorption of fat-soluble

vitamins. Pancreatic enzymes including amylase chymotrypsinogen, trypsinogen and lipase hydrolyse carbohydrates, proteins, and lipids into oligomer units. In addition, the pancreas secretes bicarbonate to maintain neutral pH (6-7) in the small intestine. Thus, the duodenum is a major site for the breakdown of feed. The absorption of nutrients takes place in the jejunum. Furthermore, enzymes such as saccharase and isomaltase secreted by the brush border of the small intestine hydrolyses the oligomers into monomeric units (Klasing, 1999). After passing the ileum, the unabsorbed digesta enters the large intestine. A pair of ceca is present at the junction of the small and large intestine, visible as elongated blind sacs within the intestinal wall. The digesta entering the cecae include small low molecular weight, non-viscous particles, of ileal or renal origin. The cecae harbour various microbial species involved in the fermentation of the carbohydrate from the digesta. The ceca play an important role in water and electrolyte absorption in the recycling of renal nitrogen. Other functions of the cecae include detoxification, fat digestion and adsorption. The digesta from the cecae exit into the colon (rectum) through controlled sphincters. The colon refers to the length of the intestine between ileum and cloaca. The digesta exits at the cloaca, a common passage for the digestive and urinary systems (Klasing, 1999).

2.5.2 Histological structure of the gastrointestinal tract

The epithelium of the intestine contains two fundamental structures, namely the villi and crypts. The epithelial cells of the villi contain microvilli on their apical surface. The main function of these structures is to increase the surface area for the absorption of the nutrients. Crypts are finger like invaginations of the epithelium around the villi, which play a major role in cell generation. Within the villi and crypts lie the goblet cells, regenerative cells, and absorptive cells (enterocytes). The goblet cells secrete large amounts of high molecular weight glycoproteins (mucin) (Specian & Oliver, 1991). The secreted mucin forms a protective mucous layer on the epithelial surface and aids in repair of damaged epithelial mucosa. The main function of mucous is to shield the intestinal epithelium from bile salt, digesta, digestive enzymes and mechanical abrasion by the digesta. Additionally, the mucous layer forms a barrier that protects the epithelium against enteric bacterial pathogens and toxins. Villi mediate digestion and absorption of nutrients. They contain capillary beds, where absorbed nutrients enter the blood vessels

(Imondi & Bird, 1966).

Villi and crypts contain stem and progenitor cells that self renew throughout life to maintain the barrier function. Large numbers of epithelial cells die daily due to the exposure to the harsh luminal environment. These cells are constantly expelled from the tip of the villi into the intestinal lumen. Therefore, new epithelial cells must be generated in the small intestine to compensate for a high rate of cell death on the villi. Regeneration of epithelial cells is initiated at the crypt base, where stem cells differentiate to produce progenitor cells known as transit-amplifying cells. Each transit-amplifying cell divides 2-3 times to form absorptive or secretory cells, while travelling along the surface of the villi towards the tip. Proliferation of cells ceases when the differentiated cells exit the crypts and migrate through the villi. The cell turnover is determined based on the rate at which this migration takes place. Increased cell turnover enables the renewal of crypts and villi required in response to inflammation by pathogens and secreted toxins (Imondi & Bird, 1966).

Intestinal cell turnover can be regulated either by a change in cell production rate within each crypt or by change in the number of crypts. Increase in cell death due to inflammation from bacterial pathogens or secreted toxins results in shorter villi. In this condition, there is high cell turnover due to increased crypt cell production. Besides these microstructures, there are several protective barriers present in the small intestine. The lamina propria mucosa contains glandular complexes, smooth muscle cells and connective tissues that support the villi absorptive cells. This structure provides an additional barrier to the pathogens that might infiltrate the epithelium (Imondi & Bird, 1966).

The major stimulator for the development of intestinal morphology is uptake of nutrients (feed) that increases intestinal diameter and subsequently relative intestinal weight. Previously it has been shown that changes in villus height (VH), crypt depth (CD), villus density and rate of epithelial turnover can be used to assess the affect of diet supplementation on animal health. Increases in villi height and crypt depth indicate increased nutrient absorption, disease resistance and performance (Xu *et al.* 2003).

The tight junctions are a specialized complexes that join together intestinal epithelial and

endothelial cells. They act as semipermeable barriers and regulate the diffusion of salt, ions and large solutes based on size and charge. They also allow selective migration of dead bacterial cells or components (instead of live pathogen) through the intestinal wall for recognition by the immune system. Therefore, if these intestinal barriers are compromised, the pathogenic bacteria or toxic substances can pass through and interfere with systemic functions (Stoidis *et al.* 2011)

2.5.3 Microbiota of the gastrointestinal tract

The gastrointestinal tract (GIT) of the chicken contains a diverse population of bacteria, fungi, and protozoa. Among these, bacteria are the predominant microorganism consisting of both beneficial and harmful groups. Beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* play an important role in the productivity and health of the animal. Other key functions include detoxification, modulation of the immune system and protection from pathogens. Harmful populations such as *Clostridium* and *Salmonella* produce toxins and lead to intestinal putrefaction and infections (Gong *et al.* 2002). Aerobes and facultative anaerobes including *Escherichia, Lactobacillus*, and *Streptococcus* are the first bacterial species to colonize the GIT. Growth of aerobes reduces the oxidation/reduction potential and allows obligate anaerobes such as *Bacteroides*, *Eubacterium* and *Bifidobacterium* to colonize next. Obligate anaerobes constitute a major part of the adult microflora and perform most of the fermentation process in the ceca (Dibner & Buttin, 2002).

The population of microbes varies significantly among different segments of the GIT and bacterial diversity increases from the proximal to distal ileum (Apajalahti, 2005). The localization of bacterial species to a specific segment of the GIT depends on their ability to bind to the enterocytes or mucus layer, tolerance to the GIT environment and persistence to the host immune response. Other factors including variation in the rate of passage of digesta, pH, nutrient availability, and antimicrobial substances present in the different regions can also affect the diversity of microbes (Apajalahti, 2005). For example, the flow rate of digesta in the distal ileum, ceca and colon is low compared to the small intestine, and therefore larger populations of bacteria are found in the distal segment. The slow rate of digesta movement in the distal ileum provides sufficient time for bacteria to

bind to the gut wall and carry out fermentation to establish ecological niches (Danicke *et al.* 1999). Lower bacterial diversity in the small intestine has also been attributed to the presence of antibacterial peptides and high concentrations of bile acids (low pH).

The microbial diversity in the gut shows progression in phylotypes depending on the age of the chicken. Apajalahti *et al.* (1998) showed that the complexity of the microbial population depends on the age of the chicken. They demonstrated that the bacterial density in the ceca and ileum of newly hatched chicks increased significantly from day one (10^{10} and 10^{8} cells/ g of digesta, respectively) to days three (10^{11} and 10^{9} cells per g of digesta, respectively) post hatching. They further showed that the population remained relatively stable until 30 days post hatching.

Lactobacillus and *Bifidobacterium* are the most abundant beneficial bacterial species in the GIT. These bacterial species have also been shown to competitively exclude pathogens and impart health benefits to the host. Edelman *et al.* (2003) showed selective binding of *L. crispatus* to the receptors on the ileal epithelium of chicken. They demonstrated that pathogenic *E. coli* 0789 and *L. crispatus* were able to recognize the same receptor on the epithelium. However, the high affinity of *L. crispatus* to the binding receptors competitively prevented the attachment of *E. coli* 0789. *Lactobacillus* and *Bifidobacterium* secrete digestive enzymes inducing casein phosphatase, amylase, and lipase and helps in nutrient digestion. The fermentation product of *Lactobacillus* and *Bifidobacterium* are short chain fatty acids (SCFA) such as lactic and acetic acid, which prevent the growth of pathogenic microbes by reducing the pH in the gut (Edelman *et al.* 2003).

Germ free animals show higher susceptibility to pathogens and have a weak immune systems compared to animals with established micrbiota (Zachar & Savage, 1979). It has also been shown that germ free animals have relatively lower IgA production and weak systemic antibody responses (Crabbe *et al.* 1969). This indicates that commensal microbes communicate with the host immune system to impart beneficial effects on the health and growth of the host.

2.5.4 Use of antibiotics in animal agriculture

The term ‘antibiotic’ refers to natural or synthetic compounds that can kill or

inhibit the growth of bacterial pathogens. In food animal production, antibiotics can be used as therapeutics for the treatment of diseases, as prophylactics to prevent infection, and as growth promoters (Casewell *et al.* 2003). Moore *et al*. (1946) first reported the beneficial effects of antibiotics on the production efficiency in poultry. They concluded that sulfasuxidine and streptomycin increased the growth response in chicks. Since then sub therapeutic levels of antibiotics (< 200 g/ton of feed) have been included in animal diets to achieve the growth promoting effects (U.S. Food and Drug Administration, 2000). In 2012, it was estimated that 14.6 million kg of antibiotics were sold for the use in animal agriculture (U.S. Food and Drug Administration, 2014), which was four times (3.29 million kg) the antibiotics consumed for human use in 2011 (U.S. Food and Drug Administration, 2012). Currently, commercial poultry farms have greater rearing densities and the scale of production has dramatically increased to meet consumer demand. This has increased the frequency of infectious disease outbreaks within flocks and therefore disease outbreaks have been controlled by the use of antibiotics. In the US, 32 antimicrobials are approved for use in the broiler industry without prescription from the veterinarians. Eleven of these antibiotics are used as growth promoters to increase weight gain and feed efficiency in poultry (Jones & Ricke, 2003).

The mechanism of antibiotic action as growth promoters in animals is not well understood, but its main effect has been confirmed on the microbiota within the gut. This is because some of the commonly used antibiotics do not get absorbed and have shown no effect on the growth of germ-free birds (Coates *et al.* 1955). Antibiotics exert their effect by reducing the colonization of bacteria, increasing the metabolism of beneficial bacteria and reducing the total load of bacteria in the gut, thus reducing the overall bacterial load in the gut (Collier *et al.* 2003). Sub therapeutic levels of antibiotics can also enhance immune response of the host to an invading pathogen. Roura *et al.* (1992) showed that inclusion of streptomycin and penicillin in the diets of chicks resulted in preventing immunological stress by lowering cytokines (Roura *et al.* 1992).

However, the extensive use of antibiotics in livestock came at a cost of increase in drug resistant bacterial pathogens. In 1951, Starr and Reynolds first reported a case of antibiotic resistance in bacteria from turkeys. The use of streptomycin as a growth promoter in turkey poults resulted in drug resistant coliforms within three days of

application (Starr & Reynolds, 1951). In 1994, sixty-two isolates of vancomycin-resistant *Enterococcus faecium* were obtained from non-human sources in Great Britain, among which 22 were from farm animals. This indicated that farm animals could serve as a reservoir for the development of drug resistant bacteria (Bates *et al.* 1994). Following this report, avoparcin was the first antibiotic to be banned in Europe in 1995. Consequently, the European Union banned the use of antibiotic growth promoters in 2006 (Castanon, 2007). The selection pressure caused by antibiotics on gut microbes results in the development of resistant genes, which can be transferred among different species of pathogenic bacteria by horizontal gene transfer. This results in the excessive growth of resistant bacterial pathogens such as *Clostridium, Salmonella,* and *Campylobacter* in the host resulting in harmful diseases. In addition, change in the microbial population within the gut can make the host more vunerable to infection by other environmental pathogens (Lewis *et al.* 2010). In the United States, the FDA controls the use of cephalosporin in animal agriculture. Also, there is increased interest to exclude the use of fluoroquinolones as antibiotic growth promoters (AGP). This is because these antibiotics are commonly used in human medical treatments. In North America there is a heighted public awareness of the negative effect of antibiotics in livestock production. Therefore, there is an increasing interest to develop alternatives to antibiotics (Yan *et al.* 2011). Other control measures such as competitive exclusion and vaccination have contributed significantly to reduce pathogen (*Salmonella*) infections in layer production (Filho, *et al.* 2009).

2.5.5 Prebiotics

Prebiotics are defined as a "non-digestible selectively fermented ingredients that benefits the host by stimulating the growth and/or activity of the microflora in the colon" (Roberfroid & Gibson, 1995, Patterson & Burkholder, 2003, Hajati & Rezaei, 2010). Prebiotics improve gastrointestinal health by providing a substrate for the growth and establishment of multiple species of beneficial bacteria in the gut (Cummings & Macfarlane, 2002). Prebiotics influence gastrointestinal health by a variety of mechanisms such as production of metabolites such as lactic acid, inhibiting or reducing the growth and establishment of pathogenic microbes, modifying metabolism of intestinal microbes, and stimulation of the gut-microbe mediated host immunity against microbial

and metabolic diseases (Reddy, 1999). Some examples of beneficial probiotic bacteria found in the gut microbiota of chickens include *Bifidobacteria, Lactobacillus, Ruminococcus,* and *Streptococcus*. These bacteria present in the small intestine use non-digestible polysaccharides and dietary fiber for energy (Mussatto & Mancilha, 2007).

2.5.5.1 Effect of prebiotics on gut microflora

Upon feeding, prebiotics enhance the growth of beneficial microbiota that produce metabolites, which in turn enhance the ability of animals to absorb minerals and vitamins in both the small and large intestine (Sako *et al.* 1999). The principal substrates for bacterial growth are dietary fiber and carbohydrates such as polysaccharides (pectins, hemicelluloses, gums, inulin and resistant starches), oligosaccharides (raffinose, stachyose, fructo-oligosaccharides, galactooligosaccharides and resistant dextrins), and sugars (lactulose, non-absorbed lactose and non-absorbed fructose). Most of the saccharolytic species belonging to the genera *Bifidobacterium, Ruminococcus,* and *Lactobacillus* are able to utilize and grow on such complex carbohydrates (Gibson & Roberfroid, 1995). The microbial fermentation results in the production of short chain fatty acids (SCFA) such as acetic, propionic, butyric acids and lactic acid. SCFA play an important role in the control of colonic diseases, proliferation of coenocytes and intestinal mucosa in chickens. Short chain fatty acids such as butyric, propionic acid and acetic acid also maintain mineral uptake and provide extra energy to the birds (Lan *et al.* 2005). Several reports on dietary modulation of the chicken gut microbiota indicate that the effects are variable depending on the type of prebiotics used in the study. For instance, fructo-oligosaccharides (FOS) increased the growth of beneficial bacteria, such as *Lactobacillus* (Yusrizal & Chen, 2003), but had no effect on the growth of *Bifidobacterium* in the gut of the chicken (Biggs *et al.* 2007). However, in another study, FOS supplementation significantly enhanced the growth of both *Bifidobacterium* and *lactobacillus* in broilers (Xu *et al.* 2003).

2.5.5.2 Effect of prebiotics on gut pathogens

Prebiotics are known to selectively enhance the growth beneficial bacteria, which in turn have been shown to reduce the survival of pathogens, primarily due to the competition for nutrients, via adhesion sites and production of organic acids. This is

known as competitive exclusion. For example, feed supplementation with fructooligosaccharide and galactooligosaccharide (0.5-3.0%) have been shown to modulate the gut microbiota in broiler chickens. The dietary treatment selectively increased the beneficial bacteria (*Bifidobacterium* and lactobacilli) and competitively reduced *Campylobacter jejuni* (Baffoni *et al.* 2012). Similarly, organic acids (lactic acid and SCFA) function by lowering the gut pH and inhibiting the growth of acid sensitive pathogens such as *Salmonella*. Previously, a significant reduction in cecal *Salmonella* Enteritidis count was observed in layer hens fed with low levels of FOS (Donalson *et al.* 2008). Some bacteria produce antimicrobial substances like bacteriocins, which have been shown to inhibit the growth of different enteropathogens (Lasagno *et al.* 2002). Another mechanism of pathogen inhibition is by enhancing the host immune system. Prebiotics have been shown to increase the cytokines, lymphocytes and secretory IgA and modulate the number and activity of Payer's patch immune cells in chickens (Agunos *et al.* 2007). Although the exact mechanism of the effects prebiotics have on immune function in the gut is not yet established, it is proposed that the increase in beneficial bacteria and SCFA triggers the host immune system (Chung *et al.* 2012).

2.5.5.3 Factors influencing the effect of prebiotics

The gut microbiota benefits differently from various prebiotics, for instance, inulin, and glucooligosaccharides (GOS) have been shown to increase *Lactobacillus* while MOS, xylooligosaccharides (XOS), arabinoxyoligosaccharides, and transgalacto-oligosaccharides (TOS) selectively increased *Bifidobacterium*. This indicates that beneficial bacteria have substrate specificity and their metabolic pathways adapt and alter their influence on prebiotic activity (Huebner *et al.* 2007). Moreover, several variations have been reported with prebiotic feed supplementation due to factors including environmental condition, housing type, litter age, feed composition, stocking density and strain of the bird (Geier *et al.* 2009). It has been shown that gender of the birds also influences the response to prebiotics. An interaction between sex of birds and prebiotic was reported by Yusrizal & Chen (2003). They concluded that the body weight and feed conversion ratio (FCR) of female birds were improved (10% and 9%, respectively) on oligofructose supplementation, however, no effects were reported in males (Yusrizal & Chen, 2003). The prebiotic response also depends on the inclusion level of the

supplements, the rate of fermentation of these supplements and the hygiene status of the farm. Biggs *et al.* (2007) showed that a high dosage of prebiotics (inulin and short chain FOS) negatively affected the gut microbiota and retarded the growth of the birds (Biggs *et al.* 2007). In another study, Ten Bruggencate *et al.* (2003) demonstrated that higher dose supplementation of a prebiotic (FOS) increased the susceptibility of rats to *Salmonella* infection. This was due to the rapid fermentation of prebiotics leading to a higher concentration of organic acids, which resulted in impaired barrier function (Ten Bruggencate *et al.* 2003).

2.5.5.4 Types of prebiotics used in poultry

Studies on the application of prebiotics as feed additives in poultry began in the 1980's, and products like non-digestible inulin-type fructans and Aspergillus Meal (AM) derived as a fermentation product of *Aspergillus orizae* were used as prebiotics in domesticated animals (Legette *et al.* 2012). Other prebiotics used in poultry include fructooligosaccaharides (FOS, oligofructose, inulin), malto-oligossacharide, gluco-oligosaccharides, ketoses, lactose, stachyose, and oligochitosan (Huang *et al.* 2007).

Mannan oligosacharides are derivatives of mannan and glucan obtained from yeast (*Saccharomyces cerevisiae*) cell walls. As MOS do not selectively enrich beneficial bacteria, their classification under prebiotics is still a question of debate. The primary mode of action of MOS is the binding and removal of pathogens (*E. coli* and *Salmonella*) via type 1 fimbria. MOS have been shown to decrease the colonization of *E. coli* and *Salmonella* in young chickens (Fernandez *et al.* 2002). Although not being selectively fermentable, MOS have been shown to increase the population of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, induce the immune system and modify intestinal histomorphology in a favorable way in broilers (Baurhoo *et al.* 2007).

Fructo-oligosaccharides (FOS) are linear chains of β-δ linked fructans with fructosyl units linked together by β-2,1 glycosidic linkages. FOS can be categorized based on the fructan chain length, i.e, degree of polymerization (DP). Inulin is made of 2-60 DP and synthetic fructans contain 2-4 DP. Oligofructoses have 2-9 DP and can be obtained by enzymatic hydrolysis of inulin (Huang *et al.* 2007). Inulin is commercially obtained from the roots of chicory and is found naturally in plant sources such as onion,

garlic, wheat, artichoke, and bananas. Beneficial bacteria such as *Bifidobacterium* and lactobacilli have been shown to easily ferment FOS in the lower GIT. Rebole *et al.* (2010) demonstrated the effect of inulin on broiler chcken growth performance, intestinal microflora and cecal fermentation. They concluded that although the dietary supplementation of inulin had no affect on jejunum histomorphology, it significantly increased *Bifidobacterium* and *Lactobacillus* populations in the ileum and ceca. Additionally, the concentration of n-butyric and D-lactic acids was higher in the ceca of birds fed with inulin at 10 and 20 g/kg of the diet (Rebole *et al.* 2010). Since FOS meet all three criteria for a prebiotic, they are frequently used in research as a reference to compare the candidate prebiotic. For example, Kurdi & Hansawasdi *et al.* (2015) used inulin to compare the fermentation ability of an oligosaccharide mixture from rice bran and cassava pulp. They concluded that *Bifidobacterium* utilized the oligosaccharide mixture better than inulin (Kurdi & Hansawasdi, 2015). Inulin supplementation induces host immune systems, which helps in lowering pathogens in gut. In a study by Yasuda *et al.* (2009), inulin suppressed the expression of inflammation-related genes (tumour necrosis factors), which are induced in response to lipopolysaccharides in the bacterial cell wall. Down-regulation of inflammatory genes indicates less colonization by pathogenic Gram-negative bacteria (Yasuda *et al.* 2009). Likewise, seaweeds are rich in dietary fiber and remain undigested in the upper gastrointestinal tract. Hence, they can be utilized as a substrate for the fermentation by beneficial bacteria. Although seaweeds such as the *Ascophyllum nodosum* product Tasco® have been investigated as a prebiotic source in poultry, it will be interesting to evaluate the effect of red seaweeds. As the oligosaccharide composition varies largely between red and brown seaweeds, a study on red seaweeds as a dietary supplement would identify their potential as a prebiotic candidate in poultry.

2.6 Colonization of *Salmonella* Enteritidis in poultry

Salmonella Enteritidis is a major cause of food borne salmonellosis in humans, and is associated mainly with the consumption of poultry and contaminated eggs. Over the past 20 years epidemiology studies show increased incidences of *Salmonella* infection, with an estimated 500 to 2000 deaths each year as indicated by the data obtained from the WHO Global *Salmonella* survey program (Betancor *et al.* 2010, Yim *et al.* 2010). In

Canada and United States, approximately 1.4 million people/year are infected with non-typhoid *Salmonella* serotypes (Chiu *et al.* 2004). In most of the outbreaks, egg and egg products have been identified as a carrier for *Salmonella* infection. This suggests a link between egg contamination and *S.* Enteritidis infection in humans. In 2003, 12.7% of all *Salmonella* cases were due to *S.* Enteritidis, which increased to 32.1% in 2009 (Nesbitt *et al.* 2012, Middleton *et al.* 2014). Moreover, The National Enteric Disease Surveillance: *Salmonella* Annual Summary, (2011) reported *S*. Enteritidis as the most dominant serotype from clinical and non-clinical sources (NEDS, 2011). Thus contamination of eggs by *S.* Enteritidis, either by penetration though the shell or during formation in the reproductive tract (directly), is a serious threat to both the egg production industry and consumers. This necessitates an understanding of the reproductive tract of laying hen and routes of egg contamination by *S.* Enteritidis.

2.6.1 Routes of egg contamination by *Salmonella* Enteritidis

There are two possible routes of egg contamination by *Salmonella*, horizontal transmission which involves penetration of *Salmonella* through the eggshell and vertical transmission which is direct contamination of egg contents through the transovarian route.

2.6.1.1 Horizontal transmission

Horizontal transmission usually occurs though contaminated moist material such as chicken manure during or after oviposition. Such moist organic matter serves as a source of nutrients, which aids in the growth and survival of *Salmonella* in the environment. When artificially contaminated eggs with feces containing *Salmonella* were stored at 25°C increase of 4-5 log CFU of *Salmonella* was observed after 3 days of incubation. This indicates that *Salmonella* can utilize feces for growth and can penetrate to the interior of eggs (Schoeni *et al.* 1995). Moreover, at low temperature and humidity (15°C and 45%, respectively), *Salmonella* on dry eggshell surfaces were shown to penetrate the intact egg. Thus, in the absence of fecal contamination, *Salmonella* can survive in adverse environmental conditions by lowering its metabolism (Messens *et al.* 2006). However, the presence of *Salmonella* in a laying hen farm does not imply the contamination of egg contents as well. In a recent study *Salmonella* in feces, cloacal swabs, eggshell and egg content were assessed from a *S.* Enteritidis positive laying hen

farm. Results indicated that feces (92%) were most frequently positive, followed by eggshells (34%) and cloacal swabs (4%). *S.* Enteritidis was not detected in the eggs. This indicates that a high level of contamination in the feces does not mean it will be present in the egg. Membrane barriers and antibacterial components of the albumen can prevent the contamination of the egg interior (Garcia *et al.* 2011).

Despite the protective physical and chemical barriers, bacteria can easily penetrate the eggshell immediately after oviposition. This is due to the development of negative pressure when the egg is exposed to environmental temperature lower than the chicken body temperature (42°C). This enables bacteria to easily migrate into the egg contents (Padron, 1990). Immature culticle and opened pores in the freshly laid eggs aid in bacterial penetration. The cuticle is the first line of defence against bacteria; as eggs age the cuticle dehydrates and shrinks, exposing the pores for penetration by bacteria (Messens *et al.* 2007). The penetration of bacteria through the eggshell also depends on factors such as shell weight, shell thickness, strain of bacteria and age of the bird (Jones *et al.* 2002). This suggests a need to control *S.* Enteritidis either at the farm level or during processing steps to prevent egg associated outbreaks.

2.6.1.2 Vertical transmission

An alternative route of bacterial migration into the egg is transovarian infection of the laying hen. It involves direct contamination of the egg interior and shell by *S.* Enteritidis as a result of infection in the reproductive tract of laying hens. The route by which *Salmonella* colonizes most frequently is still not clear, however, previous reports have shown that egg contamination in the reproductive tract is more frequent than the penetration through eggshell. *S.* Enteritidis can be isolated from the reproductive organs, when the bird is not positive for intestinal colonization (Lister, 1988). Moreover, *S.* Enteritidis can escape the host defense mechanism to survive persistently in the reproductive tract of the hen. This suggests the suggestion that *Salmonella* colonization inside the egg is more likely due to infection in the reproductive tract of the hen (Gast & Holt, 2000). The reproductive tract of hen contains the ovary, where the yolk (ova) matures and is released into the oviduct. The oviduct is divided into five sections, namely the infundibulum, magnum, isthmus, uterus and cloaca. The ovulatory follicles enter the

infundibulum, the magnum forms albumen, the isthmus forms the eggshell membranes, the uterus deposits the eggshell and the cloaca is involved in egg laying. Thus the colonization of *S.* Enteritidis in different sections of the oviduct can determine yolk or albumen contamination. Keller *et al.* (1995) studied colonization of *S.* Enteritidis in forming and freshly laid eggs, and concluded that forming eggs can be contaminated by descending infections from ovarian tissue, ascending infections from the vaginal area and cloaca, and lateral infections from the colonized oviduct (Keller *et al.* 1995). Previous studies have shown that *S.* Enteritidis can colonize more frequently the ovary than the oviduct (Gast *et al.* 2013). This is because *S.* Enteritidis can attach and multiply in the granulosa cell layer of the preovulatory follicles of the ovary (Thiagarajan *et al.* 1996). Dawoud *et al.* (2011) studied the invasion of ovarian follicles by different strains of *S.* Enteritidis. They used an *in vitro* invasion assay and determined that all tested strains of *S.* Enteritidis were able to invade the ovarian follicles after 2 h (% invasion = 0.016 to 0.034%) as compared to *E. coli* (negative control 0.0003%) (Dawoud *et al.* 2011).

In the upper oviduct of the laying hen, isthmus and magnum cells are the major sites of *S.* Enteritidis invasion. The interaction of *S.* Enteritidis with the epithelial cells of the isthmus and magnum was studied both *in vitro* and *in vivo*. During *in vitro* cell culture assay, monolayers of tubular epithelial cells of the isthmus (ICTEC) or magnum (MCTEC) were inoculated with *S.* Enteritidis and internalization in the glandular cells was observed by confocal scanning microscopy. Although *S.* Enteritidis could invade both sections, invasion was greater in the isthmus than in the magnum. Moreover, similar results were replicated in the *in vivo* loop model experiment, where higher ratios (isolated bacteria per isolated tubular gland cell) of isthmus invasion (5.3×10^{-5}) were observed than in the magnum (1.3×10^{-3}) (De Buck *et al.* 2004). This indicates that *S.* Enteritidis can enter and contaminate eggs through the albumen. The ability of *S.* Enteritidis to colonize the reproductive tract of hens depends on their virulence factors such as lipopolysaccharide and fimbriae. De Buck *et al.* (2003) showed that Type 1-fimbriae are required for the adhesion of *S.* Enteritidis to the isthmal secretions. The *fimD* mutant (involved in the assembly of fimbrial subunit) could not adhere to the isthmal secretions compared to wild type strain. This shows a correlation between Type 1-fimbriae and colonization of the isthmus by *S.* Enteritidis, which could result in vertical contamination

of eggs (De Buck *et al.* 2003). Furthermore, the expression of the very-long lipopolysaccharide O-Antigen (VL-O-Ag) of *Salmonella* plays a crucial role in the contamination of eggs. The VL-OAg expression–defective strains lacked the ability to colonise the epithelial cells of reproductive tracts, and as well could not survive in albumen. This indicates that length of lipopolysaccharide of *S.* Enteritidis is one of the essential criteria for egg contamination and colonization of the reproductive tract (Coward *et al.* 2013). Thus, egg contamination by *S.* Enteritidis occurs when both the colonization in the host and survival in the adverse environment such as egg albumen are achieved.

2.6.2 Control and treatment of *Salmonella* in poultry

Due to the development of drug resistant strains, there is a need to find alternative strategies to control the spread of *Salmonella*. Poultry facilities use disinfectants such as phenolic compounds and quaternary ammonium compounds to reduce the level of *Salmonella* in the environment. However, pest and rodent carriers can reintroduce *Salmonella* into disinfected area. Improved biosecurity and control practices can help lower incidences of *Salmonella* infection in poultry. Biosecurity refers to the control measures taken to restrict viruses, bacteria, fungi, protozoa, insects, rodents, and wild birds from entering and infecting the poultry barns (Stringfellow *et al.* 2009). Various control measures to maintain biosecurity include limiting visitor entry, preventing cross contamination of poultry houses by proper personnel and equipment practices, barrier to access the flocks, worker disinfection practices, and proper disposal of dead birds and waste. Fraser *et al.* (2010) showed that improved farm biosecurity might reduce incidences of *Campylobacter* and *Salmonella* infection in poultry. However, adoption of enhanced biosecurity requires farmers to willingly pay additional cost. Control programs introduced by organizations such as the U.S Department of Agriculture (USDA) and the Canadian Food Inspection Agency (CFIA) are Hazard Analysis of Critical Control Points (HACCP) programs. These programs implement rules and regulations required for monitoring the poultry production process at a series of critical control points such as controls at the farm level, transport, packaging and processing of poultry products, until distribution (Rajic *et al.* 2007).

Vaccination is most widely used preventive measure to control *Salmonella* in layers. Live attenuated vaccine derived from host specific *Salmonella* serotypes induces potent humoral and cell-mediated immunity including generation of IgA. Previously, numerous live attenuated vaccines have been tested in chickens. *S.* Enteritidis deletion mutant *phoP/fliC* was able to induce adaptive immunity and protection against *S.* Enteritidis in chickens (Methner *et al.* 2011). Oral vaccination with TAD *Salmonella* vac® E (live attenuated *S.* Enteritidis) reduced colonization of *S.* Enteritidis in the reproductive tract as well as egg contamination in layers (Gantois *et al.* 2006). Additionally, oral vaccination of the *Salmonella* Typhimurium Nal2/Rif9/Rtt, containing commercially available live vaccines AviPro *Salmonella* Duo and AviPro *Salmonella* VacT, to laying hens on the day of hatch reduced shedding and cecal colonization of *Salmonella*. Although vaccination can protect young chickens against infection, other methods such as biosecurity measures and use of feed additives should be implemented on farm to limit *Salmonella* infection (Kilroy *et al.* 2015). Natural antimicrobial products such as organic acids, probiotics and prebiotics have been widely investigated as feed additives to limit pathogen infection in poultry (See section 2.1.5 and 2.5.4.4).

2.7 Comprehensive approach

Worldwide, *Salmonella* contamination is a major food safety issue. Although poultry is a primary source of *Salmonella* infection, various food products are also carriers for *Salmonella* transmission. Thus, it is important to control *Salmonella* in poultry as well as prevent its transmission. While seaweeds have been previously evaluated to modulate growth and microbial populations in pigs and ruminants, red seaweeds as feed additives in poultry are yet to be explored. There is a definite gap in knowledge about its mode of action, establishment as prebiotics and effect on layer hens.

Previously, a few red seaweed compounds such as furanones isolated from *Delisea pulchra* have been shown to be effective against *Salmonella.* However, the antimicrobial effect of sugar derivatives of red seaweeds against *Salmonella* is unknown. Choi *et al*. (2014) showed supplementation of brown seaweed enhanced immunity in broilers. Similarly, feed supplementation of red seaweeds can enhance immunity in layers

and reduce susceptibility against pathogen infection, thus improving overall health of the birds.

Liu *et al.* (2013) have shown results indicating that water extracts of red seaweed *Chondrus crispus* can be used as an effective antimicrobial and immune enhancer (Liu *et al.* 2013). These studies were carried out in a model organism, *C. elegans*, against *P. aeruginosa*, so the level of inclusion of red seaweeds which would be effective in layers, both as prebiotics as well as against pathogen (*Salmonella*) is unknown. If this extended response would even occur in layer hens is yet to be determined. Moreover, no study yet has compared red seaweed with antibiotics. For the establishment of red seaweeds as an alternative to antibiotics in a challenge studies against *Salmonella*, a direct comparison with antibiotic would be important. Thus, prior to making any claim of red seaweed as a prebiotic/therapeutic, a direct challenge study needs to be carried out.

2.8 Hypothesis

The hypothesis of this thesis is red seaweed species protects *C. elegans* and layer hens against *Salmonella* Enteritidis.

2.9 Objectives

1. Screen and identify antimicrobial effects of red seaweeds.
2. Determine the effect of red seaweed extracts for the treatment of *S.* Enteritidis-infected *C. elegans*.
3. Determine the combined effect of red seaweed extracts and antibiotics.
4. Evaluate the effect of selected red seaweed species as a potential prebiotic source for layer hens.
5. Evaluate the effect of selected red seaweed species on *S.* Enteritidis-challenged layer hens.

Objectives 1 and 2 explaining the *in vitro* antimicrobial activity of red seaweed

extracts and the effect of red seaweed extracts on *S.* Enteritidis infected *C. elegans* are addressed in Chapter 3. Objective 3, to determine the combined effect of red seaweed extracts and antibiotics is addressed in Chapter 4. Objective 4, to evalutate red seaweeds as prebiotics is addressed in Chapter 5 and Objective 5 to evaluate antibacterial effects of red seaweeds on *S.* Enteritidis challenged layer hens is addressed in Chapter 6.

CHAPTER 3. Red seaweeds, *Sarcodiotheca gaudichaudii* and *Chondrus crispus* down regulate virulence factors of *Salmonella* Enteritidis and induce immune response in *Caenorhabditis elegans*.

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3.1 Abstract

Red seaweeds are a rich source of unique bioactive compounds and secondary metabolites that are known to improve human and animal health. *S.* Enteritidis is a broad host range pathogen found in contaminated chicken and poultry products, finding its way into human food chain. Worldwide, *Salmonella* foodborne illness outbreaks have become an important economic and public health concern. Moreover, the development of resistance in *Salmonella* serovars towards multiple drugs highlights the urgent need for alternative control strategies. This study evaluated the antimicrobial properties of red seaweed extracts against *Salmonella* Enteritidis using a *C. elegans* infection model. Six selected red seaweed species were tested for their antimicrobial activity against *S.* Enteritidis. Spread plate assays revealed that *Sarcodiotheca gaudichaudii* (SG) and *Chondrus crispus* (CC) (1%, w/v) significantly reduced the growth of *S.* Enteritidis. Water extracts of SG at concentrations of 0.8, 1 and 2 mg/mL significantly reduced the growth of *S.* Enteritidis (log CFU 4.5-5.3, $p < 0.05$, n=9). Similarly, CC at concentrations of 800 µg/mL, 1 and 2 mg/mL significantly reduced the growth of *S.* Enteritidis (log 5.7-6.0, $p < 0.05$, n=9). However methanolic extracts of both CC and SG did not affect the growth of *S.* Enteritidis. Addition of seaweed water extract (SWE) (200 µg/mL, CC and SG) significantly decreased (p <0.05, n=96*)* the biofilm formation and reduced the motility of *S.* Enteritidis. Moreover, SWE (CC and SG) suppressed the relative expression of the quorum sensing gene *sdiA* and of Salmonella Pathogenesis Island – 1 (SPI-1) associated genes *sipA* and *invF*, indicating that SWE might reduce the invasion of *S.* Enteritidis in the host by attenuating virulence factors. Furthermore, CC and SG water extracts significantly improved the survival of infected *C. elegans*, likely by impairing the ability of *S.* Enteritidis to colonize the digestive tract of the nematode. Additionally, SWE increased the survival of *C. elegans* following exposure to *S.* Enteritidis by enhancing the expression of immune responsive genes. As the innate immune response pathways of *C. elegans* and mammals show a high degree of conservation, these results suggest that SWE (CC and SG) may also impart beneficial effects on animal and human health.

3.2 Introduction

Food-borne *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) is the world's leading cause of egg associated salmonellosis in humans (Govaris *et al*. 2010, Sheela *et al*. 2003). *S.* Enteritidis is a broad host range pathogen carried by chicken and poultry products to the human food chain. In humans, *Salmonella* infection causes food poisoning and intestinal infections associated with mucosal inflammation and diarrhea leading to mortality (Yim *et al*. 2010). Thus worldwide, *Salmonella* outbreaks have become an important public health and economic concern (WHO, 2014). The WHO Global *Salmonella* survey program estimated that the organism causes 500 to 2000 deaths each year (Betancor *et al*. 2010, Yim *et al*. 2010). In 2003, 12.7% of all *Salmonella* cases were due to *S.* Enteritidis; in 2009 the percentage increased to 32.1%. In Canada and United States, each year approximately 1.4 million people are infected with non-typhoid *Salmonella* serotypes (Nesbitt *et al*. 2012, Middleton *et al*. 2014). Moreover, The National Enteric Disease Surveillance report from 2011 indicated *S*. Enteritidis as the most dominant serotype from clinical and non-clinical sources (CDC, 2012), in addition to being one of the top three non-typhoidal serovars in Canada (Galanis *et al*. 2012).

In chickens, *S.* Enteritidis colonizes the gastrointestinal tract, from where infection can extend to organs such as the ovaries and the oviduct, to eventually localize inside the egg and embryo (Guard-Petter, 2001). The ability of *S.* Enteritidis to establish persistent infection in avian tissues, including the egg is largely responsible for its presence in the human food chain (Revolledo *et al*. 2009, Yim *et al*. 2010).

S. Enteritidis invades its host using bacterial organelles (flagella and fimbrie) and virulence factors associated with motility, quorum sensing and biofilm formation (De Kievit & Iglewski, 2000, Parker & Guard-Petter, 2001, Brossard & Campagnari, 2012). Upon consumption of contaminated water or food, flagellar motility enables bacteria to recognize and adhere to the host epithelium (Pontier-Bres *et al*. 2012). Bacteria penetrate the intestinal epithelium by suppressing signal transduction pathways leading to host cytoskeleton rearrangement. This is followed by the delivery of effector protein into the host and suppression of the host immune response to establish persistent infection (Groisman & Mouslim, 2000, Brown *et al*. 2005). The survival capabilities of *S.* Enteritidis are enhanced by the formation of biofilms on variety of biotic and abiotic

surfaces. Biofilm formation constitutes a matrix of complex polysaccharides, in which are closely embedded one or more organisms adhered to the surface (Prouty *et al*. 2002).

Salmonella infection can be fatal in immunocompromised patients if not treated with antibiotics. Fluoroquinolones and cephalosporin are most commonly used antibiotics to treat infections caused by *Salmonella* serovars. However, the development of resistance in *Salmonella* serovars towards multiple drugs highlights the urgent need to design alternative strategies (Acheson & Hohmann, 2001). Previously, bacteriophages, antimicrobial peptides, and essential oils have been used as a potential alternatives to antibiotics (Fratamico & Cooke, 1996, Joerger, 2003).

Salmonella infects *Caenorhabditis elegans* (Aballay *et al*. 2000). *C elegans* is an anatomically simple, genetically tractable nematode with advantages of small body length (1.5 mm adults), rapid generation time (3 days) and large brood size (approximately 300 progeny per gravid adult) (Aballay & Ausubel, 2002, Sifri *et al*. 2005). Several studies have shown that bacterial pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio sp*., *Salmonella* Typhimurium, *E. coli* O157:H7, and *Enterococcus faecalis* have similar pathogenic mechanisms in nematodes and higher animals (Aballay *et al*. 2000, Aballay & Ausubel, 2002, Breger *et al*. 2007). This pathosystem has been used as a high throughput model to screen compounds with potential anti-infective and anti-microbial properties, applicable to cure infections in higher animals and humans (Moy *et al*. 2009). Additionally, Tenor *et al*. (2004) have shown *C. elegans* as an attractive model to study the interaction between *Salmonella* effector protein and host innate immunity. This is due to the overlap between virulence factors of *Salmonella* required for both nematode and human pathogenesis (Tenor *et al*. 2004). *C. elegans* respond to *Salmonella* infection with an innate immune response by activating the p38 mitogen-activated protein kinase (PMK-1) and subsequently by synthesizing antimicrobial peptides that are similar to those in the immune responses in humans (Aballay *et al*. 2003, Alegado & Tan. 2008). Recently, components of cultivated red seaweeds have been shown to improve the immune response of *C. elegans* to *Pseudomonas aeruginosa* (PA-14) through the induction of PMK-1 and Daf-2/daf-16 insulin signalling pathways (Liu *et al*. 2013).

Red seaweeds are a rich source of bioactive compounds and of secondary metabolites including lipids, polysaccharides, proteins, polyphenols, and minerals, which impart several health benefits (Pujol *et al*. 2002, Bansemir *et al*. 2004, Yuan *et al*. 2005, Lins *et al*. 2009, Gómez-Ordóñez *et al*. 2012, Souza *et al*. 2012). Edible red seaweeds, *Sarcodiotheca gaudichaudii* and *Chondrus crispus*, are abundant along the coasts of the eastern Pacific Ocean and the western Atlantic Ocean (Gabrielson, 1982, Guiry & Guiry, 2015). Certain strains of red seaweeds are commercially cultivated in an on-land facility by Acadian Seaplants Limited (Hafting *et al*. 2012). Previously it has been shown that the red seaweed derived brominated furanones reduced swimming motility and flagellar biosynthesis in *Salmonella* serovar Typhimurium, and showed biofilm inhibiting activities (Janssens *et al*. 2008). Furthermore, enzymatic extracts of red seaweed *Chondrus crispus* were identified effective against HSV-1 virus, indicating potential antiviral activity of sulphated polysaccharides in the extracts (Kulshreshtha *et al*. 2015). In another study, feed supplementation of red seaweeds *Sarcodiotheca gaudichaudii* and *Chondrus crispus* reduced the prevalence of pathogenic bacteria such as *Clostridium perfringens* in the gut of the chicken. In addition, the relative abundance of beneficial bacteria such as *Bifidobacterium longum* and *Streptococcus salivarius* was also found to be increased (Kulshreshtha *et al*. 2014).

Here, we report the effects of water extracts of red seaweeds *Sarcodiotheca gaudichaudii* and *Chondrus crispus* on *Salmonella* Enteritidis using the *C. elegans* infection model. In addition, we also examined the effects of water extracts on biofilm formation, motility, quorum sensing signalling and virulence factors in *S.* Enteritidis.

3.3 Materials and Methods

3.3.1 Preparation of seaweed extract (SWE)

Red seaweeds (*Chondrus crispus*, *Gymnogongrus devoniensis, Palmaria palmata*, *Sarcodiotheca gaudichaudii, Solieria chordalis* and *Sarcodiotheca spp.*) were provided by Acadian Seaplants Limited, Dartmouth, Nova Scotia, Canada. The extraction procedure is summarized in Scheme 1 (Appendix Scheme S1). Briefly, sun dried seaweeds were ground to a fine powder using a coffee grinder. Seaweed water extracts (SWE) were prepared by adding 5 grams of algal powder to 20 mL distilled

water (DW) the slurry was incubated at 50°C for 3 h with shaking at 140 rpm (New Brunswick Scientific, Enfield, CT, US). After centrifugation at 10,000g for 15 min the supernatant was recovered and the residual pellet was re-extracted three times. The resulting supernatants were pooled and freeze dried (Freeze dryer, Thermo Fisher Scientific Inc., Labconco Corporation, Kansas City, MO, US). Dilutions of SWE were prepared by dissolving 0.2, 0.4, 0.8, 1 and 2 g of soluble freeze dried extract in 1 mL distilled water and used as working concentration in all the experiments.

3.3.2 Bacterial strains, growth condition and *C. elegans*

A nalidixic acid resistant strain of *S.* Enteritidis was provided by the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, ON, Canada. Half strength tryptic soy agar (TSA) medium (BD Difco, NJ) supplemented with nalidixic acid at a concentration of 32 µg/mL was used for bacterial growth (Ebers *et al*. 2009). *C. elegans* strain Bristol N2 was maintained on modified nematode growth medium (0.35% peptone instead of .25%) at 20°C seeded with *Escherichia coli* OP50 as a food source (Aballay & Ausubel, 2001). Nalidixic acid resistance was used as a marker to selectively grow resistant strains of *S.* Enteritidis on TSA supplemented with nalidixic acid. Bacterial strains were grown overnight at 37°C and were equilibrated to OD_{600} = 0.1 (1×10^8 cells/mL) to maintain uniform bacterial cell count. All experiments were repeated three times with 6 biological replicates.

3.3.3 Bacterial inhibition test

Seaweeds were screened for antimicrobial activity against *S.* Enteritidis by the spread plate technique (Buck & Cleverdon, 1960). One hundred microliters of fresh overnight culture of *S.* Enteritidis (OD_{600} = 0.1) was spread plated on the TSA plates containing ground seaweed (1% w/v). The plates were incubated at 37˚C for 24 h and log CFU/mL was calculated to ennumerate bacteria. Seaweeds showing maximum inhibition (least log CFU/mL) were selected for preparation of seaweed extract.

Broth inoculation method: A *S.* Enteritidis suspension of 100 µL (OD_{600} = 0.1) was added to 200, 400, 800 µg/mL of SWE and the total volume was made up to 10 mL with tryptic soy broth. Culture tubes were incubated at 37°C for 24 h. The growth of *S.* Enteritidis was

measured spectrophotometrically at OD_{600} and bacteria were enumerated after serially diluting the treatments and bacterial dilution plating on TSA plates.

Agar well diffusion method: Antimicrobial activity of SWE was also evaluated by the agar well diffusion assay with some modification (Bennett *et al*. 1966). Twenty milliliters of TSA (45°C) was poured into sterile 15 cm petri dishes and *S.* Enteritidis was spread-plated. Ten millimeter wells were bored using a sterile cork borer and known concentrations of SWE were added into the wells in the plates. The plates were incubated for 24 h at 37°C and antimicrobial activity was measured using a vernier caliper to determine the zone of growth inhibition. water was used as a negative control.

3.3.4 Effect of SWE on bacterial motility- Swimming and Swarming

The ability of SWE to alter bacterial motility was tested as described by Rashid & Kornberg, (2000) with some modifications (Difco bacteriological agar instead of agarose). A single colony of bacteria from overnight grown culture was spotted using a sterile toothpick on swim (0.3 %, wt/vol agar) and swarm (0.5 % wt/vol agar) plates containing known concentrations of SWE. All plates were sealed with parafilm to prevent dehydration and incubated at 30°C for 14-15 h.

3.3.5 Biofilm formation assay

Overnight grown *S.* Enteritidis culture was diluted 1:100 in tryptic soy broth containing known concentrations of SWE. Two hundred microliters of the sample were dispensed into 96 well polyvinyl chloride microtitre plates. The plates were incubated statically at 28°C for 24 h. Biofilm formation was quantified by staining the wells with 20 µL of crystal violet (0.14% (w/v) (in water) at room temperature for 20 min (Prithiviraj et al. 2005). The wells were washed three times in distilled water to remove excess crystal violet (CV). CV stained cells were eluted with 95% ethanol and optical density was measured at OD_{600} using a spectrophotometer (Spectronic 21, Bausch & Lomb, NYI, NY, USA).

3.3.6 Effect of SWE on expression of virulence and quorum sensing related genes

For gene expression analysis, *S.* Enteritidis with an initial OD_{600} of 0.1 was cultured at 37°C TSB in the presence and absence (control) of SWE with shaking at 160

rpm. Bacterial cells were harvested by centrifugation at 12000g for 10 min. Total RNA was extracted using Trizol (Invitrogen, MA, USA) as described by the manufacturer. The RNA was quantified by NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Wilmington, DE, USA) and the quality was assessed by agarose gel electrophoresis. RNA from each biological replicate was used for cDNA synthesis using the High Capacity cDNA reverse transcription kit (Applied Biosystems). The relative transcript levels of quorum sensing, virulence, and flagella associated genes were quantified using StepOnePlus Real time PCR (Applied Biosystems, Burlington, ON, Canada). The reaction mix contained 2 ng of cDNA, 5µL Promega GoTaq SYBR green master mix (Promega North America, Madison, WI, USA) and 300 nM of each gene specific primer (Appendix Table S1). *16SrRNA* and *tuf-A* genes were used as internal controls and the relative expression levels were determined by ΔΔ CT method (Liu *et al*. 2013).

3.3.7 *C. elegans* killing assay

Modified nematode growth medium was used to establish the *C elegans* - *S.* Enteritidis pathosystem as described by Aballay & Ausubel (2002). The *C. elegans* killing assay was conducted by two methods as described below:

1. *Incorporating SWE into the media:* Treatment plates were prepared by adding SWE to nematode growth media (NGM) to a final concentration of 200, 400 and 800 µg/mL. The *C elegans* population was synchronized by placing adult nematodes on NGM plates to lay egg for 4-6 h. Eggs were incubated for two days at 20±2°C to ensure uniform adult population. Thirty to 40 synchronized L4 (young adult stage) nematodes were used for each assay. Heat - killed *S.* Enteritidis (HK-*S.* Enteritidis) and *E. coli* OP50 (HK- *E. coli*) were used as control and 70 µM fluorodeoxyuridine (FuDR) was used to prevent the development of progeny. The plates were incubated at 25°C and scored for live *vs*. dead worms every 24 h. A worm was considered dead when it failed to respond to plate tapping or a gentle touch with a platinum wire. Worms killed as a result of being stuck to the wall of the plate were excluded from the analysis. Nematodes were subjected to a combination of three pre-treatments with SWE to target virulence of bacteria and immune response of C elegans as described below:

a) Pre-treatment of bacteria with SWE: Synchronized worms were infected with S. Enteritidis grown overnight on NGM plates containing 200, 400, 800 µg/ml of SWE to test its efficacy in reducing bacterial virulence.

b) Pre-treatment of nematodes with SWE: Synchronized populations of worms were maintained on NMG plates from egg stage containing 200, 400, 800 µg/ml of SWE. Pre-treated L4 nematodes were transferred to S. Enteritidis treatment plates.

c) c) Pre-treatment of bacteria and nematodes with SWE: Synchronized worms from egg stage maintained on NGM plated were infected with S. Enteritidis grown overnight on NGM plates containing 200, 400, 800 µg/ml of SWE

Adding SWE over the media: *S. Enteritidis* was grown on modified NGM plates and 200, 400, 800 µg/ml of SWE was added over the media along with food source. The killing assay was performed with three combination of pre-treatment as described above.

3.3.8 Bacterial colonization assay- *Salmonella* Enteritidis CFU within the *C. elegans* gut

The number of *S.* Enteritidis cells from the *C. elegans* gut was determined according to the modified method previously described by Prithiviraj et al., (2005). For each replicate, 6 adult *C. elegans* were picked from the treatment plates and transferred into a 1.5 ml microfuge tube containing 500 µL of M9 buffer supplemented with 20 µg/mL gentamicin and washed three times to remove bacteria from *C. elegans* surfaces. The nematodes were disrupted in a microfuge tube containing 50 µL of M9 medium with 1% Triton X-100 using a microfuge pestle. The resulting slurry was serially diluted and plated on TSA medium (containing nalixic acid) and the numbers of colony forming units were counted.

3.3.9 Effect of SWE on expression of immune response genes in *C. elegans*

C. elegans were infected with *S.* Enteritidis as described in section 3.3.7. Approximately 100 worms per treatment (SE infection and *S.* Enteritidis infection + SWE) were harvested after 5 days of exposure to *S.* Enteritidis. Worms were transferred into 1.5 mL microfuge tubes and washed three times in M9 buffer to eliminate excess bacteria. Excess buffer was pipetted out and total RNA was extracted using Trizol (Invitrogen) following the manufacturer's protocol. RNA quality and quantity determination, cDNA synthesis and quantitative real time PCR were performed as

previously described. The immune responsive gene specific primers used for this experiment are listed in Appendix Table S2. Relative expression levels were determined by the ΔΔ CT method and *ama-1* was used as internal control.

3.3.10 Statistical Analysis

A completely randomized design was followed to analyze effects of application method, concentration and antimicrobial assays. All experiments were performed three times with at least three biological replicates. Data were analyzed using ANOVA one-way analysis of variance with a *P* value of 0.05 using the statistical software Minitab and SAS. If significant main effects were found with ANOVA, the tukey's procedure was used to compare differences among the least-square means. The standard deviation (SD) of each mean (SEM) was reported with the mean. Differences were considered significant when *P* was <0.05.

3.4 Results

3.4.1 Seaweed extracts reduces the growth of *Salmonella* Enteritidis

To identify the antimicrobial potential of red seaweeds, six selected powdered seaweeds were added to TSA and tested against *S.* Enteritidis by the spread plate technique. At an inclusion level of 1% w/v, *Sarcodiotheca gaudichaudii* (SG) and also *Chondrus crispus* (CC) significantly reduced growth of *S.* Enteritidis (Figure 3.1). Since CC and SG were effective at lower concentrations, these two seaweeds were selected for all further assays. Water and methanol extracts were prepared from SG and CC and were tested against *S.* Enteritidis by well diffusion and broth inoculation methods. In the well diffusion plate method, antimicrobial activity was determined by measuring the clearing zone of *S.* Enteritidis growth. The SG (water extract) inhibited the growth of *S.* Enteritidis in a concentration dependent manner (0.4, 0.8, 1, 1.6 and 2 mg/mL) and the zone of inhibition varied from 3 to 13 mm (Figure 3.2 a & b). The SG water extract did not exhibit any antimicrobial activity at or below to 200 µg/mL. For CC, a clear zone of growth inhibition (4 to 9 mm) was observed at 1, 1.6 and 2 mg/mL. However, concentrations of CC water extract below 800 µg/mL did not display any antimicrobial activity, therefore only water extracts were used for all further experiments. The antimicrobial activity of both CC and SG water extracts were further verified by a liquid culture inhibition test.

SG at 200 and 400 µg/mL did not affect the growth of *S.* Enteritidis. However, the bacterial titers of *S.* Enteritidis were significantly reduced at concentrations 0.8, 1 and 2 mg/mL (log CFU 4.5-5.3, $p < 0.05$, n=9). SG at a concentration of 2 mg/mL showed higher (5.13 log CFU/mL) *S.* Enteritidis count compared to 1 mg/mL (4.3 log CFU/mL) (Figure 3.2 d). For CC, the growth of *S.* Enteritidis was significantly reduced at higher concentrations 800 µg/mL, 1 & 2 mg/mL (log 5.7-6.0, $p < 0.05$, n=9) but lower concentrations had no effect on the growth (Figure 3.2).

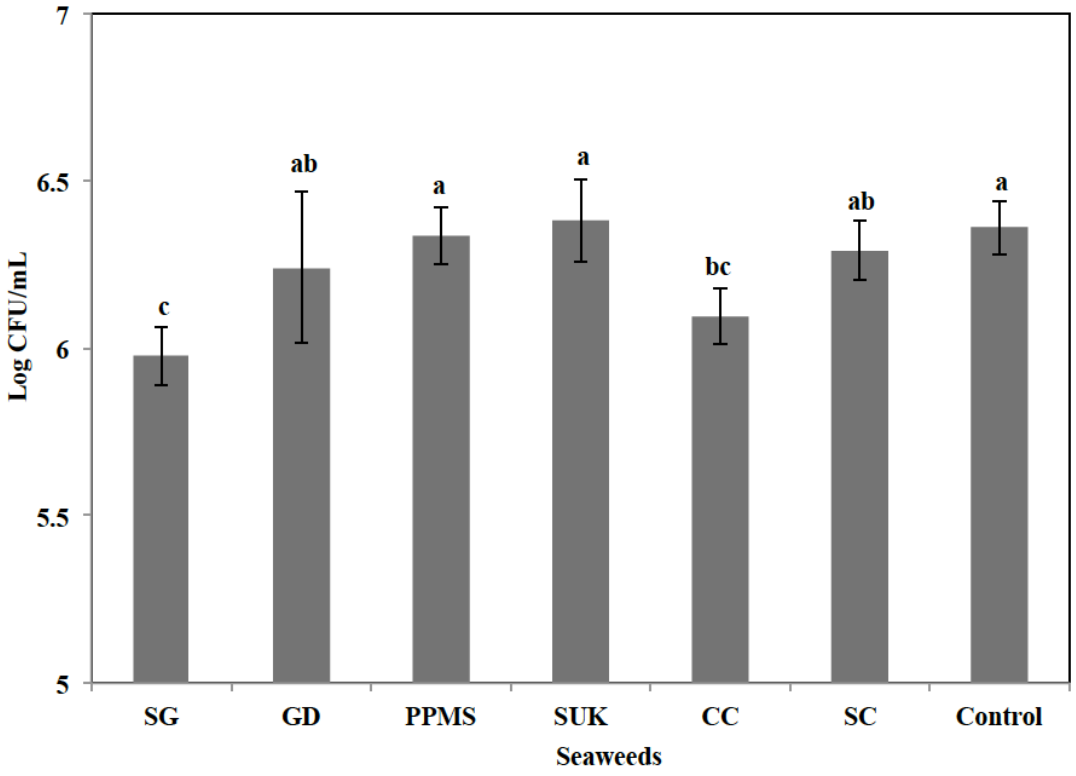
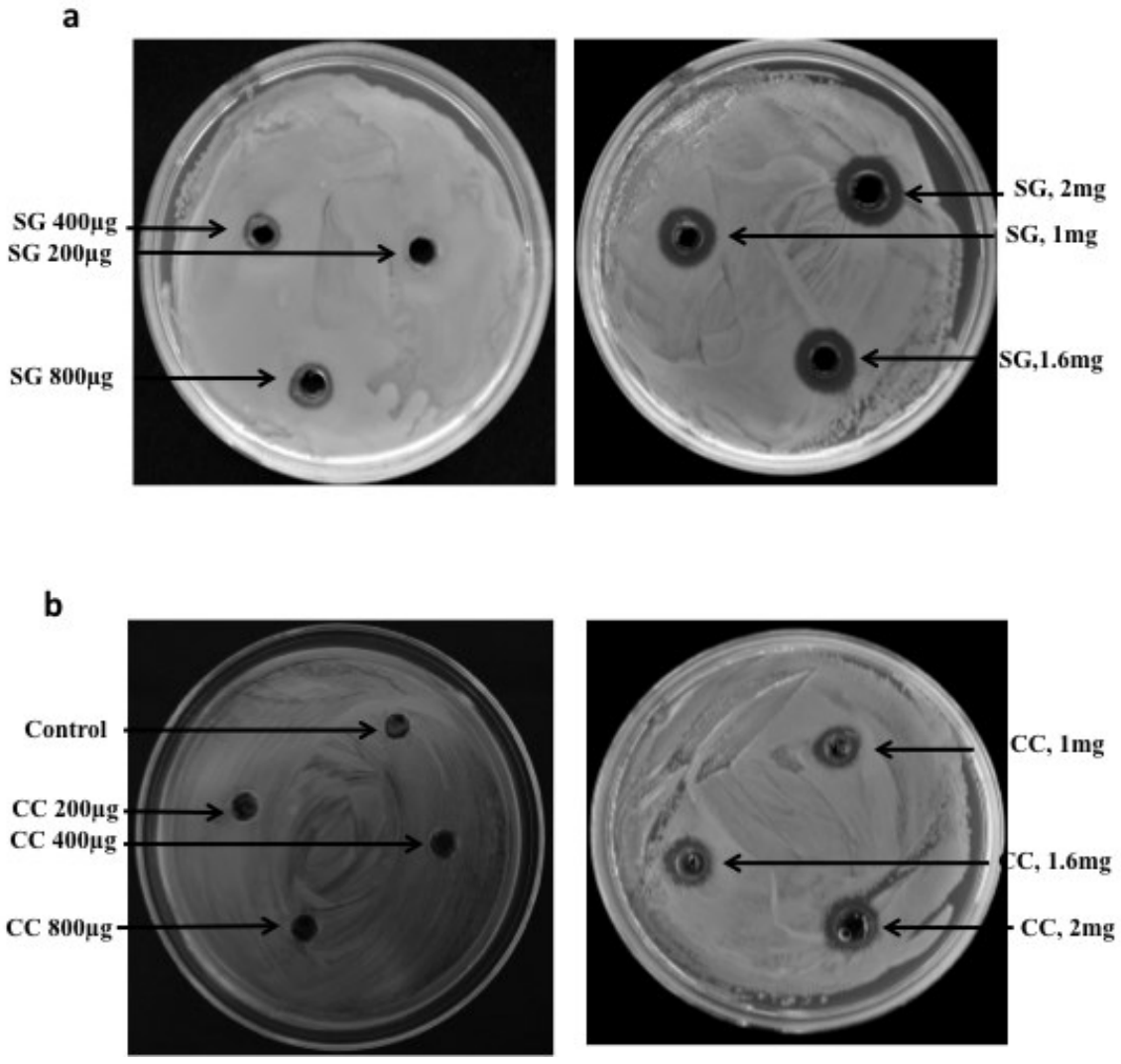


Figure 3.1. Effect of red seaweeds on the growth of *Salmonella* Enteritidis (*S.*Enteritidis). Six red seaweed species *Chondrus crispus* (CC), *Gymnogongrus devoniensis* (GD), *Palmaria palmate* (PPMS), *Sarcodiotheca gaudichaudii* (SG), *Solieria chordalis* (SC) and *Sarcodiotheca spp* (SUK) were tested against *S.* Enteritidis. A one hundred µl of fresh overnight culture was spread plated on TSA plates containing ground seaweed (1% w/v). Log CFU/mL was calculated after incubating the plates at 37˚C for 24 h. Values with different superscript letters (Tukey multiple mean comparison) are significantly different (one-way Anova; $p<0.05$). Values represent mean ± standard deviation from three independent experiments (n=9)



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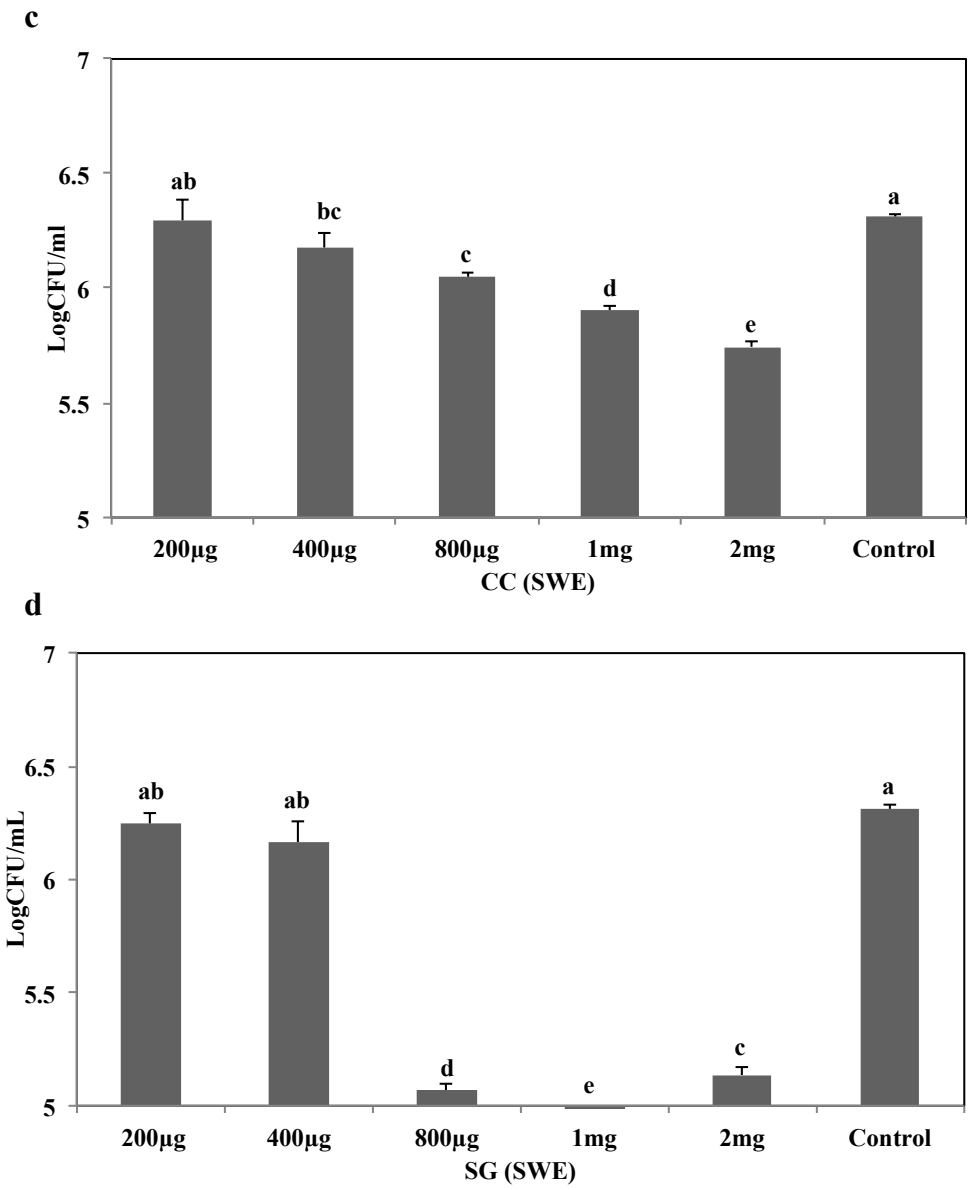


Figure 3. 2. Antimicrobial effect of red seaweed water extracts (SWE) on *S.* Enteritidis. *Chondrus crispus* (CC) and *Sarcodiotheca gaudichaudii* (SG) were tested at different concentration against *S.* Enteritidis by the agar well diffusion method and the liquid culture broth inoculation method. Solid agar showing the zones of growth inhibition at different concentration of a) SG b) CC. Liquid culture showing the bacterial colony count at different concentrations of c) CC extract and d) SG extract. Values with different superscript letters (Tukey multiple mean comparison) are significantly different (one-way Anova; $p<0.05$). Values represent mean ± standard deviation from three independent experiments (n=9)**.**

3.4.2 SWE inhibits biofilm formation of *Salmonella* Enteritidis

Biofilms increase the survival of bacteria in adverse environmental conditions and contributes to the virulence. We tested if SWE reduces biofilm formation by *S.* Enteritidis. Addition of SWE (200 µg/mL, CC and SG) significantly decreased (p <0.05, n=96) biofilm formed by *S.* Enteritidis (Figure 3.3). The presence of CC and SG in the culture medium resulted in biofilm formation equivalent to an optical density of 0.06±0.004 and 0.05±0.005 respectively, which was 3-4 fold lower when compared to the control (OD_{600} =0.17±0.01) (Figure 3.3).

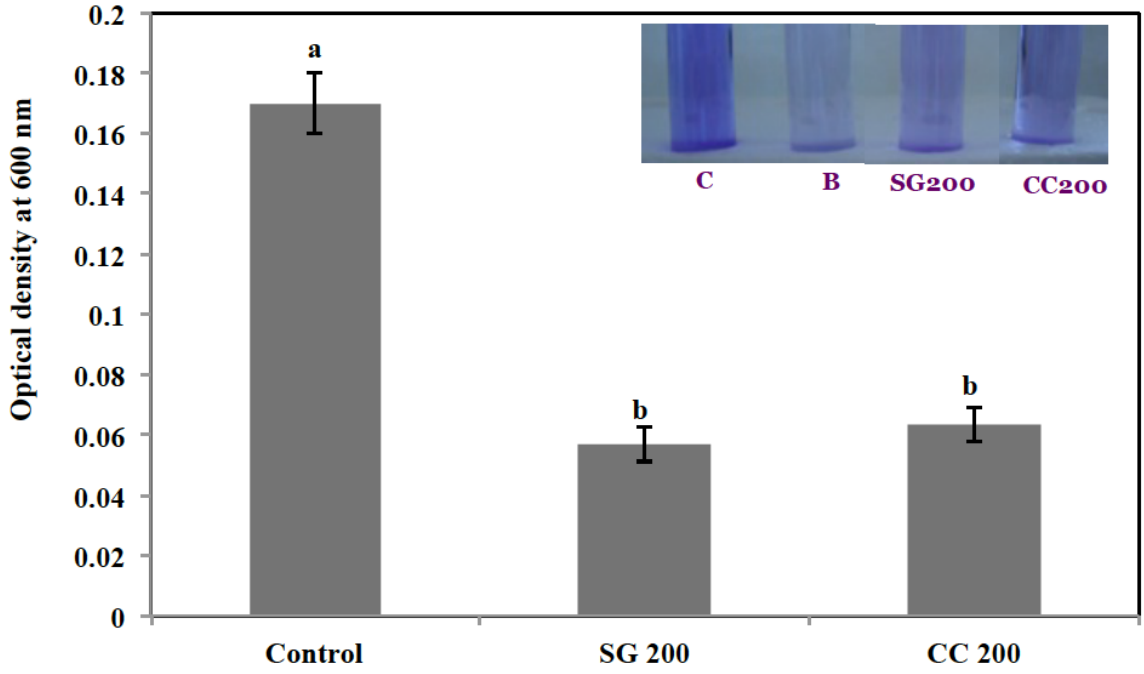


Figure 3.3. Effect of SWE treatments on biofilm formation of *S.* Enteritidis.
S. Enteritidis culture was statically grown for 24 h at 37°C in polyvinyl chloride microtitre plates in the presence of 200 µg/mL SWE (SG or CC). Biofilm formation was quantified by staining with crystal violet at an optical density of 600 nm. Values with different superscript letters (Tukey multiple mean comparison) are significantly different (one-way Anova; p<0.05). Values represent Mean ± Standard deviation from three independent experiments. Picture insert: C, positive control; B, negative control; SG200, 200 µg/ml SG extract; CC200, 200 µg/mL CC extract.

3.4.3 SWE affects motility of *Salmonella* Enteritidis

Salmonella motility plays a key role in the initial establishment and colonization of host tissues. Therefore, we tested if SWE affects *S.* Enteritidis motility. Motility tests were performed as described by Rashid & Kornberg (2000) with some modifications. Compared to the control, adding SG (200 µg/mL) water extract showed a significant reduction (70-90%) in swimming (helical rotation of flagella on semisolid agar plates) and swarming (multicellular translocation on semisolid agar plates) motility (Figure 3.4 a and b). However, CC (200 µg/mL) water extract did not significantly affect the motility of *S.* Enteritidis compared to SG or control (Figure 3.4a and b). CC water extract reduced the swimming motility by 5-10% (Figure 3.4a) and swarming motility by 20-25% (Figure 3.4b) compared to control plates.

3.4.4 SWE suppress the expression of virulence and quorum sensing related genes in *Salmonella* Enteritidis

Virulence factors including Type three secretion system, filaments, and flagella are required for the initial attachment and subsequent internalization of *S.* Enteritidis in the intestinal epithelium. *S.* Enteritidis regulates the gene expression patterns in response to change in population density by quorum sensing. Therefore, the effect of SWE (CC and SG) on the expression of virulence and quorum sensing genes was determined. SWE (CC and SG) suppressed the expression of genes without affecting the internal control (housekeeping gene, *tuf A* and *16SrRNA*) (Figure 3.5). The relative expression of quorum sensing transcriptional activator *sdiA* which encodes for SdiA (LuxR homologue), *Salmonella* pathogenicity island-1 (SPI-1) regulator *hilA* and flagellar hook associated *fliD* genes were repressed by 4-13 fold ($p < 0.001, n=9$). Similarly, SPI-1 associated genes (*sipA* and *invF*) were down regulated by 16-20 fold by the SG water extract, and 4-8 fold by the CC water extract ($p < 0.001, n=9$) (Figure 3.5).

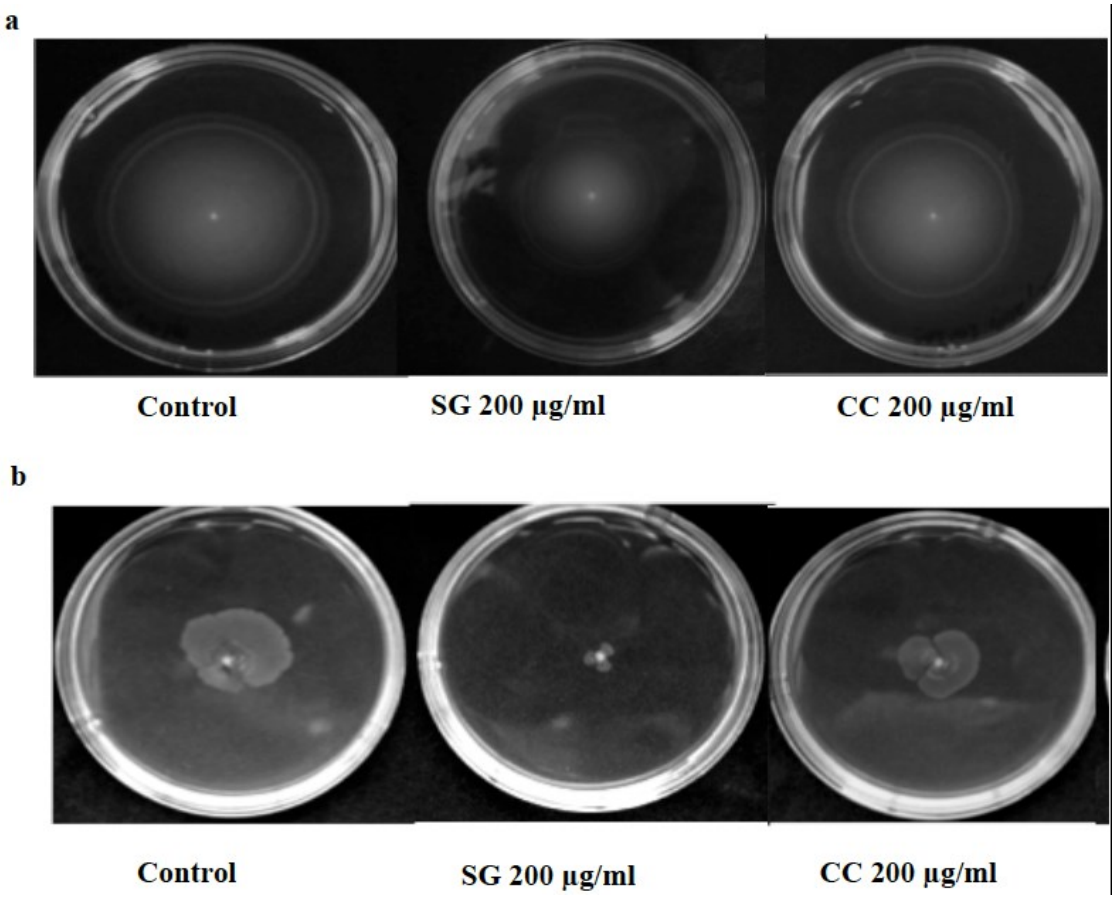
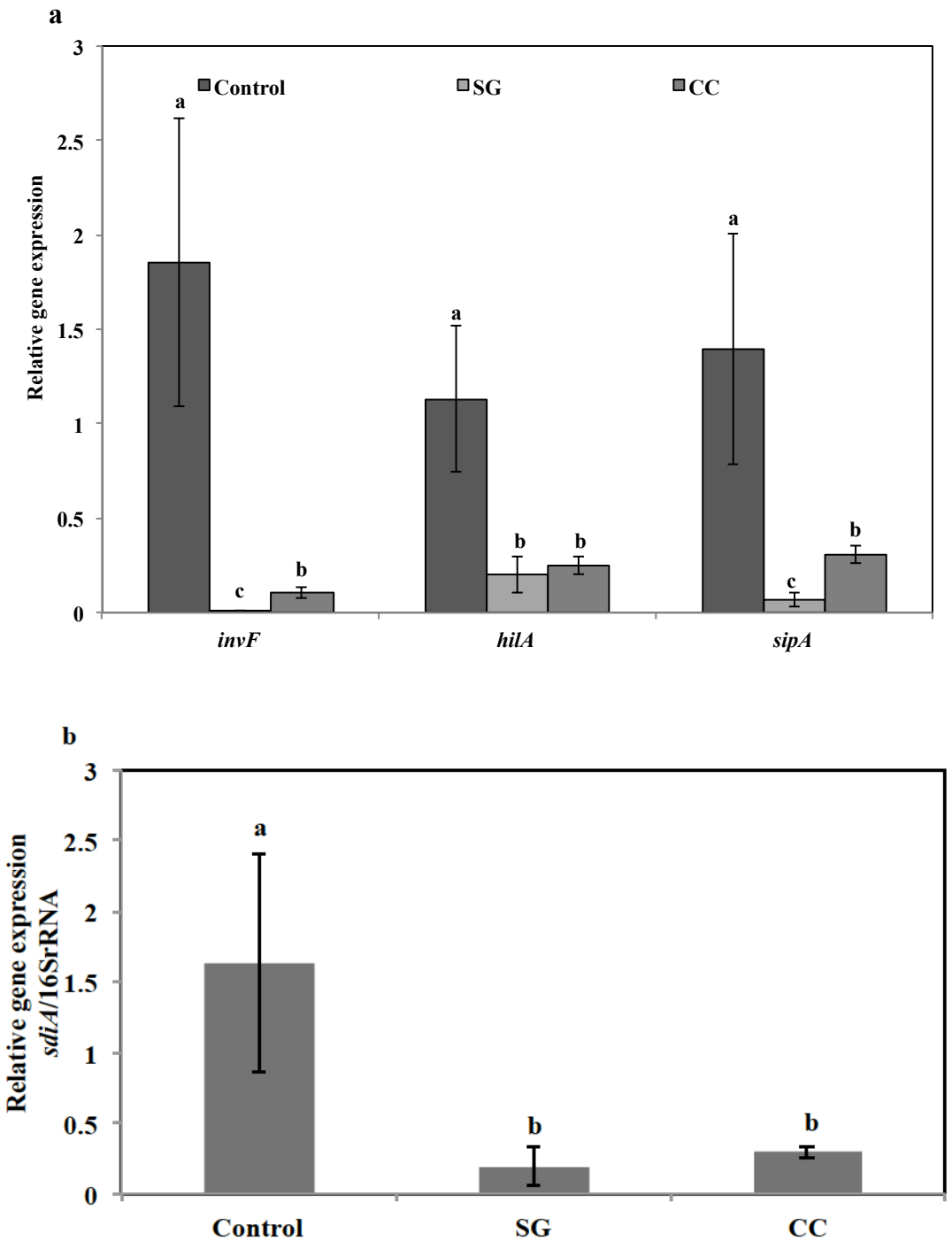


Figure 3.4. Effect of SWE treatments on the motility of bacteria.
Effect on swimming motility of *S.* Enteritidis was determined by adding 200 µg/mL SWE into the agar plates. Single purified colony was inoculated with a toothpick from an overnight TSA plate onto a swim plate (tryptone broth plus 0.3% agar) to observe for effect on motility after overnight incubation at 30°C. b) Effect of SWE on swarming motility showing deficient movement of cells when inoculated onto swarm plates (Difco bacto-agar, 0.5%) after 24 h incubation at 30°C.



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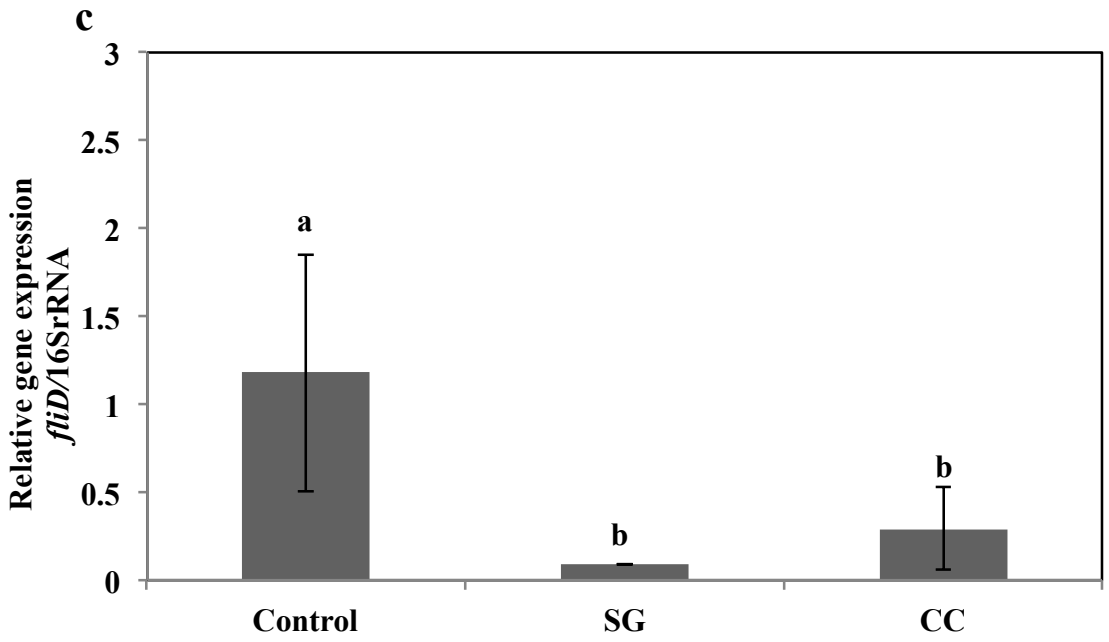

Figure 3.5. Effect of SWE treatments on the relative expression of virulence, motility and quorum sensing genes.
SPI-1encodes genes (*hilA*, *sipA* and *invF)* required for the invasion of *S.* Enteritidis into the host epithelium (Type 3 secretion system) b) Effect of SWE on the relative gene expression of *sdiA* gene (homolog of quorum-sensing regulators LuxR) c) Effect of SWE on the relative gene expression of *fliD* gene required for polymerization of flagellin on the tip of growing flagella. Values represent mean ± standard deviation from three independent experiments; each experiment had three biological replicates.

3.4.5 SWE protects *C. elegans* from infection by *Salmonella* Enteritidis

Since two seaweeds exhibited antimicrobial activity *in vitro*, we tested their effect on the model organism *C. elegans*. Under experimental conditions, 13±1 days were required for a L4 stage nematodes to be killed by *S.* Enteritidis colonization. *C. elegans* were cultured on *E. coli* OP50 as food source and SWE (CC and SG) as added to the food source from the early L1 stage. On day 1 of the L4 stage, the worms were exposed to *S.* Enteritidis in the presence and absence of SWE (CC and SG; 200, 400, 800 µg/mL). The SWE were added to the culture medium and the food source on NGM plates. The

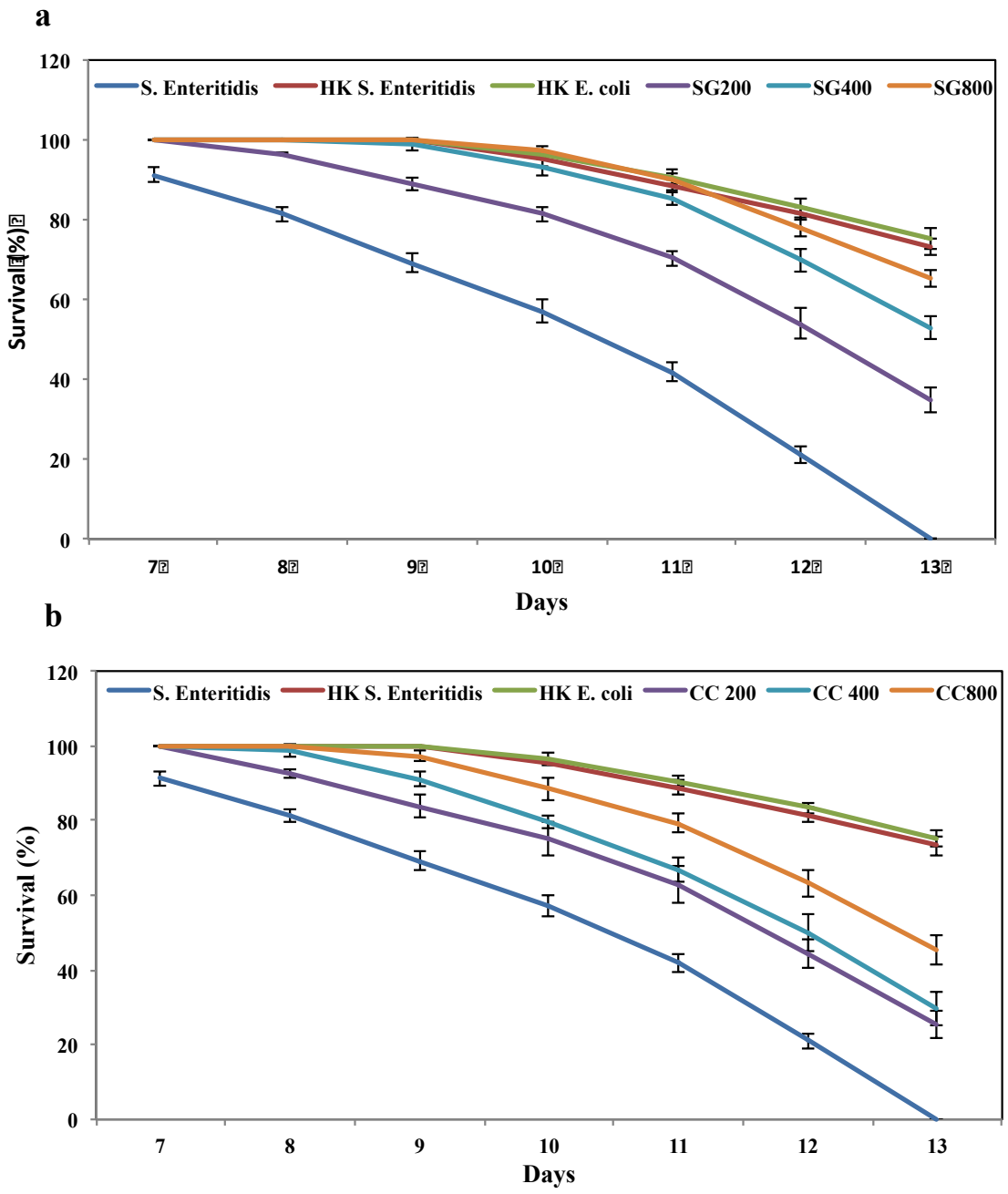

Figure 3.6. Effect of SWE treatments on the survival of nematodes infected with *S.* Enteritidis.

Three concentrations of SWE (200, 400 and 800 µg/mL) were used with. Worms were grown with seaweed water extract as food supplements and were exposed to *S.* Enteritidis. a) SG treatment and b) CC treatment. HK- *S.* Enteritidis, Heat killed *S.* Enteritidis; HK- *E. coli*, Heat killed *E. coli.* Values represent Mean ± Standard deviation from two experiments with six biological replicates.

survival percentages of the worms were recorded each day, and infected worms without seaweed supplement served as control. SWE (CC and SG) increased the survival percentage of the worms, when used either as a food source for the worms (pre-treatment of worms with SWE) or as an inhibitor of *S.* Enteritidis in the culture medium (pre-treatment of bacteria with SWE) (Appendix Table S3). However, the combination treatment (pre-treatment of bacteria and worms with SWE) showed the highest rates of protection of the worms from *S.* Enteritidis infection (Figure 3.6 a & b). For the SG water extract, the percentage survival was increased ($p<0.0001$, $n=18$) by 34.69±3.06 % at 200 µg/mL and 52.85±2.75% and 65.35±2.35% at 400 and 800 µg/mL, respectively (Figure 3.6a). Likewise, the CC water extract also resulted in survival rates higher than control. The CC water extract increased the survival of *S.* Enteritidis infected worms by 25.41±3.80%, 29.54±4.35% and 45.32±3.95% with the treatment of 200, 400 and 800 µg/mL (Figure 3.6 b), respectively. Compared to the control, both CC and SG water extracts significantly increased the survival of the infected worms, however treatment with SG showed higher survival rates ($p<0.0001$). Moreover, no significant differences were observed in the application of SWE incorporated either into the medium or over the NGM growth medium (Appendix Table S3). *C elegans* did not show any developmental abnormalities when fed with diet supplements of SWE along with food source *E coli* OP50 and the rate of feeding was uniform in all treatments.

3.4.6 SWE reduces accumulation of *Salmonella* Enteritidis in *C. elegans*

The increase in survival of *S.* Enteritidis-infected *C. elegans* by SWE supplementation could have been due to an impaired ability of *S.* Enteritidis to colonize the digestive tract of the nematode. Hence, the numbers of viable bacteria in *C.elegans* were enumerated by standard plate count. Higher concentrations (400 and 800 µg/mL) of SG water extract were effective in reducing the colony count (log CFU=4.11±0.99, 2.71±0.46 respectively) of *S.* Enteritidis in the worms. For the CC water extract, 800 µg/mL was significantly effective (log CFU= 3.3±0.13, $p< 0.05$, $n=9$) in reducing the bacterial CFU within the *C elegans* gut. However, there was no significant difference observed in bacterial accumulation between 400 and 800 µg/mL of CC water extract (Figure 3.7).

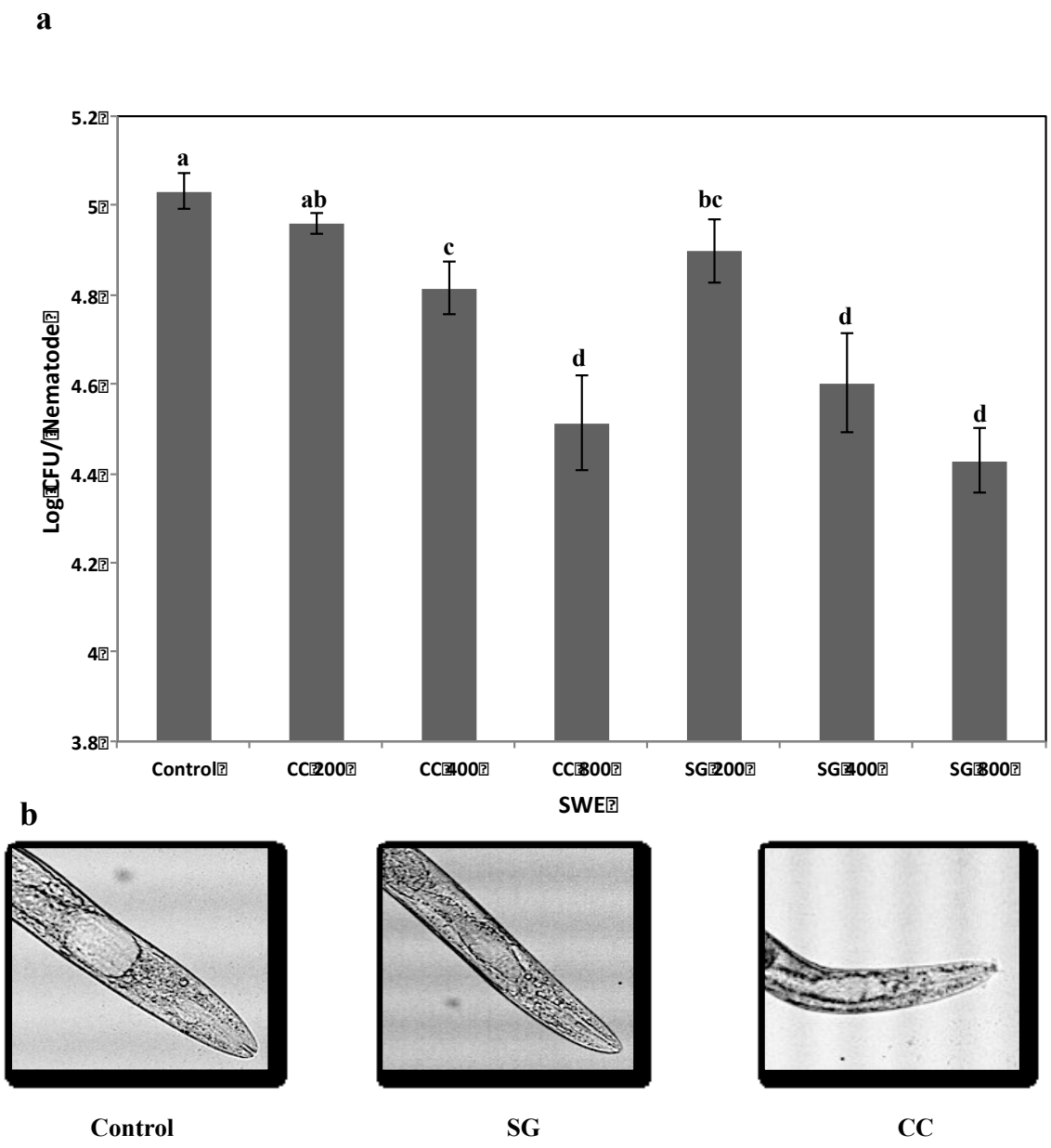

Figure 3.7. *S.* Enteritidis colony counts in the gut of *C. elegans*.
a) Effect of SWE on bacterial CFU count in the *C. elegans* gut. b) Microscopic images showing the gut of *C. elegans* infected with *S.* enteritidis. In control the gut is swelled while much less swelling was observed when infected nematodes were treated with SWE (magnification 20x). Values with different superscript letters are significantly different (Tukey multiple mean comparison, $p<0.05$). Values represent mean ± standard deviation from two experiments with 6 biological replicates.

3.4.7 SWE induces immune response genes in *Salmonella* Enteritidis infected and non-infected *C. elegans*

Apart from inhibition of virulence factors, another mechanism by which SWE can increase the survival of *C. elegans,* following *S.* Enteritidis exposure, is by enhancing the immune system of the worm. Therefore we tested the effect of SWE (CC and SG) on the expression of *C. elegans* immune response genes by quantitative real time PCR. SWE (CC and SG) activated most of the tested immune genes under infected (fed on SWE and exposed to *S.* Enteritidis) and non-infected conditions (fed on SWE). For SG, the tested immune response genes *spp-1* (saponin like protein), *abf-1* (antibacterial protein), *f49f1.6* (ShK domain-like, PMK-1), and *f38a1.5* (lectin family protein) were 2 to 15 fold up-regulated after 5 days of infection by *S.* Enteritidis. The expression of *f49f1.6* and *f38a1.5* was 2-5 fold up-regulated in the non-infected worms (fed on SG) (Figure 3.8a, $p<0.001$). Supplementation with CC water extract increased the expression of *spp-1* 12 times, of *abf-1* 6 times, of *f49f1.6* 7 times and of *f38a1.5* 17 times in the *S.* Enteritidis infected worms (Figure 3.8b, $p<0.001$). Moreover, CC water extract supplementation also increased the expression of tested genes by 3-4 fold in the non-infected worms. The level of expression of immune genes was higher with CC compared to SG supplementation (Figure 3.8 a & b).

3.5 Discussion

In this study, we report the antimicrobial activity of the cultivated red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii,* against the enteric pathogen *Salmonella* Enteritidis. Water extract of CC and SG reduced biofilm formation, motility and down regulated the expression of genes encoding virulence factors of *S.* Enteritidis. Moreover, the extracts also protected the soil nematode *C. elegans* from killing by *S.* Enteritidis. In growth inhibition assays, the higher concentrations of CC extract (1 and 2 mg/mL) were the most effective in reducing the growth of *S.* Enteritidis, indicating a dose dependent response of CC water extract on the reduction of the colony count of *S.* Enteritidis. However, for SG, although higher concentrations (1 and 2 mg/mL) significantly reduced bacterial titers, an increased in colony count was observed with 2 mg/mL compared to 1 mg/mL. This indicates that the threshold concentration of antimicrobial compounds in the crude extract could be less than 2 mg/mL. Red seaweeds

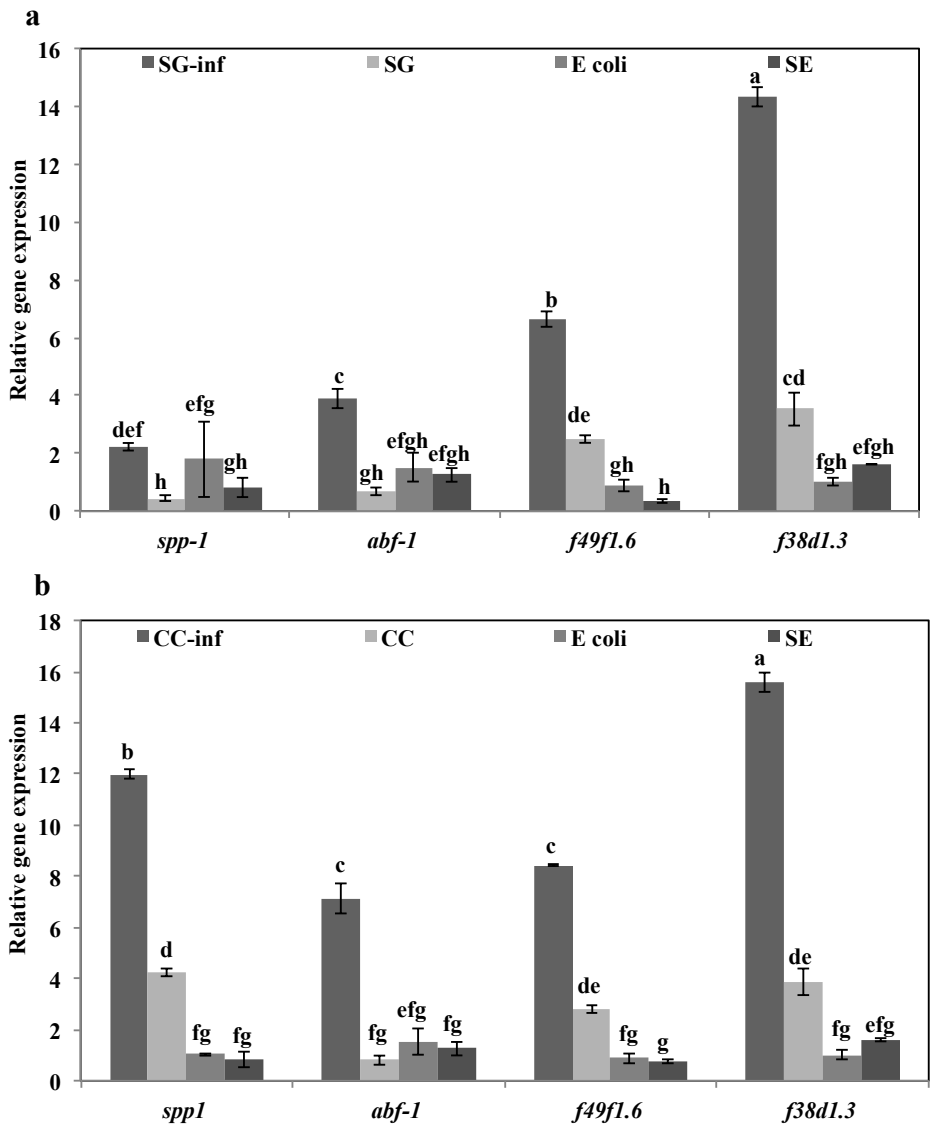

Figure 3.8 Effect of SWE on the relative expression of immune responsive genes of *C. elegans*.
Expression of the immune response genes *spp-1* (saponin like protein), *abf-1* (antibacterial protein), *f49f1.6* (ShK domain-like, PMK-1) and *f38a1.5* (lectin family protein) after 5 days of exposure to **a) SG** or **b) CC** was analyzed by quantitative reverse transcription PCR. **SG-inf**, *C. elegans* fed on SG and infected with *S.* Enteritidis; **SG**, *C. elegans* fed on SG; ***E. coli***, *C. elegans* fed on heat killed *E. coli*; **SE**, *C. elegans* fed on *S.* Enteritidis. Values with different superscript letters are significantly different (Tukey multiple mean comparison, $p<0.05$). Values represent Mean ± Standard deviation from three independent experiments; each experiment had three biological replicates.

are rich in sugars such as D-galactose and α-D- glucose (Kim, 2011). Thus, these sugars present in the extracts can serve as a carbon source at higher concentrations (<2 mg/mL) that could enhance the growth of *S.* Enteritidis.

After entering hosts by the oral-fecal route, *S.* Enteritidis uses several strategies to colonize and persist in the cell. *S.* Enteritidis utilizes flagellar motility including swimming and swarming to move towards the favorable environment for colonization and produce biofilm and virulence factors to cause disease (Bogomolnaya *et al*. 2014). Previous studies have shown a strong correlation between biofilm formation, antimicrobial resistance and persistent infection (Wang *et al*. 2013). *Salmonella* adhere and survive on surfaces for a prolonged period by forming biofilms. Biofilms protect *Salmonella* from several stresses including antimicrobials, temperature and the host immune system. Several chemical agents such as sodium hypochlorite, alkaline peroxide and benzalkonium chloride are commonly used to eliminate *Salmonella* biofilms. However, limitations such as corrosion, toxicity and development of microbial resistance, restrict the use of such compounds. Hence, alternative strategies are required to interfere with the *Salmonella* biofilm formation. Our results showed that SWE (CC and SG) effectively reduced the build up of biofilms by *S.* Enteritidis on polystyrene at a concentration (200 µg/mL) that did not affect the growth of planktonic cells. This could be due to the presence of quorum sensing inhibitors in the SWE. Quorum sensing (QS), cell-to-cell bacterial communication systems involve signaling molecules such as autoinducers (acylated homoserine lactones, AHL). Quorum sensing mediates virulence, motility and biofilm formation in human pathogens including *Salmonella* (Jesudhasan *et al*. 2010, Choi *et al*. 2012). Previously, halogenated furanones of red algae *Delisea pulchra* were shown to be an antagonist of AHL -mediated gene expression. The furanones inhibited quorum sensing in Gram-negative bacteria by interfering with AHL mediated gene expression of the LuxR protein (Manefield *et al*. 1999).Similarly, synthetic furanones improved *P. aeruginosa* clearance from the lungs in mice by inhibiting bacterial quorum sensing (Wu *et al*. 2004). Janssens *et al*. (2008) identified that brominated furanones of *Delisea pulchra*, were capable of inhibiting *S. enterica* serovar Typhimurium biofilm formation. They concluded that the expression of the target gene of quorum sensing systems, such as the AHL receptor SdiA, was not altered on treatment

with brominated furanones. Biofilm formation was reduced by repressing the expression of the global flagellar regulator *flhD* (Janssens *et al*. 2008). In the present study, expression of *sdiA* (activator of SdiA) was repressed by both CC and SG (Figure 3.5b). The algal extracts likely impaired quorum sensing and thus contributed to reduced biofilm formation. However, the specific seaweed compounds responsible for repressing quorum-sensing regulators remain to be identified. The presence of functional flagella is required for both biofilm formation and motility in Gram-negative bacteria. Depending on environmental conditions, flagella are involved in initial reversible attachment as well as release of motile cells from the mature biofilm (Chelvam *et al*. 2014). In present study, SWE (200µg/mL, CC and SG) reduced the swimming and swarming motility of *S.* Enteritidis. Additionally, SWE also down-regulated the expression of the *fliD* gene that is required for polymerization of flagellin. This indicates that restricted *S.* Enteritidis motility in the presence of SWE could be due the inability of flagellin molecules to assemble onto the hook (Yokoseki *et al*., 1995). Although both SWE (CC and SG) reduced *S.* Enteritidis motility, cells treated with SG extracts formed smaller swarms on motility agar plates. This could be due to the combined affect on both flagellar biosynthesis and quorum sensing (Manefield *et al*. 1999, Janssens *et al*. 2008,) Flagellar - based motility contributes to the virulence of pathogens through adhesion, biofilm formation and translocation of virulent protein via Type 3 secretion system (T3S). The Type 3 secretion system enables *S.* Enteritidis to invade and survive within the cell (Galan, 2001). SWE (CC and SG) reduced the expression of SPI-1 encoded virulence factors *hilA, invF* and *sipA* (Figure 3C). A previous study showed that obacunone, a triterpenoid from citrus, repressed SPI-1 of *Salmonella enterica serovar* Typhimurium mediated through *hilA* (Vikram *et al*. 2012). Another study revealed that *Chondrus crispus* water extract (CCWE) reduced the virulence factors and QS genes in *Pseudomonas aeruginosa* (PA-14) (Liu *et al*. 2013). Interestingly, in both the studies, the extracts did not show a direct effect on the growth of bacteria. However, in the present study, higher concentrations (800 SG µg/mL, 1 and 2 mg/mL) of SG and CC extract showed direct antimicrobial effects. The growth inhibitory activity might have been due to an alteration of the cell wall integrity of *S.* Enteritidis. Higher concentrations of the

extracts likely changed the permeability of the cell, resulting in cell lysis and leakage of intracellular content (Hierholtzer *et al.* 2013).

The ability to invade and colonize the intestinal tract is an important characteristic required for pathogenicity by *S.* Enteritidis (Aballay *et al*. 2000). In the present study, we used a *C. elegans* infection model to investigate the effects of SWE on *S.* Enteritidis pathogenicity and worm innate immunity. SWE (CC and SG) significantly increased the survival of *C. elegans* infected with *S.* Enteritidis. Additionally, the highest concentration of SWE (800 µg/mL CC and SG), significantly reduced the accumulation of *S.* Enteritidis in the *C. elegans* gut (Figure 3.7). The reduced *S.* Enteritidis colonization could have been partially due to the decrease in the ability of bacteria to attach to the surface of the intestinal epithelium of *C. elegans* (Aballay *et al*., 2000). The reduction in bacterial attachment might have been due to the affect of SWE on biosynthesis of flagellar components. Compared to the control, the low concentration of SWE (200 µg/mL CC and SG), significantly improved the survival of worms, however it did not affect the population of bacteria in the gut. These data suggest that low concentrations of CC and SG water extract increased the survival of the worms by repressing the expression of SPI-1 genes *hilA* and *infF* that are essential for virulence and killing in *C.elegans* (Tenor *et al*. 2004). *C. elegans* immune response up-regulates the expression of defense related genes to combat infection caused by invading pathogens. In the present study, both CC and SG water extracts induced the expression of immune related genes such as *f49f1.6* (regulated by PMK-1), *spp-1*, *abf-1* and lectin family protein *f38a1.5* induced during *Salmonella* infection (Alegado & Tan, 2008). However, the level of expression of the genes was higher with CC water extract indicating a stronger enhancing effect of CC extract on the *C. elegans* immune system (Figure 3.8). Indeed, the expression of immune-related genes was also induced without infection, indicating that SWE can augment immune responses in *C. elegans* (Alegado & Tan, 2008, Liu *et al.* 2013).

In conclusion, SWE (CC and SG) inhibited the growth, motility and biofilm formation of *S.* Enteritidis. Furthermore, gene expression analysis showed that SWE inhibited the quorum sensing, virulence and motility related genes. This indicates that one possible mechanism of *S.* Enteritidis inhibition by SWE could be by interfering with

flagellar biosynthesis. Another possibility is that SWE inhibited quorum sensing due to presence of compounds that are structurally similar to auto-inducers. Additionally, SWE reduced *S.* Enteritidis colonization in *C. elegans* and increased the survival of infected worms. Both CC and SG increased the survival of *C. elegans*, however the level of activity seems to be different. SG was more effective as an antimicrobial in reducing *S.* Enteritidis invasion, whereas CC induced immune responsive genes to enhance the immunity of *C. elegans* thereby increasing their survival. Further studies are required to verify these assumptions and to contribute to the understanding of the inhibitory mode of action of SWE on *S.* Enteritidis. Moreover, further investigations are needed to identify the purified compounds responsible for the antibacterial activity. Taken together, the results indicate that CC and SG water extracts show antimicrobial effects on *S.* Enteritidis and improve the survival of *S.* Enteritidis infected *C. elegans*.

Connection Statement between Chapter 3 and 4

In United States, the annual approval of new antibiotics for marketing has declined significantly. According to the Infectious Diseases Society of America (IDSA), only two new antibiotics have been approved since 2009. However, the infection caused by antibiotic resistant bacteria continues to increase resulting in significant mortality. The antimicrobial potential of red seaweed water extract was identified in a previous study (Chapter 3). As described in Chapter 3, red seaweed extract reduced the virulence, motility and biofilm formation of *S.* Enteritidis. Therefore, in the following study we hypothesize that reduced virulence of bacteria by seaweed extract could predispose bacteria to the existing antibiotics. Reduction in virulence, motility and biofilm formation could increase the suseptibility of bacteria to antibiotics. In the study described in Chapter 4, we identified the efficacy of the combination of SWE and their pure components with tetracycline against *S.* Enteritidis. The relative expression of the genes regulating the multidrug efflux pump (AcrAB) was determined using Real time PCR. The efflux related genes (*marA, ramA* and *acrB* were down regulated when *S.* Enteritidis was treated with a combination of seaweed pure components and tetracycline. The use of SWE in combination with tetracycline as a therapeutic approach can potentiate the activity of existing antibiotics. In the future, this could help in increasing the lifespan of existing antibiotics.

The findings in this chapter are included in a report of invention submitted to Dalhousie University.

CHAPTER 4. *In vitro* antimicrobial effect of red seaweeds and components of red seaweeds in combination with tetracycline against *Salmonella* Enteritidis

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4.1 Abstract

In recent years, excessive use of antibiotics has resulted in an increase of drug resistant strains of pathogenic bacteria, limiting the treatment of human diseases. The antimicrobial effect of water extracts of two red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii* in combination with tetracycline and streptomycin against *Salmonella* Enteritidis (SE) was studied. Streptomycin showed a stronger antimicrobial effect as compared to tetracycline with MIC_{25} (1 µg/mL) and MIC_{50} (1.63 µg/mL). Addition of *Chondrus crispus* (CC) water extract at a concentration of 200 µg/mL with tetracycline (MIC_{25} and MIC_{50}) significantly enhanced the antibacterial activity (log CFU/ mL 4.7 and 4.5 at MIC_{25} and MIC_{50} respectively). *Sarcodiotheca* (SG), water extract at 400, and 800 µg/mL ($p=0.05, n=9$) in combination with tetracycline showed complete inhibition of bacterial growth. Combination of floridoside (purified seaweed component) and tetracycline (MIC_{25} & MIC_{50}) *in vitro* revealed that only the lower concentration (15µg/mL) of floridoside potentiated the activity of tetracycline. Sub-lethal concentrations of tetracycline (MIC_{50} & MIC_{25}) in combination with floridoside exhibited antimicrobial activity that was comparable to full strength tetracycline (23 µg/mL). Further, the relative transcript levels of efflux-related genes of *S.* Enteritidis, namely *marA*, *arcB* and *ramA,* were significantly repressed by the combined treatment of floridoside and tetracycline compared to control MIC treatments (MIC_{25} and MIC_{50}). Taken together, floridoside isolated from red seaweeds can be used to potentiate the activity of antimicrobials (antibiotics) by repressing the efflux pump activity. To our knowledge, this is the first report of antibiotic potentiation by red seaweeds and a pure seaweed component against *S.* Enteritidis.

4.2 Introduction

Antibiotics are used in the treatment of infectious diseases caused by bacteria in humans, animals and plants. Treatment of gastrointestinal infections, except systemic infection such as typhoid fever, does not require antibiotics except in immunocompromised patients and those having underlying illness (Chalon *et al.* 2012). However, in the past few years the use of antibiotics even for mild gastroenteritis has increased. Thus, the increased use of antibiotics due to the over-prescription by physicians and/or lack of patient compliance have lowered the sensitivity of bacteria to

antibiotic therapy. Moreover, *Salmonella* are zoonotic, thus the use of antibiotics in farm animals adds to the ever increasing number of drug resistant strains of *Salmonella* (Funk *et al.* 2007). According to recent reports by World Health Organization and the U.S. Centers for Disease Control and Prevention, every year more than 2 million people get infected with antibiotic-resistant bacteria, which lead to approximately 23,000 deaths (Eurosurveillance Editorial Team, 2013, World Health Organization, 2014). The drug resistance of pathogenic bacteria has developed since the middle of the last century, an era when antibiotics were used extensively to treat human and animal diseases. It is likely that the emergence of drug resistant strains of pathogenic bacteria is due to the large scale use of antibiotics in medicine and agriculture (Amabile-Cuevas, 2013).

Antimicrobial resistance in bacteria develop by a number of biochemical mechanisms such as formation of biofilms (as observed in clinical conditions of periodontal pockets and cystic fibrosis (CF of the lung). Other mechanisms include chromosomal mutations and horizontal transfer of antibiotic resistance genes (Hawkey, 2000). Antibiotic resistance also develops due to the inactivation of antibiotics by bacterial enzymes and is also caused by mutation in the antibiotic target, modification in the outer membrane lipid bilayer and porin permeability, and sequestration of antibiotics within the biofilms of bacteria (Nikaido, 1994, Mah *et al.* 2003, Nikaido, 2009). Therefore, there is an urgent need to find effective alternatives that can be used to treat infections caused by drug resistant *Salmonella* strains in humans and farm animals.

Some antimicrobial therapies involve the use of antimicrobial peptides, cell membrane permeabilizers, molecular chaperones DNA synthesis and efflux pump inhibitors. However, despite being effective in *in vitro* studies, none of these strategies have advanced to clinical trials (Khan *et al.* 2012). According to the most recent report from the Infectious Diseases Society of America (IDSA), there are only seven new drugs in the pipeline (phase 2 or phase 3 trials) for the treatment of infections caused by Gram-negative pathogens. Even more alarming is that only three of these compounds could be described as novel. The rest are new compounds in already existing classes of antibiotics. More importantly, these drugs under development in the pipeline might not make it to approval by FDA and are not guaranteed to work against resistant strains of pathogens

(Boucher *et al.* 2013). An alternative approach to finding new antibiotic classes is to potentiate the activity of already existing antibiotics using combined therapies. Several antimicrobial peptides, molecules, plant extracts and essential oils have been shown to potentiate the activity of antibiotics such as chloramphenicol, ciprofloxacin, and tetracycline against Gram-positive and Gram-negative bacteria (Hussin & El-Sayed, 2011, Singh *et al.* 2013).

Tetracyclines are broad-spectrum bacteriostatic antibiotics that interfere with protein translation by inhibiting the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Tetracycline forms a complex with Mg^{2+} and blocks aminoacyl-tRNA binding, and thus inhibits protein synthesis (Hierowsk, 1965). Essential oils from *Salvia* species have been shown to potentiate the efficacy of tetracycline by inhibiting efflux pumps in *Staphylococcus epidermis* (Chovanová *et al.* 2015). Moreover, organic extracts of pomegranate, myrrh, and thyme significantly increased the efficacy of tetracycline against both Gram-positive and Gram-negative pathogens. This suggests that combination with natural compounds could be used to extend the lifespan of "fading" antibiotics (Hussin & El-Sayed, 2011).

Floridoside 2-*O*-α-D-galactopyranosylglycerol) is a neutral heteroside found in red algae. It plays an important role in osmotic acclimation and provides resistance to osmotic stress in red algae (Reed *et al.* 1980). Floridoside also has potent medicinal properties and has been shown to possess antiviral and antitumor activities (Pardoe & Hartley, 2001). Earlier, Khan et al. (2012) reported alginate, a polysaccharide found in brown seaweeds, potentiates the antimicrobial activity of antibiotics against pathogens such as *Pseudomonas*, *Acinetobacter*, and *Burkholderia* spp. (Khan *et al.* 2012). Here, we describe the combined effect of extracts of red seaweeds *Chondrus crispus* and *Sarcodiotheca gaudichaudii* and compounds isolated from red seaweeds along with antibiotics (tetracycline and streptomycin) against *Salmonella* Enteritidis.

4.3 Materials and Methods

4.3.1 Bacterial strain, chemicals and antibiotics

Reagents and growth conditions required to culture *Salmonella* are detailed in section 3.3.2. The antibiotic discs tetracycline (TE30; 30 μg), streptomycin (S10; 10 μg), erythromycin (E15; 15 μg), novobiocin (NB30; 30 μg), penicillin (P10; 30 μg), and triple sulfa (SSS25; 15 μg),) were purchased from Becton (BBL™ Sensi-Disc™), Dickinson and Company Franklin Lakes, NJ, USA. Acadian Seaplants Limited, Dartmouth, NS, Canada kindly provided the seaweeds. The extracts were prepared as described in section 3.2.1. Tetracycline and streptomycin were obtained from Sigma Aldrich (Oakville, ON, Canada). Stock solutions of antibiotics and seaweed extract were prepared and stored at -20 °C. Other chemicals and media used in this study were purchased from Difco Laboratories, Baltimore, MD, USA.

4.3.2 Antibiotics sensitivity assay

Susceptibility of *S.* Enteritidis to antibiotics was determined using the disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) with some modifications (CLSI, 2012). Briefly, the bacterial culture (OD_{600} = 0.1, 1×10^8 cells/mL) was spread on a tryptic soy agar plate, before placing the antibiotic discs. Plates were incubated at 37°C for 16-18 h, and the diameter of zone of growth inhibition was measured. The diameter of the paper disc was subtracted giving the growth-free zone of bacterial inhibition.

4.3.3 Determination of MIC of antibiotics

The susceptibility of *S.* Enteritidis to the antibiotics tetracycline and streptomycin was tested by a broth inoculation method (Ferraro, 2001). The testing of MICs (MIC_{25} and MIC_{50}) was performed in triplicate with an inoculum of 1×10^8 cells/mL. MICs were determined as the lowest concentration of antibiotics required for complete inhibition of bacteria after incubation at 37°C for 16-18h in an incubator shaking at 200 rpm. MATLAB R2010a (curve fitting tool) was used to determine minimum inhibitory concentrations (MIC_{25} and MIC_{50}) of antibiotics.

4.3.4 Combined effect of SWE and antibiotics on *Salmonella* Enteritidis

The combined effect of extracts of *C. crispus and S. gaudichaudii* and antibiotics (tetracycline and streptomycin at MIC_{25} and MIC_{50}) were evaluated *in vitro* by a liquid culture inhibition test. To 10 mL of tryptic soy broth, seaweed extract (SWE) and 100 µL *Salmonella* Enteritidis (OD_{600} = 0.1, 1×10^8 cells/mL) were added and the final concentrations of seaweed extracts in 10 mL with tryptic soy broth were 200, 400, 800 µg/mL. Culture tubes were incubated at 37°C for 24 h. The growth of *S.* Enteritidis was determined by plating the serially diluted culture on TSA plates to enumerate the colony forming units (CFU).

4.3.5 ^{1}H nuclear magnetic resonance spectroscopy

Water extracts from seaweeds were prepared as described in section 3.3.1. The extracts were sent to the National Research Council (NRC), Halifax, NS, Canada for the isolation of components. The detailed methodology of the extraction process is described in Scheme S2 (Appendix Scheme S2) (provided by NRC). A ^{1}H nuclear magnetic resonance (1H NMR) spectrum of crude seaweed extracts was obtained using a Bruker Advance DRX200 NMR spectrometer (East Milton, ON, Canada).

4.3.6 Antimicrobial effect of seaweed components on *Salmonella* Enteritidis

Pure compounds (isethionic acid, citrulline, taurine and floridoside) isolated from seaweeds and identified by ^{1}H NMR/MS were tested *in vitro* against *S.* Enteritidis by the liquid culture method as described in section 3.3.3. Fifteen µg/mL of pure compound was added to TSA broth and inoculated with *S.* Enteritidis. Antimicrobial activity was determined as a measure of log CFU/ mL.

4.3.7 Combined effect of floridoside and tetracycline on *Salmonella* Enteritidis

Synergistic interactions of floridoside and tetracycline (MIC_{25} & MIC_{50}) were evaluated *in vitro* by the liquid culture inhibition test as described in section 3.2.3. Briefly, the bacterial cells were grown in the presence of different combination of floridoside (15 µg/mL) + tetracycline (MIC_{25}, 4 µg/mL), floridoside (15 µg/mL) + (MIC_{50}, 7.9 µg/mL). Tetracycline (MIC_{25} and MIC_{50}) and floridoside (15 µg/mL) were used as controls. Antimicrobial activity was determined as a measure of log CFU/ mL.

4.3.8 Effect of floridoside and tetracycline on expression of efflux pump related genes

Gene expression analysis was carried at time intervals of 45, 90 and 180 min to understand the mechanism of the combined effect of tetracycline and SWE. The relative transcript abundance of multidrug efflux pump genes were quantified using the StepOne Plus Real time PCR system (Applied Biosystems) as described in section 3.3.6. The gene specific primers used for this experiments are listed in Appendix Table S4.

4.3.9 Statistical Analysis

A completely randomized design was followed for all assays. The experiments were performed three times with three biological replicates. Data were analyzed using ANOVA one-way analysis of variance with a *P* value of 0.05 using the statistical software Minitab and SAS. Log transformation was applied to the non-homogenous data before analysis. If significant main effects were found with ANOVA, the Tukey's procedure was used to compare differences among the least-square means. The standard deviation (SD) was reported with the mean. Differences were considered significant when $P < 0.05$.

4.4 Results

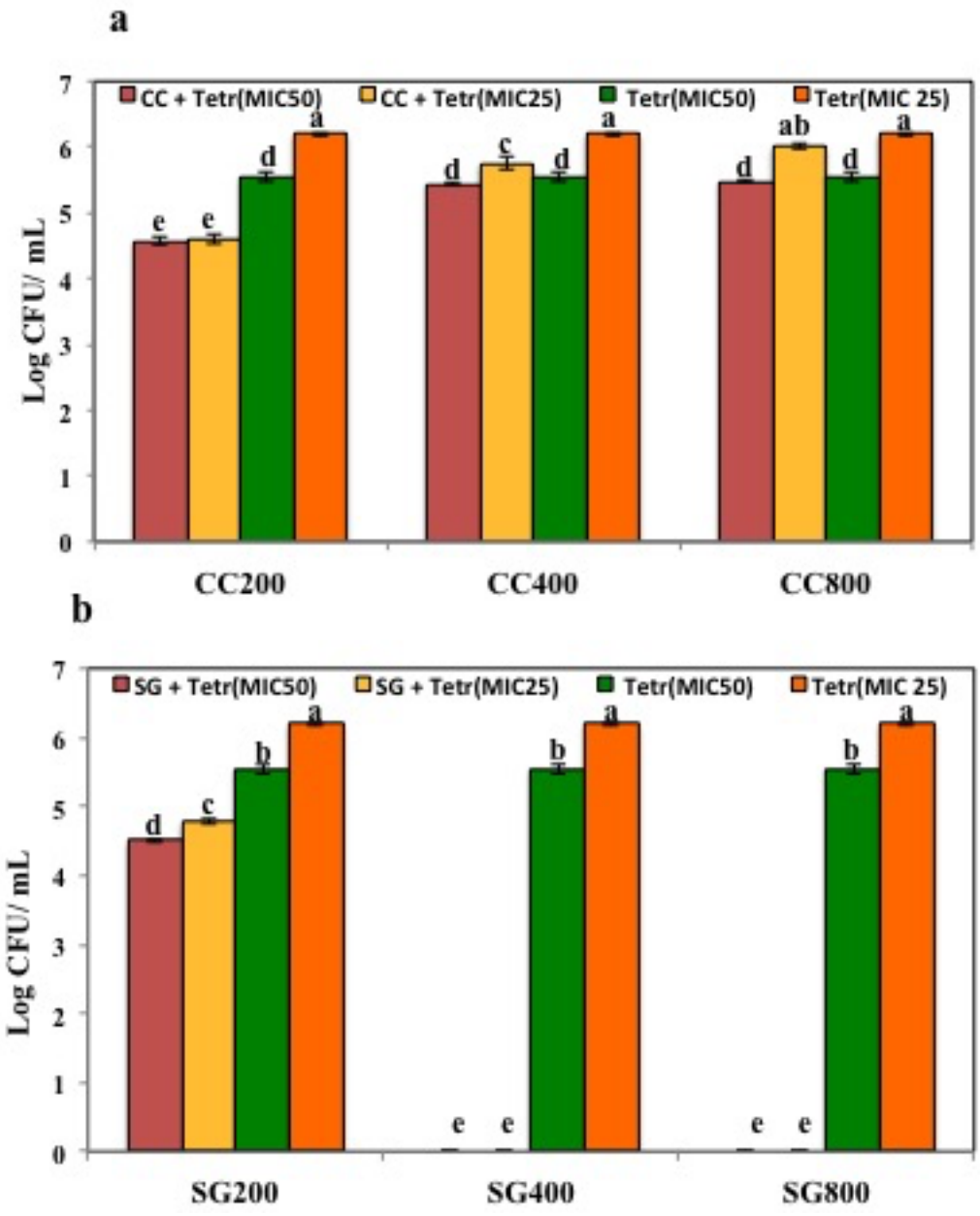
4.4.1 Screening of antibiotics against *Salmonella* Enteritidis

The efficacy of antibiotics against *S.* Enteritidis was determined by disc diffusion ,method via determining the zone of growth inhibition. Antibiotics tetracycline, streptomycin, penicillin, erythromycin, triple sulfa and novobiocin were tested against *S.* Enteritidis. Among the antibiotics tested, tetracycline (30μg) and streptomycin (10μg) exhibited zones of inhibition of 22.5 and18 mm, respectively). On the basis of zone the of inhibition interpretation chart, tetracycline and streptomycin were chosen for further studies.

4.4.2 Determination of minimum inhibitory concentrations (MIC_{25} and MIC_{50})

The minimum inhibitory concentration (MIC_{25} and MIC_{50}) of the selected antibiotics (tetracycline and streptomycin) was determined by the MATLAB curve fitting tool. For tetracycline, the MIC for 50% of the strain (MIC_{50}) was ≤ 4 μg/mL and 25% of

the strains (MIC_{25}) was ≤ 7.9 µg/mL. Streptomycin exhibited higher antimicrobial activity against *S.* Enteritidis compared to tetracycline with MIC_{25} and MIC_{50} of 1 and 1.63 µg/mL, respectively.



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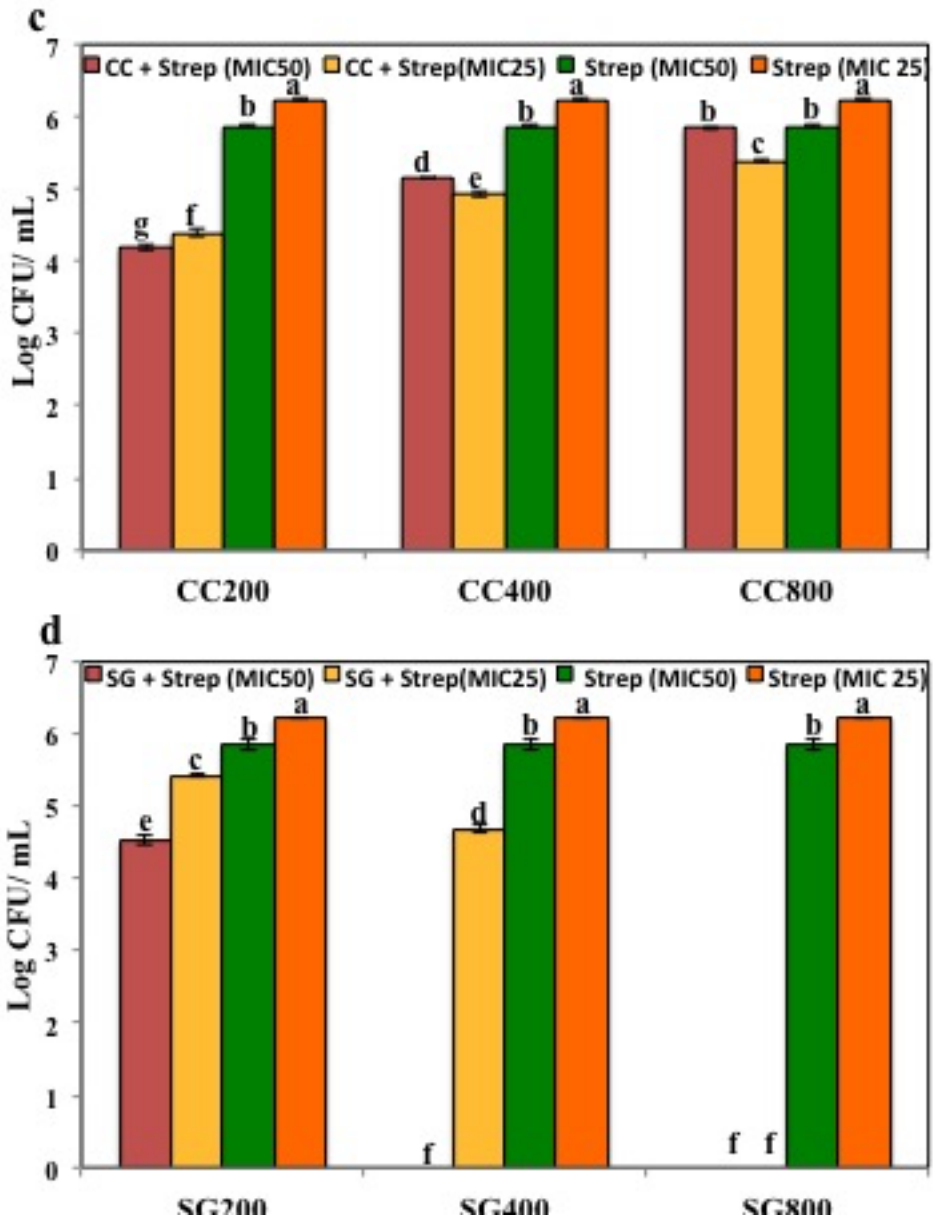


Figure 4. 1. Combined effect of antibiotics (tetracycline and streptomycin) and seaweed extracts on *S*. Enteritidis

a. *Chondrus crispus* and tetracycline, b. *Sarcodiotheca* and tetracycline, c. *Chondrus crispus* and streptomycin, d. *Sarcodiotheca* and streptomycin. Values with different superscript letters are significantly different ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments ($n=9$). CC +Tetr (MIC50), Combination of tetracycline at MIC_{50} and *Chondrus crispus*. CC +Tetr (MIC25), Combination of tetracycline at MIC_{25} and *Chondrus crispus*; Tetr (MIC50), Tetracycline at MIC_{50}; Tetr (MIC 25), Tetracycline at MIC_{25}; SG +Tetr (MIC50), Combination of tetracycline at MIC_{50} and *Sarcodiotheca*; SG +Tetr (MIC25), Combination of tetracycline at MIC_{25} and *Sarcodiotheca*; CC +Strep (MIC50), Combination of streptomycin at MIC_{50} and *Chondrus crispus*. CC + Strep (MIC25), Combination of streptomycin at MIC_{25} and

Chondrus crispus; Strep (MIC50), Streptomycin at MIC_{50}; Strep (MIC 25), Streptomycin at MIC_{25}; SG + Strep (MIC50), Combination of streptomycin at MIC_{50} and *Sarcodiotheca*; SG + Strep (MIC25), Combination of streptomycin at MIC_{25} and *Sarcodiotheca*;

4.4.3 SWE potentiate the effect of antibiotics on *Salmonella* Enteritidis

The combined effect of SWE (CC and SG) with antibiotics was determined by a liquid culture inhibition test. Antibiotics (tetracycline and streptomycin) at MIC50 and 25 were combined with 200, 400, 800 µg/mL SWE (SG &CC) (Figure 4.1). The combination of tetracycline and CC at 400 µg/mL (log CFU 5.4 at MIC_{50}, *p=0.01, n=9*) and 800 µg/mL (log CFU 6.1 at MIC_{25} and 5.8 at MIC_{50}, *p=0.01*, *n=9*) did not affect the growth of *S.* Enteritidis compared to tetracycline alone (log CFU 6.1 and 5.5 at MIC_{25} and MIC_{50} respectively, *p=0.01, n=9*). However the combination of tetracycline at MIC_{25} and 400 µg/mL of CC were effective in reducing *S.* Enteritidis growth. Moreover, the lowest concentration of CC (200 µg/mL) and tetracycline (MIC_{25} and MIC_{50}) was the most effective in reducing the bacterial growth (log CFU 4.7 and 4.5 at MIC_{25} and MIC_{50} respectively) (Figure 4.1a). For SG, the response was dose dependent, the higher concentration of SG (800 µg/mL, *p=0.05, n=9*) in combination with tetracycline showed complete inhibition of bacterial growth (Figure 4.1b). With 200 µg/mL of SG the bacterial growth was reduced (log CFU 4.8 and 4.5 at MIC_{25} and MIC_{50} repectively), significantly lower that MIC controls (log CFU 5.5) (Figure 4.1b). The antimicrobial effect of SWE (CC and SG) and streptomycin (MIC_{25} and MIC_{50}) was similarly tested. Similar trends were observed for streptomycin and SWE (CC& SG) against *S.* Enteritidis (Figure 4.1 c and d). The combination treatment with lowest concentration of CC (200 µg/mL, log CFU 4.1 &4.3 at MIC_{50} and MIC_{25} respectively, p =0.05, *n=9*) and higher concentration of SG (800 µg/mL, log CFU 0 at MIC_{50} and MIC_{25} respectively, p =0.05, *n=9*) were most effective (Figure 4.1c and d). In a comparison of the inhibitory effects of both antibiotic combinations with SWE, tetracycline showed better combined effects and was used in further experiments.

4.3.5 ^{1}H nuclear magnetic resonance spectroscopy of seaweeds water extracts

The NMR analysis identified four major compounds namely isethionic acid, citrulline, taurine and floridoside in the water extracts of CC and SG (Appendix Figure S1).

4.4.4 Seaweed compound (floridoside) affects *S.* Enteritidis growth

The susceptibility of *S.* Enteritidis to the purified seaweed compounds (isethionic acid, citrulline, taurine and floridoside) was tested by the liquid culture method. Floridoside and isethionic acid (15µg/mL) significantly reduced the colony count (log CFU 6.21 and 6.33 respectively, p=0.09, n=9) of *S.* Enteritidis compared to control (log CFU 6.5, p=0.09, n=9). However, no statistically significant difference was observed in CFU of *S.* Enteritidis on treatment with citrulline and taurine (Figure 4.2). Between two effective seaweed compounds (floridoside and isethionic acid), floridoside showed highest antimicrobial activity and was selected for the further experiments.

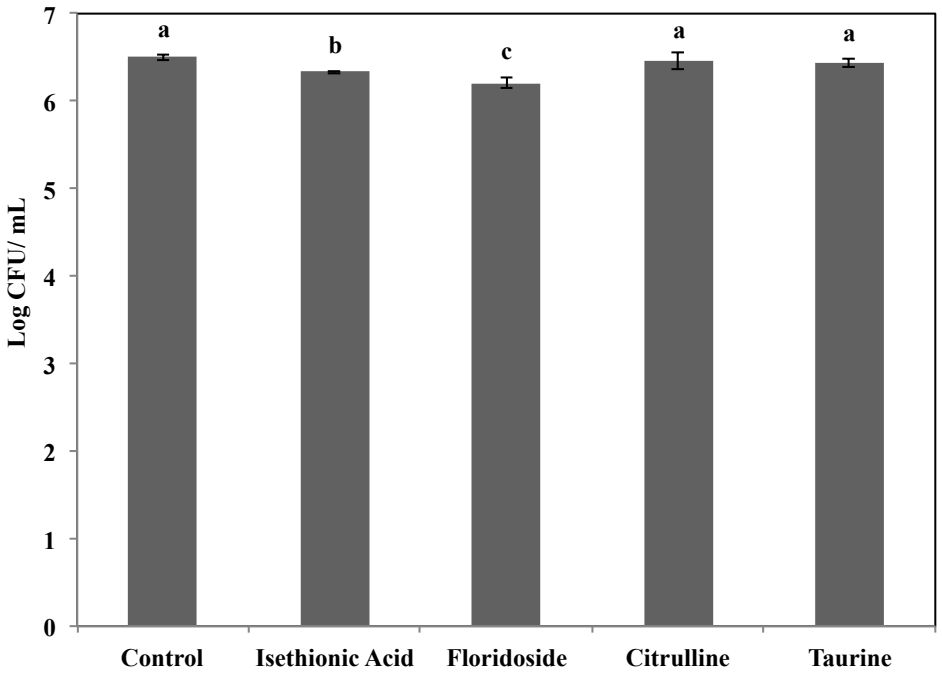


Figure 4. 2. Antimicrobial effect of pure compounds from SWE on the growth of *S.* Enteritidis.
Values with different superscript letters are significantly different ($p < 0.05$). Values represent mean ± standard deviation from three independent experiments ($n=9$).

4.4.5 Floridoside potentiates the activity of tetracycline against *S.* Enteritidis

Different concentrations of floridoside (15-100 µg/mL) in combination with tetracycline (MIC_{25} & MIC_{50}) were tested for their antimicrobial activity using a broth dilution method (Figure 4.3). Floridoside at 15 µg/mL potentiated the activity of tetracycline at both MICs ((log CFU 4.3-5.2 ($p < 0.05$, $n=9$). Sub lethal concentration of tetracycline (MIC_{50} & MIC_{25}; 4 & 7.9 µg/mL, respectively) in combination with floridoside (15µg/mL) exhibited antimicrobial activity comparable to full strength tetracycline (23 µg/mL). Compared to MICs alone, the combination of tetracycline (MIC_{25} & MIC_{50}) and 25 µg/mL of floridoside inhibited the growth (log CFU/mL 6.05 and 4.7, $p < 0.05$, $n=9$) of *S.* Enteritidis (Figure 4.3). The numbers of bacterial aggregates at higher concentrations of floridoside (50 & 100 µg/mL) in combination with tetracycline were not significantly different than the control ($p > 0.05$, $n=9$).

4.4.6 Floridoside and tetracycline suppress the expression of efflux pumps related genes

Gene expression analysis was conducted to understand the inhibitory mechanism of combined effect of tetracycline and floridoside on *S.* Enteritidis. Real-Time PCR analysis showed that the combination of floridoside and tetracycline (MIC_{25} & MIC_{50}) suppressed the expression of efflux-related genes after 90 mins of treatment (Figure 4.4). The relative transcript level of *marA*, which encodes a global regulator of multidrug efflux pumps was repressed by 2-15 fold compared to control MIC treatments (Figure 4.4). Similarly, the *arcB* gene encoding the transporter component of the main efflux pump (AcrAB) and *ramA,* a transcriptional activator of protein RamA involved in multidrug efflux pumps, were down-regulated by 18-25 fold and 14-20 fold, respectively ($p < 0.001$, $n=9$) (Figure 4.4). This indicates that floridoside might favour the accumulation of tetracycline in the cell by repressing the expression of efflux pump genes.

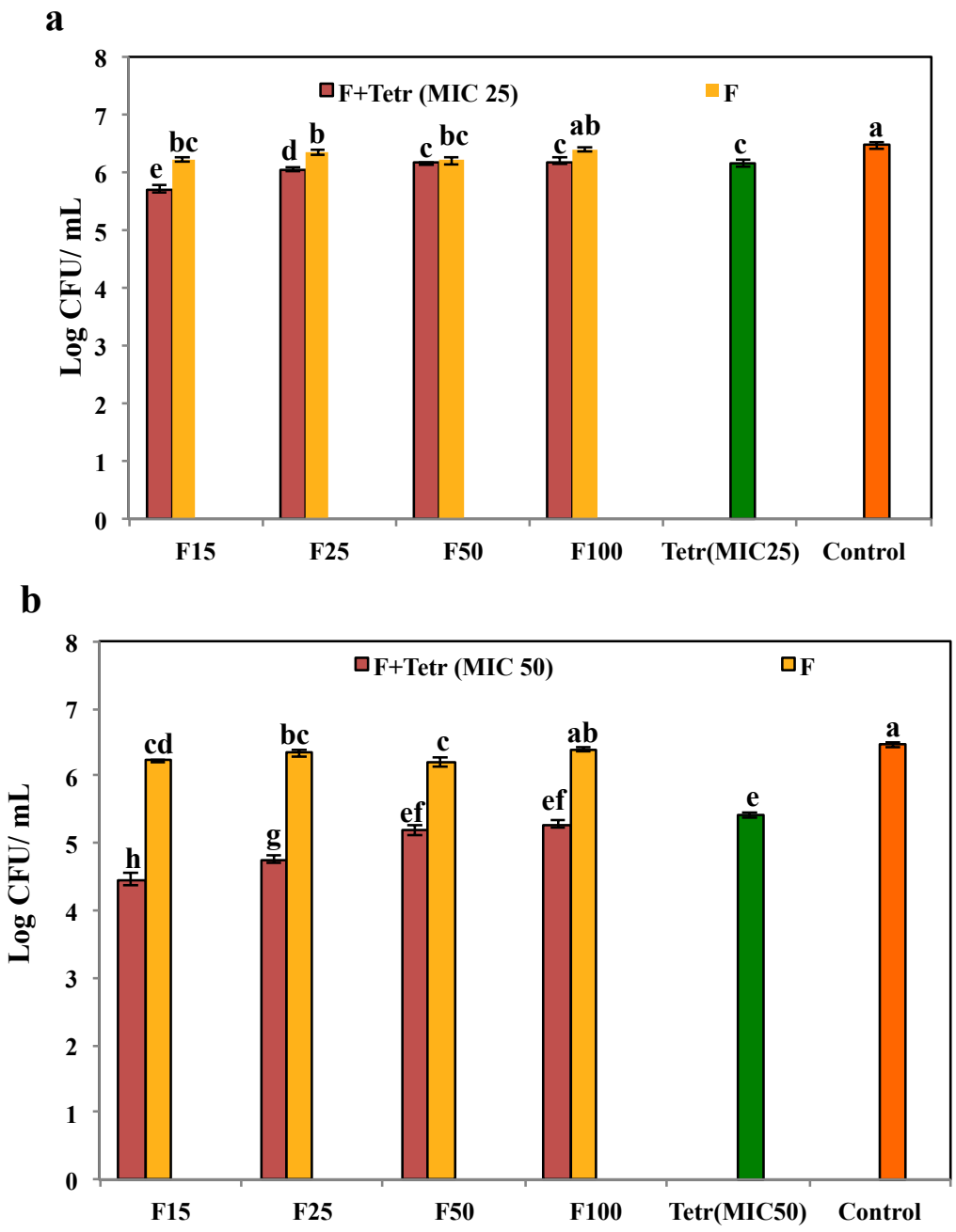
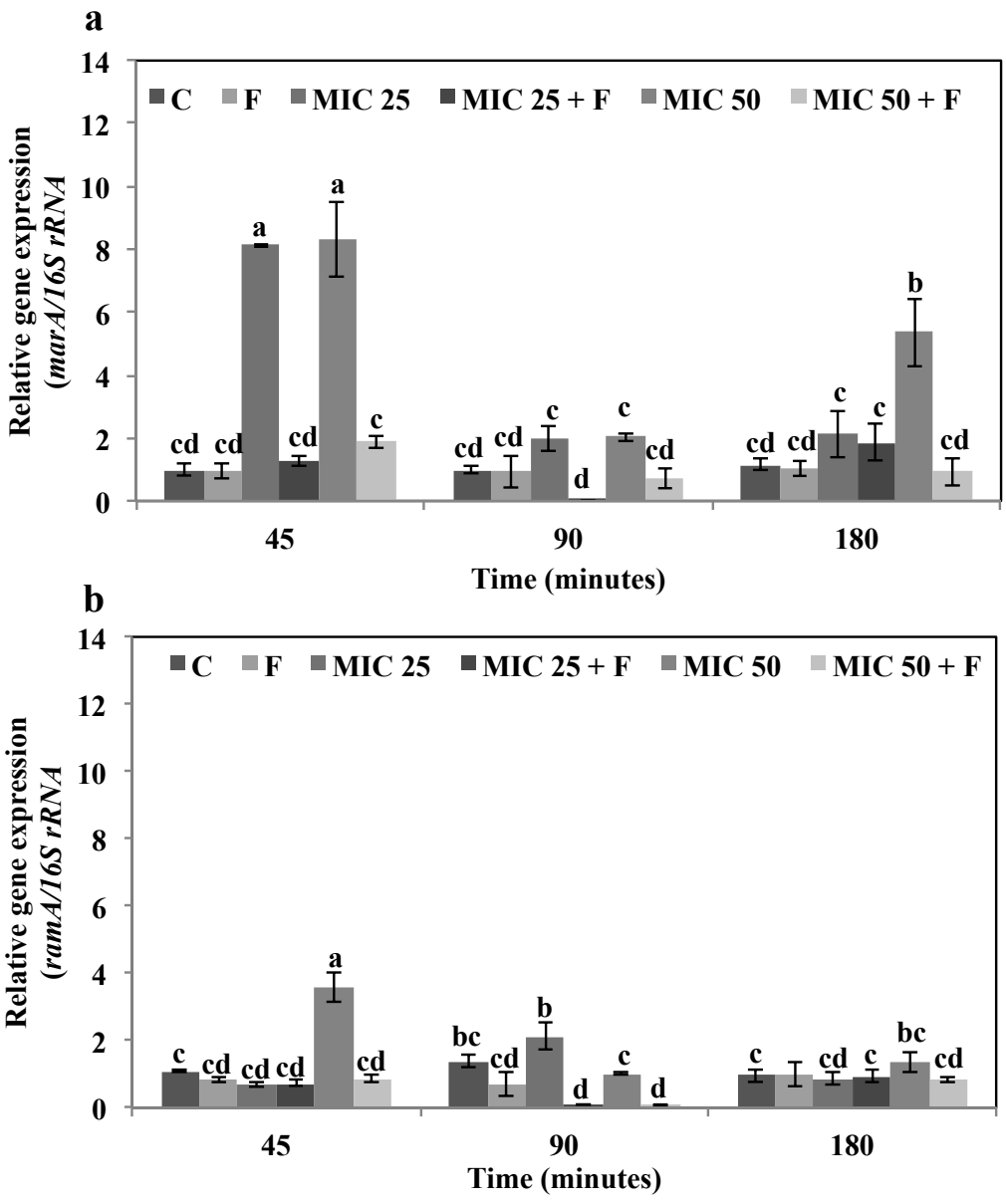


Figure 4. 3. Combined effects of floridoside and tetracycline on the growth of *S.* Enteritidis.
a. MIC_{25} b. MIC_{50} . Values with different superscript letters are significantly different ($p < 0.05$) . Values represent mean ± standard deviation from three independent experiments ($n=9$). F, Floridoside; Tert (MIC 25) , Tetracycline at MIC_{25}; F + Tetr (MIC 25), Combination of tetracycline at MIC_{25} and floridoside; Tetr (MIC 50), Tetracycline at MIC_{50}; F + Tetr (MIC 50), Combination of tetracycline at MIC_{50} and floridoside.



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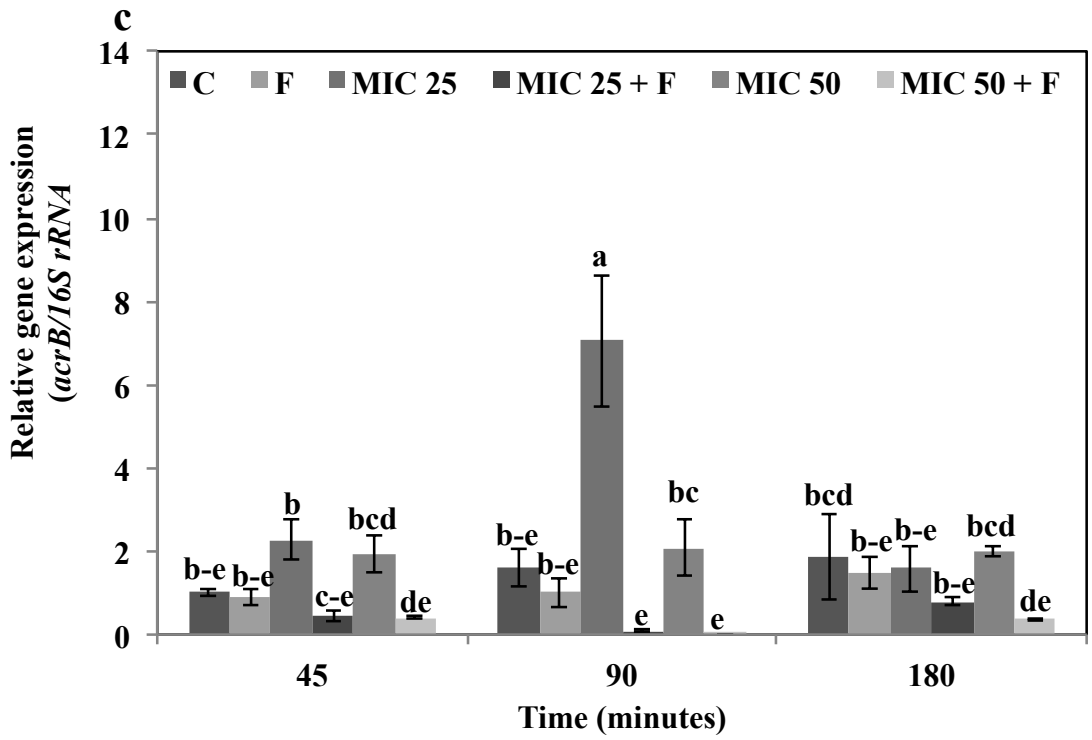

Figure 4.4. Effect of floridoside on the expression of efflux pumps related genes of *S.* Enteritidis.
a. *marA* b. *ramB* c. *acrA* after 45, 90, and 180 min of treatment with floridoside (15µg/mL and Tetracycline (MIC_{25} & MIC_{50} , 4 and 7.9 µg/mL). Values with different superscript letters are significantly different ($p < 0.05$) .
C, Control; F, Floridoside; MIC 25, Tetracycline at MIC_{25}; MIC25 + F, Combination of tetracycline at MIC_{25} and floridoside; MIC 50, Tetracycline at MIC_{50}; MIC50 + F, Combination of tetracycline at MIC_{50} and floridoside. Values represent mean ± standard deviation from three independent experiments (n=9).

4.5 Discussion

Antimicrobials used in food animals contribute in the selection and dissemination of drug resistant zoonotic food borne pathogens such as *Salmonella* Enteritidis. Non-typhoid *Salmonella* has become resistant to drugs including ampicillin, chloramphenicol, quinolones, and sulphonamide (Su *et al.* 2004). Currently, due to lesser financial incentives, pharmaceutical companies have limited their research on the development of new antibiotics. The main aim of the present study is to identify compounds from seaweeds that can improve the efficacy of existing antibiotics.

Bacterial resistance to antimicrobial drugs can be related to their ability to form

biofilms and secrete virulence factors. Previously, it has been shown that, among other functions, the matrix of biofilms prevents the access of antibiotics to the bacterial cells by sequestering them in the periplasm (Mah *et al.* 2003). Furthermore, several studies have also indicated the coselection of virulence traits with antimicrobial drug resistance by integration of virulence and resistance plasmids. Up-regulation of virulence improves the fitness of the pathogen, and has been shown to contribute to drug resistance (Guerra *et al.* 2004). In the present study, we determined the ability of SWE (CC and SG) to potentiate the activity of existing antibiotics (tetracycline and streptomycin) by using conventional broth dilution and MIC assays. We observed that combination of CC and SG water extract with antibiotics significantly reduced the growth of *S.* Enteritidis by 3-6 fold compared to the antibiotics alone (Figure 4.1). Previously, we have shown that SWE (CC and SG) reduced biofilm formation and down-regulated virulence gene expression of *S.* Enteritidis (Chapter 3). Therefore, the increase in bacterial susceptibility to antibiotics could likely be due to the effect of SWE (CC and SG) on biofilm formation and secreted virulence factors.

Floridoside is a neutral heteroside isolated from red algae and serves as a soluble carbon reserve for cellular processes. Floridoside from red seaweeds has also been researched for its potential medicinal and pharmaceutical applications. Park *et al.* (2007), isolated floridoside from red alga *Ahnfeltiopsis flabelliformis* and discovered its anti-quorum sensing activity. They identified that a mixture of seaweed compounds containing betonicine, floridoside and isethionic acid was capable of inhibiting AHL signalling in the quorum-sensing inhibition assay (Park *et al.* 2007). A year later, the same research group isolated the individual compounds and tested their effects on cell growth and quorum sensing in reporter stain *A. tumefaciens*. They observed that although the isolated floridoside had no effect on cell growth and quorum sensing, its combination with other isolated seaweed compounds significantly inhibited AHL activity (Liu *et al.* 2008). In another study, Janssens *et al.* (2008) tested the effect of red seaweed compound furanones with tetracycline on the viable cell count of *Salmonella* biofilms. They concluded that pre-treatment of furanones reduced the viable cells in *Salmonella* biofilms by 50- to 2,100-fold. This indicates that application of furanones increased the susceptibility *Salmonella* to the antibiotics (Janssens *et al.* 2008). In the present study, we

tested the effect of floridoside and tetracycline against *S.* Enteritidis. Results showed that floridoside potentiated the activity of tetracycline against *S.* Enteritidis. Interestingly, in *Pseudomonas aeruginosa* quorum sensing has been suggested to mediate antibiotic resistance. Accumulation of quorum sensing auto inducers (C4-HSL) in the medium has been shown to increase the transcription of the multidrug resistant pump MexAB-OprM (Maseda *et al.* 2004). Moreover in *E. coli*, the quorum sensing regulator SdiA has been shown to control multidrug resistance by functioning as a positive regulator of the multidrug resistance pump AcrAB. Overproduction of SdiA has been shown to increase the levels of AcrAB leading to multidrug resistance (Rahmati *et al.* 2002). Previously, we have shown that crude seaweed extracts down regulated the expression of *sdiA* (Chapter 3) and floridoside has also been reported as a quorum sensing inhibitor (Park *et al.* 2007). Therefore, the possible mode of action of floridoside could be the inhibition of bacterial quorum sensing resulting in increased susceptibility of *Salmonella* to the antibiotics. Moreover, as quorum-sensing inhibitors do not cause bacterial cell death, the selection pressure for development of resistance could be immensely reduced in the pathogenic bacteria.

Tetracycline inhibits protein synthesis in bacteria by binding to the 30S subunit of the ribososme. Bacteria can acquire tetracycline resistant by enzymatic inactivation of the drug or by increasing efflux pump activity. Multidrug efflux pumps are membrane proteins that utilize cellular energy to transport antibiotics from the cells to the external environment (Littlejohn *et al.* 1992). In the present study, the relative transcript level of efflux related genes of *Salmonella* Enteritidis namely *marA*, *arcB* and *ramA,* were significantly repressed by the combined treatment of floridoside and tetracycline compared to control antibiotics alone (Figure 4.4). Reduced expression of efflux-related genes indicates a decrease in the efficiency of *Salmonella* to efflux tetracycline from the cells (Poole, 2001). Thus in the presence of floridoside, the efflux of tetracycline would have been reduced, resulting in the accumulation of tetracycline to a level which can inhibit protein synthesis in the cell, thus eventually leading to cell death.

To conclude, this research indicates that extracts and pure compounds from seaweeds can be used to enhance the activity of antibiotics. The extracts and compounds

can work in combination with the sub-lethal doses of tetracycline and streptomycin to potentiate their antimicrobial activity. The proposed mode of action of combined effect is that floridoside might inhibit the quorum sensing in *Salmonella*, repressing the efflux related gene expression, resulting in the accumulation of tetracycline within the bacterial cell, ultimately leading to cell death. Taken together, these finding show that seaweed compounds can be used to increase the lifetime of existing antibiotics. Further research needs to be carried out to understand the structure-activity relationship of floridoside and tetracycline, which enhanced the antimicrobial activity aginst *Salmonella*. This will further help to determine specific targets of floridoside in *Salmonella* that results in cell death and verify the role of quorum sensing in the inhibitory activity of floridoside and tetracycline.

Connection Statement between Chapter 4 and 5

In poultry, antibiotics are used at a sub therapeutic level to promote growth in broilers and as therapeutics to control disease-causing pathogens. Use of antibiotics in poultry is linked to the increase in antibiotic resistant strains, which pose a serious threat to the effective treatment of bacterial infections. Therefore, there is an increasing interest by poultry producers to find alternatives to antibiotics. Since seaweed extracts reduced the colonization of *S.* Enteritidis in *C. elegans* and enhanced their immune responsive genes (Chapter 3), we hypothesized that seaweeds could be used as an alternative to antibiotics in poultry. Prior to the challenge study, a prebiotic trial was carried out to evaluate the effect of seaweeds on growth and performance of layer hens. The following study indicates that *Chondrus crispus* and *Sarcodiotheca gaudichaudii* selectively increased the abundance of beneficial bacteria and non pathogenic strains in the gut. The fermentation product of beneficial bacteria, i.e. short chain fatty acids (SCFA), were significantly higher in birds fed on red seaweeds. This indicates that seaweed served as fermentable substrate for intestinal microbiota and could be used as a prebiotic source for chickens.

Chapter 5. Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, affect performance, egg quality and gut micro flora of layer hens

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This is a pre-copyedited, author-produced PDF of an article accepted for the publication in Poultry Science following peer review. (Appendix B)

5.1 Abstract

The aim of this study was to evaluate the effect of the inclusion of red seaweed supplementation to standard poultry diets, on production performance, egg quality, intestinal histology and ceacal short chain fatty acids in Lohmann Brown Classic laying hens. A total of 160 birds were randomly assigned to 8 treatment groups. Control (C) hens were fed a basal layer diet, positive control hens (INU 2) were fed a diet containing 2% inulin and six treatment groups were fed a diet containing one of the following; 0.5, 1 or 2% *Chondrus crispus* (CC0.5, CC1, CC2) or one of the same three levels of *Sarcodiotheca gaudichaudii* (SG0.5, SG1, SG2). Dietary supplementation had no significant effect on the feed intake, body weight, egg production, fecal moisture content and blood serum profile of the birds. The feed conversion ratio, per gram of egg was significantly better (P=0.001) for 2 % CC and SG treatments. Moreover, 1% SG supplementation increased egg yolk weight (P=0.0035) and birds with 1% CC supplementation had higher egg weight (P=0.0006). The 2% SG and CC group had greater (P <0.05) villus height and villus surface area as compared to the control birds. Seaweed supplementation increased the abundance of beneficial bacteria, e.g. *Bifidobacterium longum* (4 -14 fold), *Streptococcus salivarius* (4 -15 fold) and importantly reduced the prevalence of *Clostridium perfringens* in the chicken's gut. Additionally, the concentration of short chain fatty acids, including acetic acid, propionic acid, n-butyric acid and i-butyric, were significantly higher (P <0.05) in both CC and SG treatments, than the control. In conclusion, dietary supplementation using red seaweed inclusions can act as a potential prebiotic source to improve performance, egg quality and overall gut health in layer hens.

5.2 Introduction

Antibiotics have been used in poultry feed to control disease-causing pathogens in layer chickens and as a growth promoter in the broiler industry (Baurhoo *et al.* 2007; Li *et al.* 2007; Awad *et al.* 2009). The prolonged and indiscriminate use of antibiotics in livestock led to concerns such as development of antibiotic-resistant strains of pathogens, high concentrations of antibiotic residues in meat and meat products, and undesirable

changes in the microbial communities of animal gastrointestinal tracts (Filazi *et al.* 2005; Shargh *et al.* 2012; Tellez *et al.* 2012). As a consequence, since January 1, 2006, the European Union banned the use of antibiotics as growth promoters in broiler chicken production (Castanon, 2007). In North America, there is an increased public awareness of the negative effect of antibiotics in livestock production. Therefore, there is strong interest in the development of alternatives to antibiotics (Yan *et al.* 2011). Control measures such as competitive exclusion cultures and vaccination have contributed significantly to reduce pathogen (e.g, *Salmonella*) infections in layer hens (Filho *et al.* 2009). Additionally, ingredients with antimicrobial properties such as peptides, bacteriophages, probiotics, and prebiotics have been investigated for use in the poultry industry (Hinton & Mead, 1991, Joerger, 2003).

Prebiotics are defined as "non-digestible food ingredients that affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995). In other words, prebiotics improve gastrointestinal health by providing a substrate for the growth and establishment of beneficial bacteria within the gut (Cummings and Macfarlane, 2002). Prebiotics influence gastrointestinal health by a variety of mechanisms such as production of metabolites, namely lactic acid, inhibiting or reducing the growth and establishment of pathogenic microbes, modifying metabolism of intestinal microbes, and stimulation of gut-microbe-mediated host immunity against microbial and metabolic diseases (Reddy, 1999). Prebiotics also improve epithelial cell integrity by stimulating epithelial stem cell differentiation and angiogenesis. Some examples of beneficial probiotic bacteria found in the gut microflora of chickens include *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Streptococcus*. These bacteria, which are present in the small intestine, use nondigestible polysaccharides and dietary fiber for energy (Mussatto & Mancilha, 2007). Prebiotics improve the activity of the beneficial microbiota that produces metabolites, which in turn enhance the ability of animals to absorb minerals and vitamins in both the small and large intestine (Sako *et al.* 1999).

The prebiotic effects of nondigestible oligosaccharides such as inulin, mannan-oligosaccharides, fructo-oligosaccharides, and galacto-oligosaccharides as feed

supplements have been reported in studies involving layer hens (Li *et al.* 2007, Shang *et al.* 2010). In addition, health-promoting oligosaccharides, derived from seaweeds, have been evaluated for their use in poultry (Dibaji *et al.* 2014). Results of a recent study with broilers suggested that supplementing seaweed into the diet enhanced health and productivity, largely by increasing the growth of beneficial gut-microbiota in the lower gastrointestinal tract and by alleviating inflammation resulting from overstimulation of the innate immune system (Abudabos *et al.* 2013; Evans & Critchley, 2014). Algal biomass and algal products are widely used as animal feed supplements in several countries. It is estimated that about 30% of the algal biomass produced is used as animal feed (Richmond, 2004).

Gudiel-Urbano & Goñi, (2002) reported that red and brown seaweeds have prebiotic properties that alter metabolic activity of beneficial microbiota and reduce the prevalence of pathogenic bacteria in rats. Seaweeds such as *Porphyra yezoensis*, *Laminaria japonica*, and *Hizikia fusiformis* have been shown to alter nutrient digestibility by binding to bile salts and inhibiting uptake of fats resulting in lower levels of blood cholesterol in humans (Wang *et al.* 2001). Additionally, supplementation of layer hen diets with the seaweed *Macrocystis pyrifera* resulted in an elevated levels of n-3 fatty acids in the egg (Carrillo *et al.* 2008). Moreover, feeding seaweeds has resulted in an increase in the growth rate and nutrient uptake in chickens and ducks (El-Deek & Brikaa, 2009).

Edible red macroalgae (i.e, *Chondrus crispus*, *Palmaria palmata*, *Porphyra*sp, and *Mastocarpus stellatus*) are commercially harvested along the Pacific and Atlantic coast. Also, certain selected strains of red seaweed, such as *C. crispus*, are grown on land (Hafting *et al.* 2012). Red seaweeds are rich in phycocolloids (these can be carrageenans, agar) and phycobiliproteins. Red seaweeds are a good source of dietary fiber, minerals, vitamins, phlorotannins, carotenoids, amino acids, and several health-promoting compounds, providing a source of raw material for the nutraceutical and pharmaceutical industries (Holdt & Kraan, 2011).

Seaweed polysaccharides can be considered as an ideal prebiotic due to properties such as a) selectivity by beneficial bacteria but not pathogenic strains, b) nondigestibility

(i.e, resistant to digestive enzymes), and c) fermentability as a substrate for intestinal microbiota. *In vitro* studies showed that the glycerol galactoside of the red alga *Pyropia yezoensis* was resistant to action of saliva, pancreatic and digestive enzymes (Muraoka *et al.* 2008). Several algal polysaccharides are fermented by specific subsets of gut microbes; for example, agarose-derived neoagaro-oligosaccharides were fermented by beneficial gut bacteria *Lactobacillus* sp. and *Bifidobacterium* sp. but not by pathogenic strains such as *Escherichia coli* and *Enterococcus* sp. (Hu *et al.* 2006). Similarly, fecal microbiota have been reported to selectively ferment alginate-derived oligosaccharides (Michel *et al.* 1999).

The potential activity of prebiotics can be analyzed by determining the fermentation end products of the gut microbiome [i.e., short-chain fatty acids (SCFA)]. In the colon, SCFA are formed by fermentation of undigested carbohydrates and proteins. They play an important role in the control of colonic diseases, proliferation of colonocytes, and health of the intestinal mucosa in chickens. Short-chain fatty acids such as butyric, propionic, and acetic acids also aid in mineral uptake and provide extra energy to the birds (Lan *et al.* 2005). Michel *et al.* (1999) studied the effect of alginate and laminarin-derived oligosaccharides on SCFA in human fecal flora. There was no difference in the total SCFA; however, the concentration of propionate increased significantly in the treatments as compared with fructo-oligosaccharide control. This study indicated that seaweeds could be fermented by intestinal microbiota to produce beneficial by-products such as SCFA.

The aim of the present study was to investigate the effect of dietary inclusion of 2 red seaweed species (i.e., *C. crispus* or *Sarcodiotheca gaudichaudii*) on layer productivity, histomorphology of integrity of the intestinal mucosa, as well as the composition of the microbiota, in the small intestine, and ceca of layer hens. A basal diet and a ration containing inulin (an established prebiotic) served as negative and positive controls, respectively.

5.3 Materials and Methods

5.3.1 Birds and housing

A total of 160 commercial, laying hens (Lohmann Brown Classic) at 67 weeks of age were used in a 30-days trial. All birds were randomly assigned to one of 32 wire cages in the upper tiers, of a two-sided, 3-tier battery cage system, 5 birds per cage. Feed and water were available *ad libitum* throughout the trial. A controlled environment was established with 16 hours of light per day and the temperature was set at 25°C. All experimental procedures were carried out in accordance with the Council of Animal Care guidelines (CCAC, 2009).

5.3.2 Preparation of seaweed-supplemented feed and experimental design

Cultivated samples of the red seaweeds *Chondrus crispus* (CC) and *Sarcodiotheca gaudichaudii* (SG) were provided by Acadian Seaplants Limited, Dartmouth, NS, Canada. The seaweeds were grown in a salt water artificially in an on-land cultivation facility. Freshly harvested biomass was dried at 50°C for 24 h and ground to a powder (mesh size, 0.4 mm) using a micro Wiley mill, standard model 3 (Arthur H Thomas Co, Philadelphia, PA, USA). Oligo – Fiber™ DS2 inulin (Cargill Inc, Wayzata, MN, USA), a commercial prebiotic, was used as one of the controls in this experiment. The dried seaweeds (SG and CC) were incorporated into chicken feed at concentrations of 0.5, 1 and 2% (w/w). Experimental diets were made to be iso-caloric by varying the percentage of carbohydrate and protein sources. Two control treatments were included in the experiment; a basal layer diet served as the negative control while the basal diet containing 2% (w/w) inulin served as the positive control. The compositions of diets used in the experiment are given in Table S5 (Appendix Table S5). Each treatment was randomly assigned to 4 cages; therefore each cage of five birds was considered an experimental unit.

5.3.3 Layer performance and egg quality

Eggs were collected daily and the numbers recorded throughout the experiment. Feed intake and body weights (by cage) were determined on days 0, 14, and 28 of the experiment. Feed was weighed each day before adding into the feeders and feed consumption was calculated by weighing feeders at the end of each observation period.

Additionally, four eggs per cage were collected on days 0, 14, and 28. Weight, specific gravity, and other quality parameters were determined for collected eggs. From this data, the Feed Conversion Ratio (FCR) for each cage was calculated using the following formula:

FCR= *Feed consumed (kg)/ Average egg weight * Total number of eggs (kg)*

The eggs were subsequently broken to determine albumen height (using a QCH albumen height gauge, Technical Services and Supplies, York, UK), yolk weight, eggshell weight and eggshell breaking strength (using TA.xt Plus, Texture Technologies Corp, New York, NY, USA). The albumen was separated from the yolk and shell. Thereafter, albumen weight was calculated by subtracting the yolk weight and eggshell weight from the weight of the egg. Shells with their membrane intact were rinsed and dried for 48 h and dry shell weights were measured.

5.3.4 Excreta moisture content

On day 15 and 27 of the trial, three independent samples of excreta were collected with a sterile spatula from 16 randomly selected cages, two cages per treatment). Care was taken to avoid inclusion of feed spillage and bird feathers with the excreta samples. Each collection was weighed and placed in a zip-lock bag. Subsequently, each sample was dried in a forced air oven at 70°C for 24h and weighed. Percent moisture was calculated using the following formula (Miles *et al.* 2011; Van der Hoeven-Hangoor *et al.* 2013):

Moisture percentage of material n (Mn) =

{(Wet weight of the sample (Ww)-Weight of the sample after drying (Wd)}/Wwet weight of the sample (Ww)} x 100

5.3.5 Collection of blood samples and chemical analysis

Blood samples were taken from the brachial vein of birds on day 28 in Vacutainer tubes containing sodium heparin and were used for determination of the plasma component (Lan *et al.* 2005). Approximately 8 mL of blood was collected from 32 randomly selected birds (4 birds/ treatment), using a 21-gauge needle. Blood plasma was isolated by centrifuging the tubes at 3,000 × *g* for 10 min at 4°C. The separated supernatant, obtained

as plasma, was transferred into individual vials and stored at -20°C for chemical analysis. The blood plasma analysis was carried out at Diagnostic Services, Atlantic Veterinary College, University of Prince Edward Island.

5.3.6 Histomorphological analysis and sample collection

One bird per cage was randomly chosen (Day 28, n=4) and euthanized by cervical dislocation. Each bird was weighed and the ileum, ceca, spleen, liver and heart were removed. The contents of ceca were expelled by finger pressure and the empty segments were weighed. A section of 0.5 -1.0 cm of the distal ileum, immediately prior to the ileo-cecal junction was collected (Day 0, 14, 28) and fixed in buffered, neutral formalin (10% (vol/vol)) for histological studies (Awad *et al.* 2009).

The ileal samples were placed in formalin and processed for microtomy. Briefly, each sample was sliced into three sections and dehydrated in a series of alcohol with increasing concentration (70 to 100%). The tissue slices were permeated with xylene and fixed in paraffin wax. A section (0.5 μm thick) was cut with a microtome and mounted on to a glass slide. Each slide was stained by the procedure described by Drury and Wallington (1980) and was used for histological measurements. Images were scanned using a Nikon Super Cool Scan 400ED (Nikon Inc, Japan) and Sigma Scan Pro 5 (SPSS Inc, Chicago, IL, USA) was used for measuring villi height, width and area, crypt depth, and mucosal depth of the scanned images. Eight to 10 measurements of each component were made per slide. Villi heights were measured from the base of the intestinal mucosa to the tip of the villus, crypt depth was measured between the start of mucosa to the bottom end of villi and the mucosal depth was measured from one end of the crypt to the end of the serosa (Samanya & Yamauchi, 2002).

5.3.7 Analysis of intestinal microbiota

The microbial population within the gut was determined by qPCR based on a method as described by Middelbos *et al.* (2007) with minor modifications. Briefly, genomic DNA was isolated from the ileum contents (stored at -80°C) using a DNA extraction kit (QIAamp DNA Stool Mini Kit, Qiagen, Valencia, CA, USA; Faber *et al*. 2012) according to the manufacturer's instructions. DNA extracted from the samples was quantified spectrophotometrically using Nano-Drop ND-2000 (Thermo scientific,

Wilmington, DE, USA). The transcript levels of species-specific 16S rRNA genes for *Bifidobacterium longum* (Tannock, 2002), *Lactobacillus acidophilus* (Haarman & Knol, 2006), *Streptococcus salivarius* (Furet *et al.* 2009) and *Clostridium perfringens* (Wu *et al.* 2011b) were quantified using the StepOnePlus Real Time PCR system (Applied Biosystems, Burlington, ON, Canada). The reaction mix contained 2ng of DNA, 5µl SYBR Green I master mix (Promega North America, Madison, WI, USA) and 300 nM of each gene specific primer (Appendix Table S6). Additionally, the total populations of ileal contents were quantified using a universal primer set (Haarman & Knol, 2006).

5.3.8 Gas Chromatographic Analysis of Cecal Contents

The SCFA in the cecal contents were analyzed as described by Martin *et al.* (2007). Briefly, 300 mg of cecal content (n=4) was homogenized in 2 mL of buffer, containing 0.1% (w/v) $HgCl_2$, 1% (v/v) H_3PO_4 and 0.045 mg/mL 2,2 dimethyl butyric acid, as an internal standard. The diluted slurry was centrifuged at 500*x g* for 30 min and the supernatant containing SCFA was collected for analysis (Martin *et al.* 2007). The sample (0.5 µL) was analyzed using a gas chromatograph (BRUKER 430), equipped with a flame ionization detector (FID). A DB-FFAP (Dipheny- Free Fatty Acid Phase) column (Agilent Technology; length 30 m, internal diameter 530 µm with 1 µm film thickness) was used in the analysis. A cleaning injection of 1.2% formic acid wash was run following each sample run. The initial temperatures for each sample run for the oven, injector and detector were 80°C, 180°C and 220°C, respectively. The system used helium as the carrier gas with a constant pressure of 10 psi. The SCFAs in the samples were identified using an external standard containing acetate, propionate, iso-butyrate, *n*-butyrate, iso-valerate and *n*-valerate and quantified using the internal standard. The concentration of SCFA in the samples was determined as described by Zhao *et al.* (2006).

5.3.9 Statistical Analysis

A completely randomized design, with 8 dietary treatments as the main factors, was utilized. Each cage of 5 birds was considered as an experimental unit, which was replicated 4 times, for each treatment group. The main effects of diets, bird age and the interaction between these effects were analyzed using ANOVA, with a P value < 0.05 using the Proc. Mixed procedure, of the SAS Institute, Inc. software version 9.3 (SAS

Institute, Inc, Cary, NC, USA). When significant effects of treatment were found, means were separated using Tukey analysis for the pair-wise comparison to differentiate treatment means.

5.4 Results

5.4.1 Feed efficiency and layer performance

The effects of red seaweed dietary supplementation on feed intake (FI), body weight (BW) and feed conversion ratio (FCR) are summarized in Table 5.1. No interactions between the dietary inclusion of the seaweeds and bird age on response variables were detected; hence only cumulative data are presented in Table 5.1. Dietary supplementation with CC and SG did not affect feed intake (FI) ($P > 0.05$) and average body weight of hens, during the period of the experiment. Initially, the data from eggs/cage were collected on day 0 and the production percentage was calculated, and this served as a base line to compare the effects of treatments over the experimental period. The baseline egg production was not significantly different for birds assigned to each treatment. The hen-day egg production was significantly higher ($P < 0.05$) in the birds fed with 2% SG (94.6) and 1% CC (90.1), compared to inulin (IN) and control birds (C; 85.3, 88.2 respectively). Consequently, 2% SG and 1% CC improved the feed conversion ratio (FCR; $P=0.001$) as compared to other treatments (1.97, 1.69, 1.72 for control, SG and CC respectively), whereas 1% SG and 2% CC were intermediate (1.91, 1.79 for SG and CC respectively) and not different from the control. Moreover, lower inclusion levels for dietary seaweed (0.5% SG & CC) reduced egg production (79, 72, respectively) and increased FCR 2.04, 2.13, respectively, of the birds. Additionally, inulin, an established prebiotic, used as positive control had no significant effect ($P > 0.05$) on layer hen performance and egg quality (Table 5.1).

5.4.2 Egg Quality

The effect of dietary seaweed supplementation on egg quality is presented in Table 5.1. Significant treatment effects were observed for the weights of total egg, yolk and eggshell. Supplementation of diets with 1% SG increased egg-yolk weight ($P=0.0035$

Table 5. 1. Effect of red seaweed dietary supplements on layer hen performance and egg quality

Diet[1]	Feed Intake, (g/d)	Egg production, (%)	FCR[2] (g/g)	BW[3], (kg)	Egg weight (g)	Yolk weight, (g)	Yolk color (a*[5])	Egg albumen height (mm)	Shell thickness, (mm)	Relative Shell weight (%)
C	109.98a	88.21abc	1.97abc	2.06a	63.43b	17.27b	11.65a	5.84a	0.40a	9.14a
IN	107.79a	85.35abc	1.98abc	2.02a	63.44b	17.38b	12.05a	5.77a	0.39a	9.31a
SG0.5	106.48a	79.64cd	2.04ab	2.12a	65.44ab	17.91ab	12.10a	6.09a	0.39a	9.70a
SG1	103.37a	81.96bcd	1.91abcd	2.04a	65.63ab	18.72a	12.20a	5.92a	0.39a	9.56a
SG2	105.16a	94.64a	1.69d	2.12a	65.11ab	17.66ab	12.20a	5.81a	0.40a	9.38a
CC0.5	104.32a	72.85d	2.17a	2.13a	65.20ab	17.63ab	11.85a	5.62a	0.41a	9.62a
CC1	105.16a	90.17ab	1.72cd	2.16a	66.82a	18.48ab	11.65a	6.04a	0.40a	9.42a
CC2	103.55a	86.07abc	1.79d	2.21a	65.21ab	17.80ab	11.45a	5.85a	0.41a	9.43a
SEM[4]	1.68	2.13	0.05	0.66	0.63	0.27	0.23	0.18	0.00	0.14
Interactions, *P-value*										
Diets	0.11	<.001	<.001	0.43	0.006	0.0035	0.2091	0.7124	0.2769	0.1666
Weeks	<.0001	0.79	0.0004	0.67	<.0001	0.0116	0.2098	0.0043	0.3298	0.0233
Weeks X Diets	0.99	0.66	0.5763	0.59	0.0860	0.2488	0.8013	0.5867	0.1579	0.4606

[1]Values are means for each treatment (Trt) group (C: control; IN: inulin; SG0.5: contains 0.5% of *Sarcodiotheca* variant; SG1: contains 1% of *Sarcodiotheca* variant; SG2: contains 2% of *Sarcodiotheca* variant; CC0.5: contains 0.5% of *Chondrus crispus*; CC1: contains 1% of *Chondrus crispus*; CC2: contains 2% of *Chondrus crispus*). Values with different superscript letters are significantly different ($p < 0.05$).

[2]FCR = feed conversion ratio. [3]BW=Body weight

[4]Standard error of the mean (4 replicates of 5 hens each per treatment). [5]Measurement of Redness-greenness

and total egg weight (*P=0.0006*), as compared to control diets; however, 0.5 and 2% SG supplementation was not different from the control. Additionally, total egg (*P=0.006*) and eggshell weights (*P=0.0006*) were higher in birds fed with 1% CC than in 0.5% and 2% CC treatments. Egg albumen height, yolk colour and shell thickness were not affected by dietary supplementation with either seaweed. There were no interactions between the dietary inclusion of seaweeds and bird age on yolk weight, eggshell thickness and weight.

5.4.3 Moisture Content

Dietary supplementation with the selected red seaweeds did not affect (*P=0.36*) the moisture content of excreta on day 14 and 25 (Table 5.2). A 2-way interaction between excreta collection days and dietary treatments was not significantly different (*P=0.59)*. However, the total moisture content of the excreta collected on day 25 was higher (78.43, *P=0.003*), as compared to day 15 (75.27). This difference could be attributed to the age of bird and abiotic factors such as temperature and humidity.

5.4.4 Histomorphological Analysis

The mean values of villus height, width, area and depths of crypt and mucosa from distal ileal samples are shown in Table 5.3. The average villus height increased (*P=0.004*) in birds fed 2% SG and CC seaweed. Crypt depths were deeper for 2% CC supplementation than other treatments (*P=0.046).* Additionally, dietary inclusions of 2% CC and SG also increased the villus width (*P=0.03)* and area (*P=0.002),* as compared to the control birds. The positive control inulin showed higher values for villus height, width, area and depth of crypt and mucosa than the control, but was not significantly different (*P >0.05*).

5.4.5 Blood Serum Profile

Serum sodium levels were reduced (*P=0.03)* in laying hens fed 2% dietary SG and CC (149, 148.7 mEq/L, respectively), as compared with the control group (156 Eq/L) fed the basal layer diet. There was no significant effect due to feed supplementation of seaweed meal on blood serum concentrations of phosphorus, chlorine, calcium, total

protein, potassium, glucose, creatine kinase, aspartate amino transferase levels and uric acid levels (Table 5.4).

Table 5. 3. Effect of red seaweed dietary supplements on histomorphological parameters of the ileocaecal junction section in layer hens

Diet	Villus height (µm)	Villus Width (µm)	Crypt depth (µm)	Mucosal Depth (µm)	VSA[1] (mm^2)
C	658.82^{b}	149.16^{b}	159.86^{b}	490.75^{b}	1.02^{b}
IN	756.63^{ab}	157.42^{ab}	185.85^{ab}	517.34^{ab}	1.47^{ab}
SG0.5	735.83^{ab}	179.50^{ab}	241.20^{ab}	476.21^{b}	1.56^{ab}
SG1	848.72^{ab}	181.56^{ab}	245.29^{ab}	590.04^{ab}	1.72^{a}
SG2	896.82^{a}	190.67^{ab}	277.63^{ab}	593.94^{ab}	1.76^{a}
CC0.5	801.22^{ab}	174.68^{ab}	293.35^{ab}	706.50^{a}	1.69^{ab}
CC1	744.92^{ab}	221.03^{a}	264.59^{ab}	663.07^{ab}	1.41^{a}
CC2	909.47^{a}	204.40^{ab}	328.80^{a}	636.24^{ab}	2.00^{a}
SEM[2]	35.58	13.08	26.57	38.10	0.13
***P*-value**	0.0044	0.0317	0.0468	0.0143	0.0023

[1]Values are means for each treatment (Trt) group (C: control; IN: inulin; SG0.5: contains 0.5% of *Sarcodiotheca* variant; SG1: contains 1% of *Sarcodiotheca* variant; SG2: contains 2% of *Sarcodiotheca* variant; CC0.5: contains 0.5% of *Chondrus crispus*; CC1: contains 1% of *Chondrus crispus*; CC2: contains 2% of *Chondrus crispus*.
[1]VSA= villus surface area , [2]Standard error of the mean (4 replicates per treatment)
Values with different superscript letters are significantly different ($p < 0.05$).

Table 5. 2. Effect of red seaweed dietary inclusion levels on excreta moisture content (%) of layer chickens[1]

Days	C	IN	SG0.5	SG1	SG2	CC0.5	CC1	CC2	SEM	Interactions, *P-value*		
										Diets	Days	Days X Diets
14	75.68[a]	71.47[a]	76.58[a]	72.57[a]	74.29[a]	78.15[a]	78.38[a]	75.07[a]	1.86	0.36	0.003	0.59
25	78.26[a]	77.27[a]	80.13[a]	78.88[a]	78.80[a]	78.38[a]	78.32[a]	77.44[a]				

[1]Values are means for each treatment (Trt) group (C: control; IN: inulin; SG0.5: contains 0.5% of *Sarcodiotheca* variant; SG1: contains 1% of *Sarcodiotheca* variant; SG2: contains 2% of *Sarcodiotheca* variant; CC0.5: contains 0.5% of *Chondrus crispus*; CC1: contains 1% of *Chondrus crispus*; CC2: contains 2% of *Chondrus crispus*). Means represent 3 excreta samples per treatment. Excreta were collected from 24 cages (5 hens/cage), sampling was done three times per cage and the total fecal samples were pooled to represent one sample unit/cage.
Values with different superscript letters are significantly different ($p < 0.05$).

5.4.6 Organ Weight

Dietary supplementation of feed with selected red seaweeds increased (*P<0.0001)* the weight of ceca, as compared to controls (Table 5.5). The cecal weights increased (*P<0.001)* in birds fed with 2% CC (13.10 g) and 2% SG (12.48 g), as compared with the control groups (7.15 g). There were no significant effects on liver, spleen, ileum or heart weights (Table 5.5)

Table 5. 5. Effects of red seaweed dietary treatments on organ weights relative to BW/Kg in layer hens (g)[1]

Organs[2]	**Spleen**	**Cecum**	**Heart**	**Ileum**	**Liver**
C	0.87^{a}	7.15^{bc}	4.90^{a}	14.84^{a}	8.75^{a}
IN	0.67^{a}	7.63^{bc}	5.71^{a}	13.24^{a}	8.13^{a}
SG0.5	0.87^{a}	6.15^{c}	5.68^{a}	13.44^{a}	7.68^{a}
SG1	0.92^{a}	7.64^{bc}	5.07^{a}	15.16^{a}	8.08^{a}
SG2	0.75^{a}	12.48^{a}	5.68^{a}	16.48^{a}	9.45^{a}
CC0.5	0.94^{a}	9.31^{abc}	5.55^{a}	14.84^{a}	9.44^{a}
CC1	0.94^{a}	10.43^{ab}	4.64^{a}	14.86^{a}	7.88^{a}
CC2	0.79^{a}	13.10^{a}	5.35^{a}	15.10^{a}	7.84^{a}
SEM	0.09	0.73	0.30	0.74	0.74
***P*-value**	0.4750	<. 0001	0.2169	0.1816	0.6736

[1]Values are means for each treatment group (IN: inulin; SG0.5: contained 0.5% of *Sarcodiotheca gaudichaudii*; SG1: contained 1% of *S. gaudichaudii*; SG2: contained 2% of *S. gaudichaudii*; CC0.5: contained 0.5% of *Chondrus crispus*; CC1: contained 1% *of C. crispus*; CC2: contained 2% of *C. crispus*).
[2]The values are reported as least squares means (n = number of birds, n = 4/treatment) Values with different superscript letters are significantly different ($p < 0.05$).

Table 5. 4. Effect of red seaweed dietary supplements on blood serum parameters of laying hens[1]

Parameters	C	IN	SG0.5	SG1	SG2	CC0.5	CC1	CC2	SEM[2]	*P*-value
Sodium (mEq/L)	156.4	151.2	150.0	155.2	149.1	151.2	150.5	148.7	1.57	0.0359
Potassium (mEq/L)	6.2	6.1	5.7	6.6	6.1	6.0	6.4	6.4	0.26	0.5806
Na: K Ratio	25.5	24.5	26.5	23.5	24.5	25	23.5	23.2	0.97	0.4227
Chlorine (mg/dL)	118.5	116.2	114.2	117.2	114.5	115.0	115.7	115.0	1.44	0.5194
Calcium (mg/dL)	25.7	25.6	29.4	24.9	32.0	28.9	28.1	28.2	1.94	0.3237
Phosphorus (mg/dL)	4.8	4.8	5.0	4.3	5.2	5.5	4.6	5.0	0.31	0.2643
Glucose (mg/dL)	259.4	259.0	244.1	244.1	239.6	261.2	235.5	234.6	7.67	0.1061
CK[3] (U/lit)	1697.7	1992.0	1774.5	2252.2	2039.5	2111.5	2730.2	1742.2	219.00	0.1315
AST [GOT][4] (U/lit)	176.0	214.5	224.1	174.2	180.7	183.5	196.7	158.5	23.07	0.7969
T.Protein (mg/dl)	4900.0	4850.0	5350.0	5450.0	5275.0	5466.0	5175.0	5050.0	170.66	0.1214
Albumin (g/dl)	1.6	1.5	1.8	1.5	1.7	1.9	1.7	1.6	0.08	0.0852
Uric acid (mg/dl)	7.6	6.8	5.3	7.4	6.6	5.9	7.1	6.6	0.70	0.4976

[1]n = 4 for each parameter mean,
[2]S.E.M. based on pooled estimate of variance
[3]CK= Creatine kinase, [4]AST [GOT]= Aspartate aminotransferase

5.4.7 Ileal Microbial Analysis

Red seaweed supplementation altered the relative abundance of beneficial bacteria; *Bifidobacterium longum, Lactobacillus acidophilus, Streptococcus salivarius* and the pathogenic bacteria, *Clostridium perfringens* in the ileal contents of the chicken (Figure 5.1). The abundance of *Bifidobacterium longum* increased (*P<0.001)* by 14 and 9 fold in 1% and 2% SG treatments, respectively and 4 fold in the 1% CC supplementation. Additionally, the relative abundance of *Streptococcus salivarius* was 15 fold higher (*P<0.001)* in 1% CC and 4 fold higher in the 2% CC and SG dietary treatments. Interestingly, all treatments including the positive inulin control decreased the prevalence of *Clostridium perfringens* compared to the negative control (*P<0.001)*. The abundance of *L. acidophilus* increased (*P<0.001*) by 4 fold in the inulin treatment and dereased in the CC treatments.

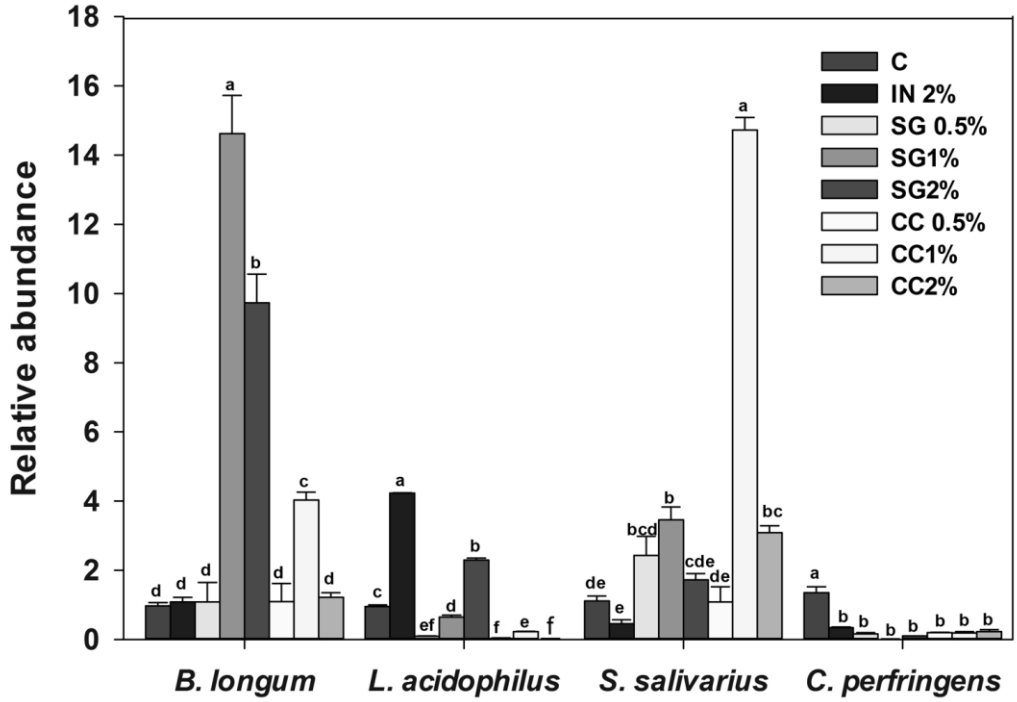

Figure 5.1. Real time PCR quantification of microbial population in the ileal contents of layer hens.
Relative abundance of *Bifidobacterium longum, Lactobacillus acidophilus, Streptococcus salivarius* and *Clostridium perfringens* in the chicken ileum. Data represent the mean of measurements from two individual chicken ileum contents. Error bars indicate standard error and different letters indicate statistically significant mean values (*P<0.001)*.

5.4.8 Short Chain Fatty Acid (SCFA) Analysis

Dietary supplementation with selected, cultivated red seaweeds affected theconcentration of some SCFA digesta contents (Figure 5.2). The concentration of acetic acid was greater (*P=0.02)* in the cecal digesta of those birds fed 1% SG and CC (52.21, 51.53 mmol/kg respectively), as compared with the basal diets 29.94 mmol/kg) or inulin (36.11 mmol/kg). Also, the concentrations of propionic acid, *n*-butyric acid and *i*-butyric acid increased by 2-3 fold in SG and CC treatments (*P=0.01, P=0.01, P=0.04* respectively). No significant differences in the concentration of *n*-valeric and *i*-valeric acids were observed in any of the treatments (Figure 5.2).

5.5 Discussion

Supplementation of layer feed with two red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, resulted in a significant improvement in layer performance and an increase in the population of beneficial bacteria with a decrease in the abundance of pathogenic *Clostridium perfringens* in the gut (Table 5.1, Figure 5.1). The role of gut microbiota in animal health is well documented. The inclusion of an oligosaccharide-rich diet, like seaweeds, in animal feed improved growth performance, immune status, and gut microbiota (Strand *et al.* 1998). The changes in gut microbiota are largely caused by complex polysaccharides in the feed that are resistant to acid hydrolysis in the upper gastrointestinal tract of higher animals (O'Sullivan *et al.* 2010); many of these polysaccharides are then fermented in the lower gastrointestinal tract by beneficial bacteria.

In the present study, addition of red seaweeds in the feed did not affect feed intake in layer hens, and similar results were observed in other studies in which the feed was supplemented with brown seaweeds, *Macrocystis pyrifera*, *Sargassum sinicola*, and a green seaweed *Enteromorpha* sp. (Carrillo *et al.* 2008). In our study, although the feed intake and the egg production were not significantly different in the treatments, close examination of the data revealed that red seaweed treatments (2% CC and SG) significantly improved FCR (Table 5.1) and an increase in egg and yolk weight in CC1

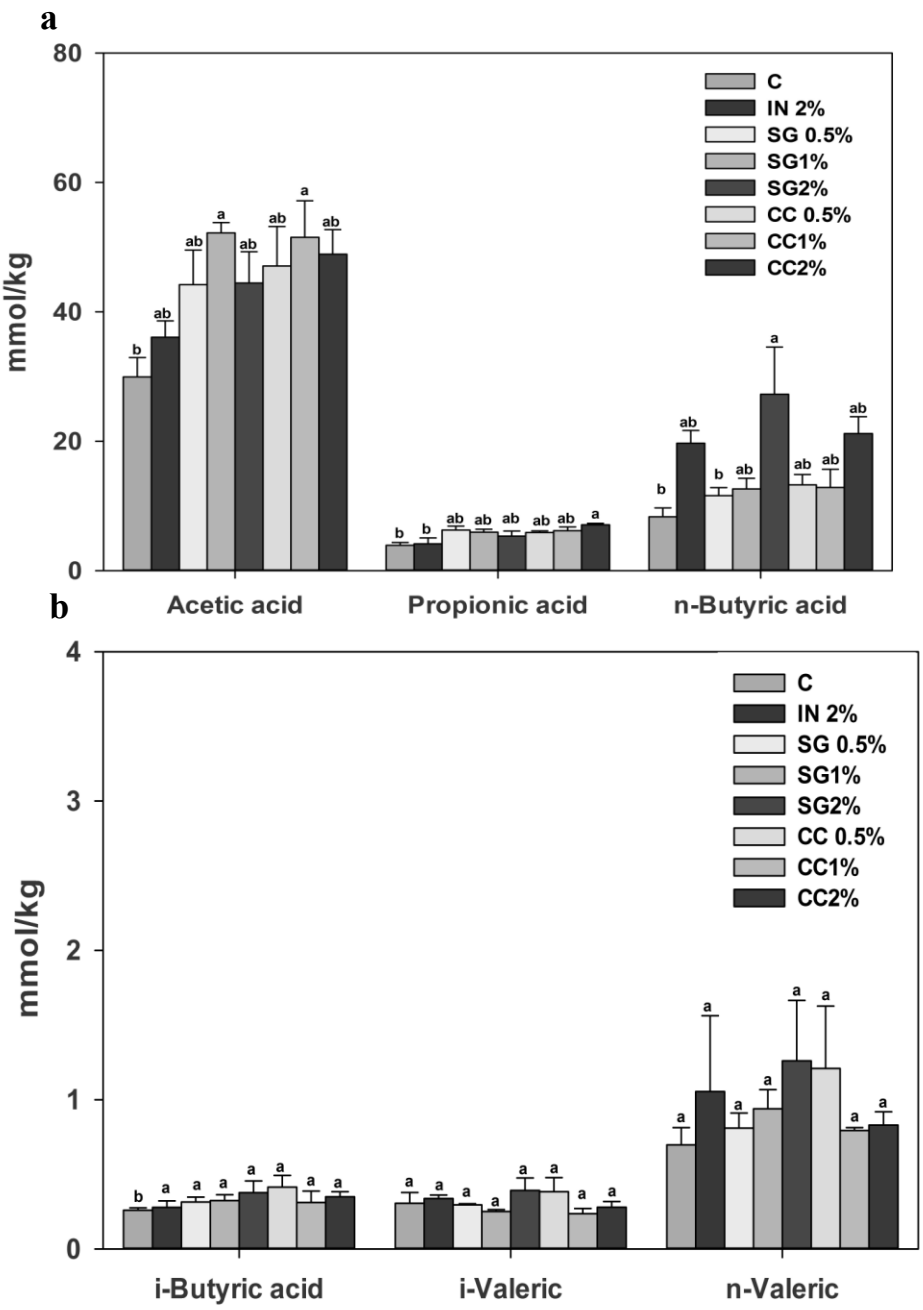

Figure 5. 2. Concentration of short chain fatty acids (mmol/kg) in the ceca digesta of layer hens.
a) Acetic, propionic and n-butyric acid b) i-Butyric acid, i-Valeric acid and n-Valeric acid. Data is representive of mean measurements from four individual chicken ceca digesta Error bars indicate standard error and different letters indicate statistically significant mean values.

and SG1 treaatments. Previous work with mannanoligo-saccharide or the red microalgae *Porphyridium* sp. did not result in an increase in FCR (Ginzberg *et al.* 2000, Bozkurt *et al.* 2012). This variation may be attributed to the age or strain (or both) of birds used in the experiment and also the type of seaweed used and inclusion rates, as well as the differences in the experimental conditions such as temperature and humidity (Geier *et al.* 2009, Zhang *et al.* 2012). The increase in egg and yolk weight with 1% level of seaweeds (1% CC and SG) might be attributed to higher yolk protein synthesis and an increase in water and mineral availability (Novak *et al.* 2004), and greater retention of feed in the gastrointestinal tract and thus a better ileal digestibility of the feed (Piray *et al.* 2007). Several reports indicated that dietary inclusion of seaweed improved egg albumen height and egg yolk color (Herber-McNeill & Van Elswyk, 1998, Ginzberg *et al.* 2000, Carrillo *et al.* 2008, Zahroojian *et al.* 2011, Carrillo *et al.* 2012).

Chondrus crispus or *S. gaudichaudii*-supplemented feed had no effect on the weight of the spleen, heart, ileum, or liver of birds. However, a significant increase in the weight of ceca was observed. An increase in the weight of the ceca correlated with an increase in the population of beneficial bacteria in the ceca. Cecal weight is an indicator of fermentation activity in the ceca (Oyarzabal & Conner, 1996). A significant increase in cecal weight was observed in broilers fed with direct-fed microbial and fructo-oligosaccharide, and a subsequent reduction in *Salmonella* infection was observed (Oyarzabal and Conner, 1996).

Seaweed-supplemented feed induced changes in the histomorphology of the small intestine; there was a significant increase in villi height and crypt depth (Table 5.3). The intestine is the major site of enzymatic digestion, and absorption of nutrients, efficiency of absorption, and hence FCR largely depends on the histomorphology of the intestine (Awad *et al.* 2008). Previous studies have shown that the intestinal histomorphology, specifically structures such as villi, crypts, and the thickness of mucosa, were altered by the composition of the diet (Samanya & Yamauchi, 2002, Nain *et al.* 2012). The increase in villi height and crypt depth is associated with healthy turnover of epithelial cell and active cell mitosis (Fan *et al.* 1997, Samanya & Yamauchi, 2002). There is evidence

suggesting a healthy gut microbiota aids healthy turnover of intestinal epithelial cells and reduce inflammation caused by pathogens and toxins (Baurhoo *et al.* 2007).

In this study, the relative abundance of probiotic bacteria *Bifidobacterium longum* and *Streptococcus salivarius* were higher in birds fed with 1% CC, 1% SG, and 2% SG. This result was similar to a previous report on the effect of dietary mannan oligosaccharides, galacto-glucomannan oligosaccharide-arabinoxylan, and dried whey in layer and broiler chickens (Baurhoo *et al.* 2007, Donalson *et al.* 2008, Faber *et al.* 2012). Beneficial microorganisms suppress the growth of pathogens by mechanisms such as competitive exclusion, secretion of SCFA, and antimicrobial peptides as well by priming the host immune system (Torok *et al.* 2008). Interestingly, a negative correlation was observed between *Bifidobacterium* sp. and *Clostridium perfringens*, suggesting *Bifidobacterium* sp. was a suppressor of *C. perfringens* (Gibson *et al.* 2005). Taken together, the dietary red seaweeds *C. crispus* and *S. gaudichaudii* increased epithelial cell turnover by altering the gut microbiota in favor of the beneficial bacteria. These results suggested that dietary supplementation with seaweeds may increase the population of probiotic bacteria and at the same time reduce the abundance of harmful enteric bacteria.

A change in the gut microbiota affects microbial SCFA. Short-chain fatty acids are major end products of microbial fermentation, which stimulate gut fluid absorption and also have proliferative effects on colonocytes (Mountzouris *et al.* 2007). The concentration of SCFA in the intestine largely depends on the fermentative substrate and microbial diversity in the gut (Cummings & MacFarlane, 1991). Seaweed in the feed significantly increased the concentration of acetate and propionate in the cecum (Gomez-Ordonez *et al.* 2012). Similarly, Deville *et al.* (2007) reported that laminarin, a brown seaweed polysaccharide, increased mucosal absorption and butyrate production. Our results on the concentration of SCFA are in agreement with these published studies. Acetic acid is primarily produced by cellulose fermentation by *Bacteriodes* and *Bifidobacterium* (Apajalahti *et al.* 2001). Thus, the higher abundance of *Bifidobacterium longum* in birds fed with seaweed might have contributed to the increased concentration of acetic acid. The higher concentrations of acetate, butyrate, and propionate observed in our study were also consistent with previous findings in layer and

broiler chickens (Apajalahti *et al.* 2001, Donalson *et al.* 2008, Rehman *et al.* 2008, Faber *et al.* 2012). Butyric acid acts as an energy substrate for epithelial growth and regulates cellular differentiation, growth, permeability, and gene expression. Moreover, SCFA lower the ceca pH resulting in reduction in the growth of pathogens such as *Salmonella* (Hinton *et al.* 1990). Faber et al. (2012) correlated the concentration of cecal SCFA to the increase in cecal weight. These authors suggested that SCFA acted as the main energy source for intestinal epithelial cells and stimulated cell growth, resulting in an increase in intestinal weight (Frankel *et al.* 1994, Faber *et al.* 2012). Thus, in our study, it is possible that the higher concentration of SCFA could have resulted in higher cecal weight.

In conclusion, supplementation of layer feed with the red seaweeds *Chondrus crispus* and *Sarcodiotheca gaudichaudii* improved the performance of layers possibly as a result of an increase in the population of beneficial bacteria and a reduction of pathogenic bacteria in the gut, improvement in villi height, crypt depth, and an increase in the concentration of SCFA. Thus, the cultivated strain of *C. crispus* and *S. gaudichaudii* could be used as a prebiotic for layer hens

Connection Statement between Chapter 5 and 6

In vitro studies showed that SWE was effective in reducing *S*. Enteritidis growth and its virulence factors. Moreover, *in vivo* studies using *C.elegans* model organisms indicated that SWE can protect worms from *S.* Enteritidis infection by either reducing the virulence of pathogen or increasing the immune responsive genes (Chapter 3). Furthermore, whole seaweed fed as dietary inclusion increased the growth and productivity and improved the overall health of layer hens. This was due to the effect of red seaweeds on the abundance of gut microbiota (Chapter 5). Since red seaweeds (CC and SG) reduced *S.* Enteritidis colonization in *C. elegans* and improved the health of layer hens as a prebiotics, we hypothesize that CC and SG can protect layer hens challenged with *S.* Enteritidis. The following study shows that, CC (4%) was effective in reducing *S.* Enteritidis colonization in feces and ceca. Birds fed on CC (4%) had higher abundance of *Lactobacillus acidophilus* and increased production of SCFA and immunoglobulin IgA. Since chickens are main reservoir of *S*. Enteritidis, this study suggests an effective alternative to control spread of *S.* Enteritidis in humans. Additionally, it is ideal for producers including organic farmers, without concerns for toxicity; it is likely to be accepted as a natural feed additive.

Chapter 6. Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii,* reduce *Salmonella* Enteritidis in layer hens

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6.1 Abstract

Salmonella Enteritidis (SE) is vertically transmitted to the egg in laying hens through infected ovaries and oviduct. *S.* Enteritidis can also penetrate the eggshell from contaminated feces when eggs pass through the cloaca. Reducing *S.* Enteritidis in laying hens is vital to provide safer eggs and minimize the spread of human salmonellosis. Antibiotics have been widely used to control bacterial diseases in broilers and laying hens. However, there is a major concern regarding the use of antibiotics due to the development of antibiotic resistance and their adverse effect on the bird's gut microflora. Thus, there has been an interest in developing alternatives to antibiotics such as the inclusion of prebiotics in the feed. In the present study the effect of feed supplemented with red seaweeds; *Chondrus crispus* (CC) and *Sarcodiotheca gaudichaudii* (SG) on the *S.* Enteritidis was examined in late phase layer hens. The treatment consisted of diet containing one of the following; 2% and 4% *Chondrus crispus* (CC2, and CC4, respectively) or *Sarcodiotheca gaudichaudii* (SG2 and SG4), and aureomycin was used as the positive control. At mid week-4, 48 birds were orally challenged with 2×10^9 CFU/mL of *S.* Enteritidis. Eggs and fecal samples were collected 1, 3, 5 and 7 days post inoculation. Birds were euthanized and organs (ceca, ovary, liver and spleen) were screened for the presence of *S.* Enteritidis, 7 days after inoculation. Results showed that seaweed reduced the negative effect on growth and egg production of *S.* Enteritidis challenged layer hens. CC4 also significantly reduced *S.* Enteritidis in the fecal samples. Fecal samples from aureomycin and CC4-supplemented birds tested negative for *S.* Enteritidis on day 5 and 7 days post inoculation (lowest detection limit $=10^{-1}$). Moreover, *S.* Enteritidis colonization in the ceca was also significantly reduced in birds fed the diet supplemented with CC (4%) and aureomycin. Blood serum profiles revealed that there were no significant differences in serum aspartate transaminase (AST) and sodium. However, the level of serum immunoglobulin (IgA) was higher in CC4 treatment. Moreover, the relative abundance of *Lactobacillus acidophilus* was significantly higher in the CC4, while the abundance of the pathogens: *Clostridium perfringens* and *Salmonella* Enteritidis were reduced compared to the control. Results indicate that feed supplemented with 4% CC is

effective in providing protection against *Salmonella* Enteritidis colonization of laying hens.

6.2 Introduction

Salmonella enterica serovar Enteritidis is a major cause of egg-associated salmonellosis in humans (Guard-Petter, 2001). Despite the ongoing implementation of several control and prevention measures, the number of *S.* Enteritidis cases has increased since 2005, making it the most common serotype responsible for *Salmonella* infections in Canada (NESP) (CDC, 2012). Major concerns associated with *Salmonella* infection in poultry include a loss in productivity, higher mortality and contamination of egg-associated poultry products consumed by humans (Chai *et al.* 2012). Therefore, due to the adverse effect on both the economy and human health, the safety of poultry products is priority for the government, poultry producers as well as consumers. Chicken serves as reservoir host for *S.* Enteritidis infection, and is carried into the human food chain through consumption of contaminated, raw, or undercooked poultry products (Guard-Petter, 2001). Previous epidemiological research has confirmed the relation between human salmonellosis and consumption of poultry products (Center for Disease Control and Prevention, CDC, 2012 and FDA, 2009)

In poultry, *Salmonella* can enter the chicken through the orofecal route and colonize the gastrointestinal tract. This leads to shedding of *Salmonella* in the feces for several weeks without any detectable clinical symptoms in the infected bird. Over the past 50 years, antibiotics have been used as growth promoters to enhance performance and to prevent disease (Barrow *et al.* 1987, Berg, 1995). However, sub-therapeutic use of antibiotics in poultry has been under scientific and public scrutiny. This is because the use of antibiotics in poultry has been linked to the increase in antibiotic resistant strains of bacteria, which pose a serious threat to the effective treatment of bacterial infections and human diseases. Henceforth, there is an increasing interest in poultry producers finding alternatives to antibiotics (Baurhoo *et al.* 2007).

The immune system of birds provides innate protection against pathogen infection. The first line of defence against *S.* Enteritidis infection is provided by the mucosal immune system of the intestine including mucosal immunoglobulin A (IgA) and mucosa associated lymphocytes and leukocytes. Systemic immune responses are required for the resistance and clearance of *S.* Enteritidis infection. Currently available vaccines induce specific immune responses in birds and protect them against pathogens. However, the development of vaccine as a strategy to control *S.* Enteritidis infection in birds has some disadvantages. Vaccines interfere with the detection of *Salmonella* by bacteriological and serological methods. Other concerns with the live vaccines include development of antimicrobial resistance and dissemination of *S.* Enteritidis in the environment (Baggesen, 2006). Therefore a natural alternative that boosts immune response in birds would be useful to combat pathogen infections. Previous studies have shown that seaweeds prime the immune system in mammals and birds (Choi *et al.* 2014). Moreover, beneficial gut microbes such as *Lactobacillus* sp. and *Bifidobacterium* sp. selectively ferment polysaccharides of seaweeds (Hu *et al.* 2006). Red seaweeds have been shown to modulate the immune response and micro flora (Chapter 5) (Kulshreshtha *et al.* 2014). However no studies have investigated their application in birds challenged with pathogens.

Several natural alternatives such as marine products, organic acids, microflora enhancers, probiotics, prebiotics, and herbal products have been researched as feed additives for poultry (Venkitanarayanan *et al.* 2013). Among these, prebiotics can be a favorable alternative because they have been shown to selectively increase the growth of beneficial microbes and inhibit pathogen colonization by competitive exclusion. Moreover, prebiotics such as oligosaccharides (mannan-oligosaccharides, galacto-oligosaccharides, fructo-oligosaccharides) in poultry feed have been shown to enhance the immune system of birds (O'Sullivan *et al.* 2010). Among various health enhancing prebiotics, seaweeds have gained interest as an alternate feed additive in poultry (Richmond, 2004). Recent studies have shown that dietary seaweed stimulates the health and productivity of birds by increasing the beneficial gut bacteria and enhancing the host

innate immune system (Abudabos *et al.* 2013; Evans & Critchley, 2014).

In the present study, layer hens challenged with *S.* Enteritidis were used to determine the efficacy of red seaweeds, *C. crispus* or *S. gaudichaudii*. We investigated the effect of two red seaweed species on the growth performance, egg production, cecal microbiota, short chain fatty acids and serum IgA production. A basal layer diet and antibiotic, aureomycin served as negative and positive controls, respectively

6.3 Materials and Methods

6.3.1 Birds and housing

A total of 96 commercial laying hens (white leghorns) at 78 weeks of age were used in this study. Birds were randomly housed in 96 wire cages and feed access to feed and water *ad libitum*. Birds were confined in an environmentally controlled room set at 16 h of light per day and the temperature was 25°C. All experimental procedures were carried out in accordance with Canadian Council of Animal Care guidelines and University of Montreal Animal Care and Use Committee guidelines (CCAC, 2009).

6.3.2 Preparation of seaweed supplemented feed and experimental design

Red seaweeds {*Chondrus crispus* (CC) and *Sarcodiotheca spp.* (SG)} used in this study were a kind gift from Acadian Seaplants Limited, Dartmouth, NS, Canada and aureomycin (chlortetracycline) was purchased from Alpharma Canada, Mississauga, ON, Canada. Two-dried seaweeds (SG & CC) were incorporated at 2% and 4% (w/w) of the total layer diet (Appendix Table S7). A basal layer diet was used as control and aureomycin was added to the basal layer diet as the positive control. There were 6 dietary treatment combinations with 16 cages per treatment, and the experimental unit was a cage/bird.

6.3.3 Layer performance and egg quality

All eggs laid were collected daily and eggs produced per cage were recorded throughout the experiment. Body weights (bird/ cage) and feed consumption were determined in week 1, 2, 3, and 4. Feed was weighed each day before adding into the

feeders and feed consumption was calculated by weighing back the feeders at the end of the time period. The data were used to calculate feed intake and egg production percentage (hen per day egg production)

6.3.4 *Salmonella* Enteritidis Challenge

Salmonella Enteritidis field strain isolated from a clinical case of salmonellosis in layer hens was obtained from the Faculty of Veterinary Medicine collection center, University of Montreal. *S.* Enteritidis was cultured in LB broth overnight at 37°C, shaking at 150 rpm. To determine the number of colony-forming units, the inoculum was diluted and plated on XLD agar for 24 h at 37°C. During week-4 post feeding, one half of the layer hens was each orally gavaged with 2 mL of the *Salmonella* culture suspended in PBS at a concentration of 10^9 cfu/mL, and the non-challenged groups were mock inoculated with sterile PBS. The infection protocol was based on results obtained from previous trials in the laboratory.

6.3.5 Colonization of *Salmonella* Enteritidis in the excreta samples

On day 1, 3, 5, and 7 post challenge with *S.* Enteritidis, three discrete samples of bird excreta were collected with a sterile spatula from all the cages. Fecal samples from two cages were pooled to represent one sample unit. Care was taken to avoid feed spillage and bird feathers from being picked along with the excreta. Each collection was weighed and placed in a zip lock bag. *S.* Enteritidis in the fecal samples was determined by blending 1 mL of pooled sample into 10 mL of sterile solution of 0.1% peptone in water in a sterile bag. The homogenates obtained were serially diluted in peptone water and plated on Xylose Lysine Deoxycholate (XLD) agar plates for the enumeration of *S.* Enteritidis. Plates were incubated for 24-48 h at 37°C and observed for the presence of *Salmonella* colonies which appeared as black on the selective medium (Goodnough and Johnson, 1991). Suspected colonies were confirmed by biochemical tests and PCR analysis.

6.3.6 Colonization of *Salmonella* Enteritidis in egg yolk

Eggs were collected daily post inoculation to test the presence of *S.* Enteritidis in the yolk. Each egg was broken aseptically and the yolk samples were placed in a sterile

Nasco Whirl-Pak (WP, Nasco, Fort Atkinson, WI) bag containing 50 mL of Hajna tetrathionate (HT) broth (BD, Franklin Lakes, NJ, USA) for 10 mins (Miyamoto *et al.* 1997). The bags were homogenized and cultured at 37 °C for 24 h. After incubation, a loopful of broth culture was spread onto mannitol lysine crystal violet brilliant green (MLCB) agar plate to detect the presence of *S.* Enteritidis. Dark colonies 3-5 mm in diameter characterized by a convex surface with a dark black center were counted as *S.* Enteritidis positives.

6.3.7 Colonization of *Salmonella* Enteritidis in the ceca samples

All birds were euthanized by carbon dioxide gas and organs (ceca, spleen, liver and ovary) were removed from each bird and placed in sterile WP bag. Spleen and ovary samples were sent to Diagnostic Services, Faculty of Medicine, University of Montreal to test for the presence of *S.* Enteritidis. The *S.* Enteritidis numbers in the ceca were enumerated by the method described by Woodword *et al.* 2005 with some modifications (Woodward *et al.* 2005). Briefly, the contents of the ceca were expelled by finger pressure into separate collection tubes and the empty segments were collected aseptically into preweighed 15 mL sterile plastic tubes. The ceca segments were weighed and homogenized by diluting in peptone water to an initial 10^{-1} dilution. The homogenates in all treatments were serially diluted in peptone water and plated on XLD agar plates for the enumeration of *S.* Enteritidis. Suspect colonies were confirmed by biochemical tests and PCR analysis.

6.3.8 Collection of blood samples and chemical analysis

Blood samples were collected from the brachial vein of birds on day 7 post *S.* Enteritidis challenge in Vacutainer tubes containing sodium heparin to determine the serum component (Lan *et al.* 2005). Approximately 8 mL of blood was collected from all the birds, using an 18-gauge needle. Blood sera was isolated by centrifuging the tubes at 3,000 × *g* for 10 min at 4°C. The separated serum was transferred into individual vials and stored at -20°C for chemical analysis. Whole blood samples were used to determine the WBC and RBC count. The whole blood and serum analyses were carried out at Diagnostic Services, Faculty of Medicine, University of Montreal.

The immunoglobulins (IgA) in serum were measured using the Chicken IgA ELISA kit (Bethyl Laboratories, Inc, Cedarlane, Hornby, ON, Canada). Serum IgA present in the sample was captured by anti-chicken IgA antibody pre-absorbed on the surface of microtiter wells. The quantity of IgA analyte present in the sample was proportional to the absorbance at 450 nm.

6.3.9 Analysis of intestinal microbiota

The population of microbes within the gut was determined by qPCR based on a method as described by Middelbos *et al.* (2007) with minor modifications. Detailed methodology is explained in section 5.3.8. Additionally, DNA samples were sent for next generation sequencing analysis to McGill University and Genome Quebec Innovation Centre, Montreal, Quebec.

6.3.10 Gas Chromatographic Analysis of Cecal Contents

The SCFA in the cecal contents were analyzed, as explained previously in section 5.3.9.

6.3.11 Statistical Analysis

Pairwise comparison was used to determine differences among treatment groups for incidences of *S.* Enteritidis colonization of the ceca, spleen, and ovary. Log and square-root transformation was applied to the non-homogenous data before analysis. The main effects of diets, bird age and the interaction between these effects were analyzed using ANOVA and repeated measures ANOVA, with a P value < 0.05 using the Proc. Mixed procedure, of the SAS Institute, Inc. software version 9.3 (SAS Institute, Inc, Cary, NC, USA). When significant effects of treatment were found, means were separated using Tukey analysis for differentiation. The standard error of each mean (SEM) was reported with the mean value. Differences were considered significant when P was <0.05.

6.4 Results

6.4.1 Red seaweed diets maintained body weight and laying activity of hens

The effects of red seaweed dietary supplements on feed intake (FI), body weight (BW)

and egg production are summarized in Table 6.1. Average feed intake and body weight of each treatment group was similar at the beginning of the trial but significantly diverged (*P <0.0001*) at the end of the trial. No interactions between seaweed inclusion and bird age on response variables were detected until week 3 of trial. However, there were significant treatment effects on BW and FI at week 4 upon inoculation of the birds with *Salmonella* Enteritidis (SE). Post infection with *S.* Enteritidis, the seaweed (SG and CC) supplemented birds maintained higher (*P <0.0001*) body weight (1486-1526 g) than control birds (1245 g). Additionally, birds fed with aureomycin, also had higher body weight (1554 g) compared to the control. Similarly, the FI of birds fed on seaweed diets was not significantly different from the control until week 3. At week 4, birds supplemented with seaweed and aureomycin showed an increase in feed intake (102.2-107.6 g/day) compared to the control birds (74.7 g/day). Feed intake of birds fed aureomycin was significantly higher in week 2 (*P=0.05)*, week-3 *(P=0.04)* and week 4 (*P=0.01*) compared to week 1.

The data from eggs/cage was collected from week 1 and the production percentage was calculated, which served as a base line to compare the effects of treatments over the trial period. The base line egg production was significantly lower in the birds fed with aureomycin and CC4 *(P=0.005*). Consequently, hen-day egg production increased from week 2 to week 4 for all the treatments except for the control. A drop in egg production rate was observed from week 1 (75.5%) to week 4 (35.7%) in birds fed on the basal layer diet (control). At week 4, egg production rate was significantly higher (*P <0.0001*) in the birds fed with SG and CC (71.4 -91.8%) compared to control birds (35.7%). Also, egg production percentage for the birds fed on aureomycin increased from week 1 (44.9%) to week 4 (80.6%) and was significantly higher than the control (35.7 in week 4 respectively) (Table 6.1). The BW, FI and egg production of birds did not differ significantly between aureomycin and seaweed treatments.

Table 6. 1. Effect of red seaweed dietary supplements on the growth and performance of layer hens

a) Feed intake b) Body weight c) Egg production

a

Diets[1]	Feed intake (g/d)			
	Weeks			
	1	2	3	4
CC2	101.3^{ab}	104.0^{ab}	103.6^{ab}	102.2^{ab}
SG2	100.9^{ab}	107.6^{a}	106.4^{a}	105.8^{a}
CC4	91.2^{abc}	104.0^{ab}	106.7^{a}	107.6^{a}
SG4	87.2^{abc}	98.5^{ab}	98.5^{ab}	104.3^{ab}
ANTB	84.0^{bc}	104.4^{ab}	104.0^{ab}	107.2^{a}
C	97.1^{ab}	102.0^{ab}	91.5^{abc}	74.7^{c}
SEM[4]	3.98			
Interactions, *P-value*				
Diets	<.0001			
Weeks	0.005			
Weeks X Diets	<.0001			

b

Diets[1]	Body weight (g)			
	Weeks			
	1	2	3	4
CC2	1532.4^{ab}	1567.9^{a}	1572.3^{a}	1526.5^{ab}
SG2	1565.7^{a}	1573.4^{a}	1570.5^{a}	1513.7^{ab}
CC4	1561.8^{a}	1571.5^{a}	1544.7^{ab}	1523.7^{ab}
SG4	1553.0^{ab}	1529.7^{ab}	1529.7^{ab}	1486.4^{ab}
ANTB	1597.8^{a}	1630.0^{a}	1609.5^{a}	1554.0^{ab}
C	1616.3^{a}	1566.1^{a}	1389^{bc}	1245^{c}
SEM[4]	32.63			
Interactions, *P-value*				
Diets	<.0001			
Weeks	<.0001			
Weeks X Diets	<.0001			

Table 6.1 continues over to next page…

c

Diets[1]	Egg production (%) Weeks			
	1	2	3	4
CC2	75.5^{abc}	79.6^{ab}	80.6^{ab}	81.6^{ab}
SG2	71.4^{abc}	86.7^{a}	82.7^{ab}	91.8^{a}
CC4	50.9^{bcd}	66.3^{abcd}	68.4^{abcd}	71.4^{abc}
SG4	71.4^{abc}	72.4^{abc}	79.6^{ab}	83.7^{ab}
ANTB	44.9^{cd}	73.5^{abc}	79.6^{ab}	80.6^{ab}
C	75.5^{abc}	87.8^{a}	74.5^{abc}	35.7^{d}
SEM[4]	6.38			
Interactions, *P-value*				
Diets	0.001			
Weeks	0.0014			
Weeks X Diets	<.0001			

[1]Values are means for each treatment (Trt) group (CC2: contains 2% of *Chondrus crispus*; SG2: contains 2% of *Sarcodiotheca* variant; CC4: contains 4% of *Chondrus crispus*; SG4: contains 4% of *Sarcodiotheca* variant; C: Control; ANTB: Antibiotics (Aureomycin). [3]Standard errors of the mean (14 replicates of 14 hens each per treatment) and different letters indicate statistically significant mean values (*P<0.0001)*

6.4.2 Red seaweed diets reduce *Salmonella* Enteritidis colonization in fecal material

All hens were clinically normal throughout the experiment and no fecal samples collected before challenge were positive for *Salmonella* Enteritidis. The effect of dietary seaweed supplementation on the level of *S.* Enteritidis in the feces of birds is presented in Table 6.2. There were no significant differences among the treatments on day 1-PI (Post Infection). On day 3-PI, supplementation of diets with CC (2 and 4%) and aureomycin significantly reduced the log CFU (*P=0.007*) of *S.* Enteritidis, as compared to control diets; however, SG supplementation (2 and 4%) was not different from control. Moreover, similar trends were observed on day 7-PI, where dietary inclusion of CC (2 and 4%) significantly reduced *S.* Enteritidis counts in the feces of birds compared to control birds. At day 5-PI, the colony counts of *S.* Enteritidis were significantly lower in

Table 6. 2. Effect of red seaweed dietary inclusions on the colonization of *S.* Enteritidis (log CFU/gram) in fecal material of layer chickens[1]

[1]Means represent 8 excreta samples per treatment. Excreta were collected from 48 cages (1 hens/cage), sampling was done three times per cage and the total fecal samples from two cages were pooled to represent one sample unit.

	Diets[2] (log CFU/gram)							**Interactions, *P-value***		
Days PI[4]	CC2	SG2	CC4	SG4	Control	ANTB	**SEM[3]**	**Diets**	**Days**	**Days X Diets**
1	4.99^{abcd}	5.53^{ab}	4.53^{abcde}	4.53^{abcde}	5.14^{abcd}	4.40^{abcde}	0.20	<.0001	<.0001	0.001
3	3.28^{efg}	4.12^{bcde}	2.53^{fg}	3.68^{defg}	5.71^{ab}	2.99^{g}				
5	3.69^{cdefg}	3.95^{cdef}	2.29^{g}	4.07^{bcde}	5.21^{abc}	2.29^{g}				
7	3.24^{efg}	4.08^{bcde}	2.29^{g}	4.06^{bcde}	4.89^{abcd}	2.29^{g}				

[2]Values represented as log CFU/gram are means for each treatment (Trt) group (CC2: contains 2% of *Chondrus crispus*; SG2: contains 2% of *Sarcodiotheca* variant; CC4: contains 4% of *Chondrus crispus*; SG4: contains 4% of *Sarcodiotheca* variant; C: Control; ANTB: Antibiotics (Aureomycin).

[3]Standard error of the mean (4 replicates of 8 hens each per treatment). Different letters indicate statistically significant mean values (*P<0.001)*

[4]Post Infection

the fecal samples of birds fed on the higher inclusion level of CC (4%) and aureomycin. However, recovery of *S.* Enteritidis from the fecal samples of birds fed on SG (2 and 4%) and lower level of CC (2%) were not significantly different than the control. No treatment effects were observed on day 1-PI on the recovery of *S.* Enteritidis from the fecal samples of birds (Table 6.2)

6.4.3 Red seaweed diet does not effect the recovery of *Salmonella* Enteritidis from the egg yolk

No *S*. Enteritidis were recovered from the egg yolk samples from seaweed treatment or control birds on day 1, 3, 5 and 7-post inoculation.

6.4.4 Red seaweed diets reduce *Salmonella* Enteritidis colonization of the ceca in layer hens

Colonization of *S.* Enteritidis in the ceca was determined at the end of the trial. The enumeration of *S.* Enteritidis revealed differences in their ability to colonize the ceca of infected birds. There was a significant reduction in the cecal *S.* Enteritidis count in birds fed with seaweed diets compared to the control birds (Figure 6.1). Dietary supplementation of CC (4%) and aureomycin significantly decreased (log CFU=1.19 and 1.3 respectively, *P=0.003)* the bacterial colonization in the ceca compared to control (log CFU=2.29). No differences were observed in the average log CFU/g ceca between the birds supplemented with SG (2 and 4%), CC (2%) and control.

6.4.5 Identification of *Salmonella* Enteritidis in fecal and cecal samples

The isolated colonies (10/treatment) from the fecal and ceca samples were identified positive for *Salmonella* Enteritidis by PCR. Among 96 birds used in the trial, 2 birds in the SG2 group showed feed withdrawal symptoms and were euthanized at the beginning of the trial. In all the treatments (16 birds/treatment), *S.* Enteritidis was recovered in the feces and ceca of only inoculated birds (8 birds/ treatment) indicating that *S.* Enteritidis was not transmitted to the contact exposed hens in the adjacent cages. Therefore samples from infected hens were only tested for organ invasion, blood characteristics, microbiota and SFCA analysis; the results obtained showed consistent and uniform *Salmonella* colonization in layer hens.

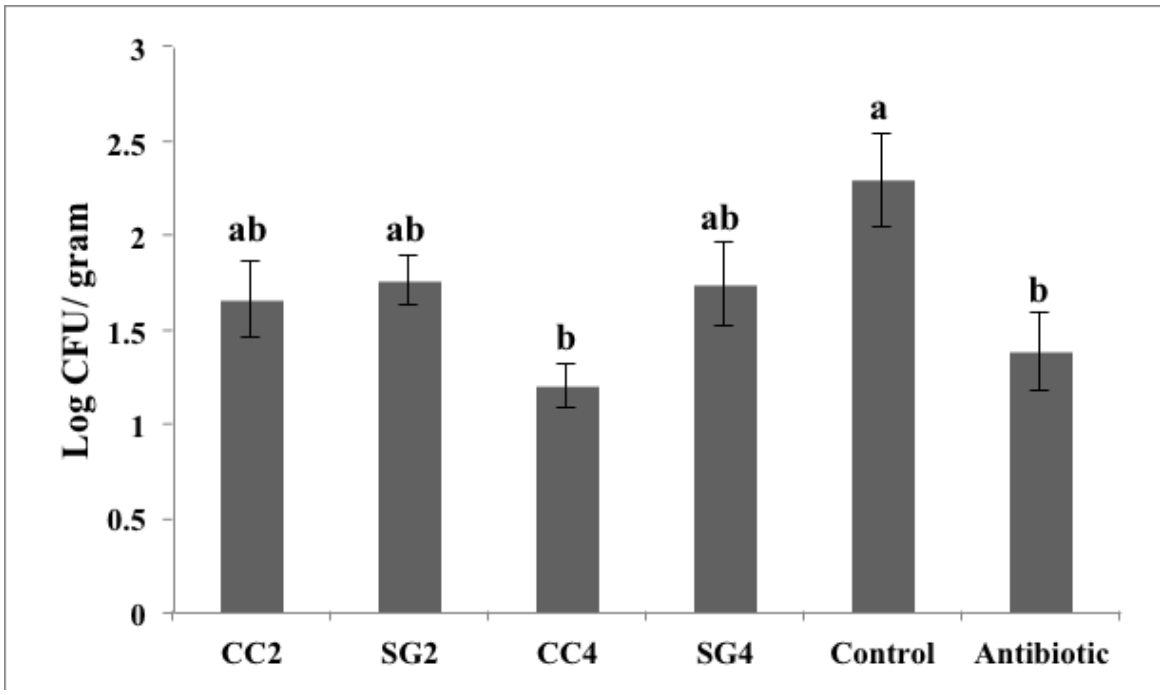


Figure 6. 1. Effect of red seaweed dietary supplements on the colonization of *S.* Enteritidis in the ceca of layer hens.
Data represent the mean of measurements from eight individual chicken ileum contents. Error bars indicate standard error and different letters indicate statistically significant mean values (*P<0.001).*

6.4.6 Red seaweed diet affects S. Enteritidis colonization in the organs

Recovery of *S.* Enteritidis from spleen and ovary samples of birds is shown in Table 6.3. Enrichment culturing of the spleen indicated higher frequencies (87-100%) of isolation of *S.* Enteritidis in hens fed with 2 and 4% of SG than among hens fed with CC 2 and 4%, 62%) and basal layer diet (control, 62%). Recovery of *S.* Enteritidis in spleen was lowest (37%) in the hens fed with aureomycin. However, the frequencies of *S.* Enteritidis recovery from ovaries did not differ significantly among the treatment groups except for SG4 (37%). Cecal samples isolated from all the birds fed on SG4 were positive for *S.* Enteritidis, control and SG2 showed 87.5% *S.* Enteritidis positive ceca, and CC4 and aureomycin had the lowest incidence (50%) of *S.* Enteritidis in the ceca (Table 6.3).

Table 6. 3. Effect of red seaweed dietary supplements on the colonization of *S.* Enteritidis in the organs of layer hens[1].

Diet[2]	*Salmonella* Enteritidis-positive hens/ Total hens (n=8)		
	Ovary	**Spleen**	**Ceca**
CC2	0%	62.5%	75%
SG2	0 %	100%	87.5%
CC4	12.5 %	62.5%	50%
SG4	37.5%	87.5%	100%
C	0%	62.5%	87.5%
ANTB	0%	37.5%	50%

[1]Values represented as percentage refers to the ratio of number of organs infected to the total number of organs.
[2]Values are means for each treatment (Trt) group (CC2: contains 2% of *Chondrus crispus*; SG2: contains 2% of *Sarcodiotheca* variant; CC4: contains 4% of *Chondrus crispus*; SG4: contains 4% of *Sarcodiotheca* variant; C: Control; ANTB: Antibiotic (aureomycin).

6.4.7 Red seaweed diet affects blood serum profile of *S.* Enteritidis infected hens

The effect of red seaweeds on red blood cells, white blood cell counts and IgA are summarized in Table 6.4. Serum IgA concentration was increased ($P < 0.001$) in CC4 (19.83 mg/mL) compared to the control (12.50 mg/mL). Moreover, SG2 treatment (33 $x10^9$/L) showed higher ($P = 0.005$) WBC counts than the control treatment (20.71 $x10^9$/L). There was no significant effect due to feed supplementation of seaweed meal on RBC count and blood serum concentrations of sodium, aspartate amino transferase (AST) and albumin levels (Table 6.4).

6.4.8 Red seaweed diets alters microbial population in ceca

Red seaweed dietary inclusion altered the relative abundance of beneficial bacteria; *Bifidobacterium longum, Lactobacillus acidophilus, Streptococcus salivarius* and pathogenic bacteria, *Clostridium perfringens* and *Salmonella* Enteritidis in the cecal

Table 6. 4. Effect of red seaweed dietary inclusion levels on blood cell counts and immunoglobulin concentration in layer hens infected with *S.* Enteritidis

Diet[1]	IgA (mg/mL)	WBC[3] (x10^9/L)	RBC[4] %	AST[5] (IU/lit)	Alb[6] g/L	Sodium (mmol/L)
CC2	16.68^{ab}	16.41^{c}	28.00^{a}	224.57^{a}	19.23^{ab}	145.23^{a}
SG2	14.98^{b}	33.00^{a}	27.33^{a}	185.71^{a}	20.46^{a}	144.20^{a}
CC4	19.83^{a}	26.29^{ab}	28.57^{a}	171.43^{a}	19.10^{ab}	145.21^{a}
SG4	10.47^{c}	18.81^{c}	26.17^{a}	235.29^{a}	16.13^{b}	139.89^{a}
ANTB	16.72^{ab}	12.69^{c}	27.88^{a}	215.43^{a}	17.04^{ab}	148.37^{a}
C	12.50^{bc}	20.71^{bc}	29.00^{a}	188.14^{a}	19.14^{ab}	144.63^{a}
SEM[2]	2.87	7.82	2.83	108.53	2.19	6.59
***P*-value**	<.001	0.005	0.51	0.8	0.008	0.3

[1]Values are means for each treatment (Trt) group (CC2: contains 2% of *Chondrus crispus*; SG2: contains 2% of *Sarcodiotheca* variant; CC4: contains 4% of *Chondrus crispus*; SG4: contains 4% of *Sarcodiotheca* variant; C: Control; ANTB: Antibiotics (aureomycin).
[2]Standard errors of the mean (8 replicates per treatment) and different letters indicate statistically significant mean values (*P<0.001).*
[3]White blood cells
[4]Red blood cell
[5]Aspartate transaminase
[6]AlbumIn

content (Figure 6.2). The abundance of *L. acidophilus* increased (*P<0.001)* by 3 fold in 4% CC treatments (Figure 6.3c). Additionally, the relative abundance of *B. longum* was 2 fold higher (*P<0.001)* in the aureomycin treatment (Figure 6.3a). The relative abundance of *S. salivarius* was significantly lower in 2 and 4% CC and SG dietary supplementations (Figure 6.3b). Interestingly, all seaweed treatments except SG2 decreased the prevalence of *C. perfringens* compared to the negative control (*P<0.001)* (Figure 6.3d). The abundance of *S.* Enteritidis was significantly reduced in the CC 2 and 4%) and aureomycin treatments (Figure 6.3e). The sequence analysis showed that Firmicutes, Bacteroidetes and Proteobacteria were the most dominant phyla in the ceca for all dietary treatments. These three predominant phyla in the ceca were significantly affected by the dietary treatments (Figure 6.3). The abundance of Bacteriodetes were found to be significantly reduced in the antibiotic treated group (26.67%, *P=0.007*) compared to the control groups (34.75%). This reduction was not observed in seaweed treatment groups, where dietary inclusion of *C. crispus* (46-6-48.4%, *P<0.05*) significantly increased Bacteriodetes compared to the control group (*P<0.0001)*. However, Firmicutes were predominant in the antibiotic-treated group (57.87%) compared to the control (51.02, *P=0.0045*) and seaweed (CC, 34-38, SG 34-50%, *P<0.05*) treated groups. *C. crispus* and antibiotic dietary inclusion did not affect the relative abundance of Proteobacteria (*P*= 0.08 and 0.95 respectively) (Figure 6.2).

6.4.9 Red seaweed diets alter Short Chain Fatty Acid (SCFA) composition in the digesta

Dietary inclusion with red seaweeds affected SCFA concentration in the digesta contents (Table 6.5). The concentration of propionic acid was higher (*P=0.003)* in the cecal digesta of birds fed 4% CC (32.64), as compared with the basal diets (13.67 mmol/kg) or aureomycin (17.60 mmol/kg). The concentration of *n*-butyric acid was higher in the aureomycin treatment (*P=0.002*) than the control. No significant differences in the concentration of acetic acid, i-butyric acid, *n*-valeric and *i*-valeric acids were observed in any of the treatments (Table 6.5).

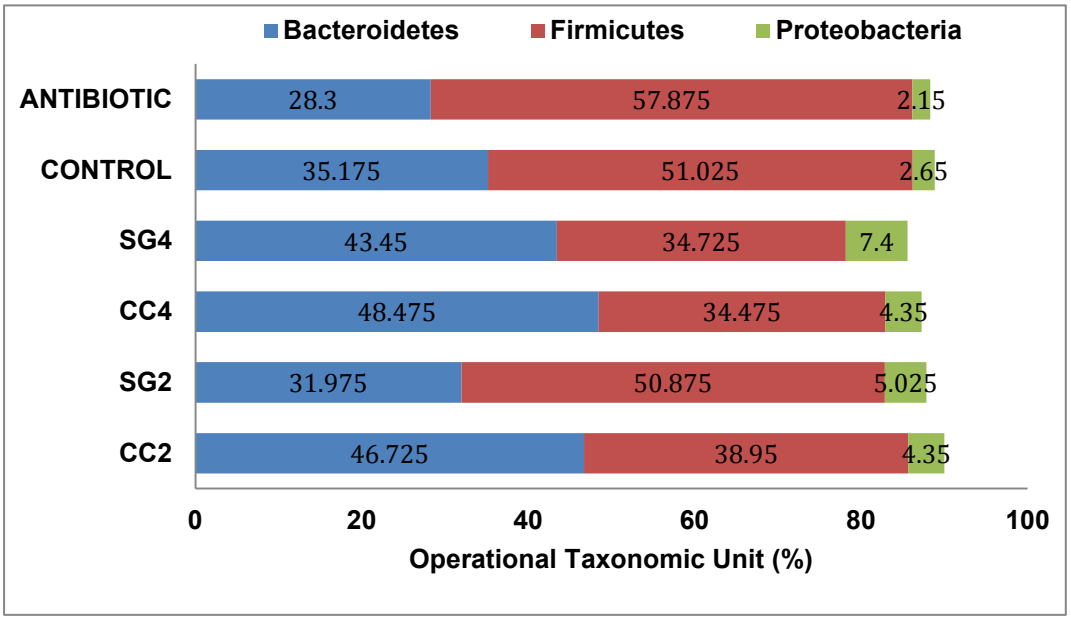


Figure 6. 2. Effect of red seaweed diets on the composition of cecal microbiota. Values are means for each treatment (Trt) group (C: control; ANTIBIOTIC: Aureomycin; SG2: contains 2% of *Sarcodiotheca* variant; SG4: contains 4% of *Sarcodiotheca* variant; CC2: contains 2% of *Chondrus crispus*. CC4: contains 4% of *Chondrus crispus*. Means represent 4 excreta samples per treatment (n=4).

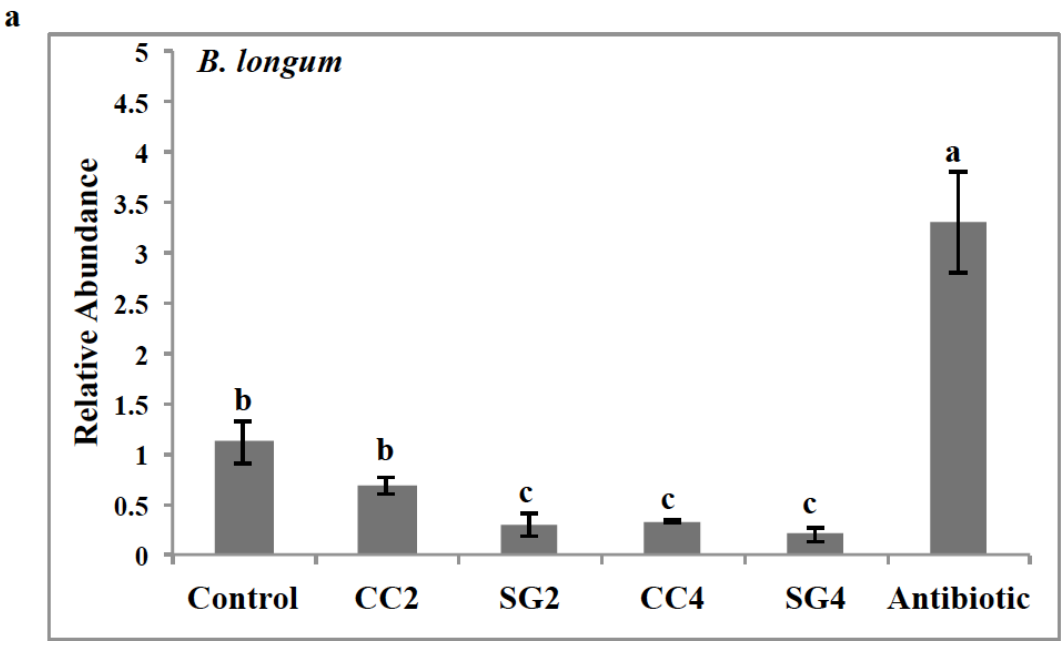
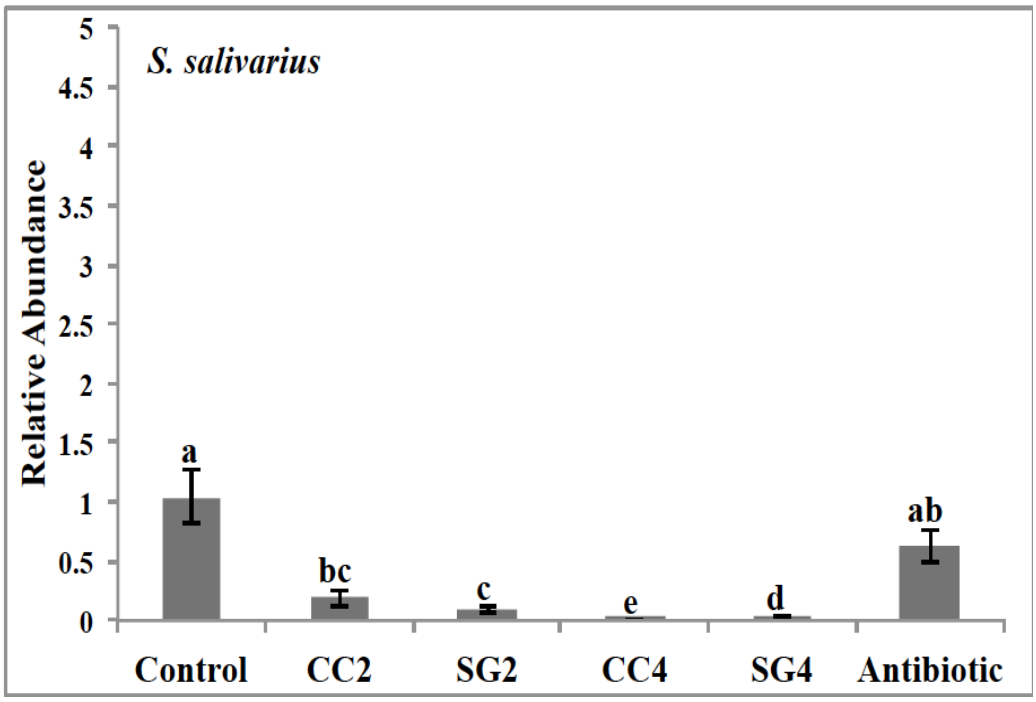


Figure 6.3 continued over to next page…

b



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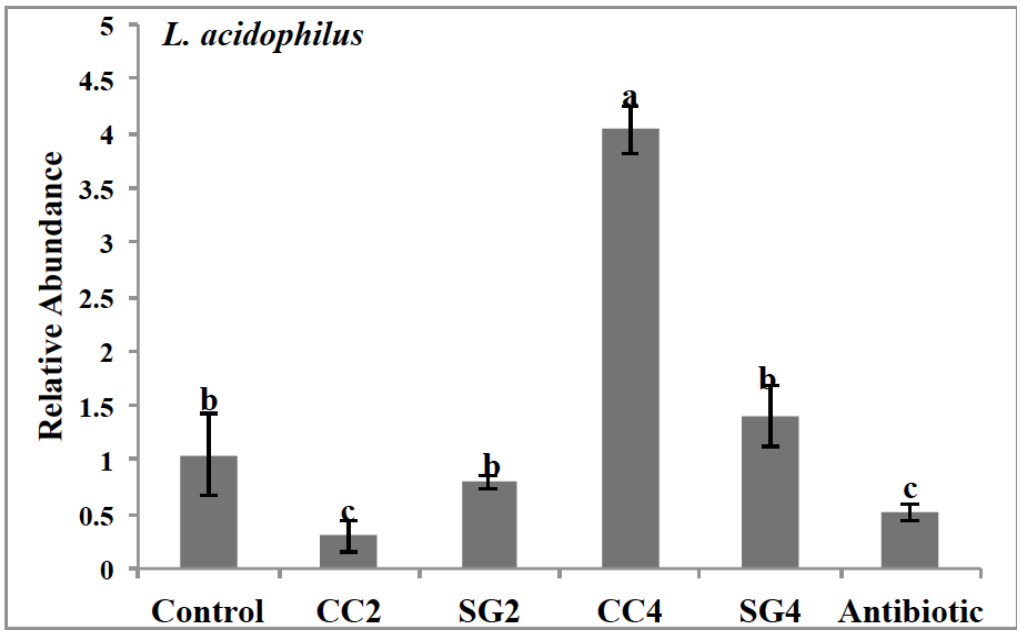

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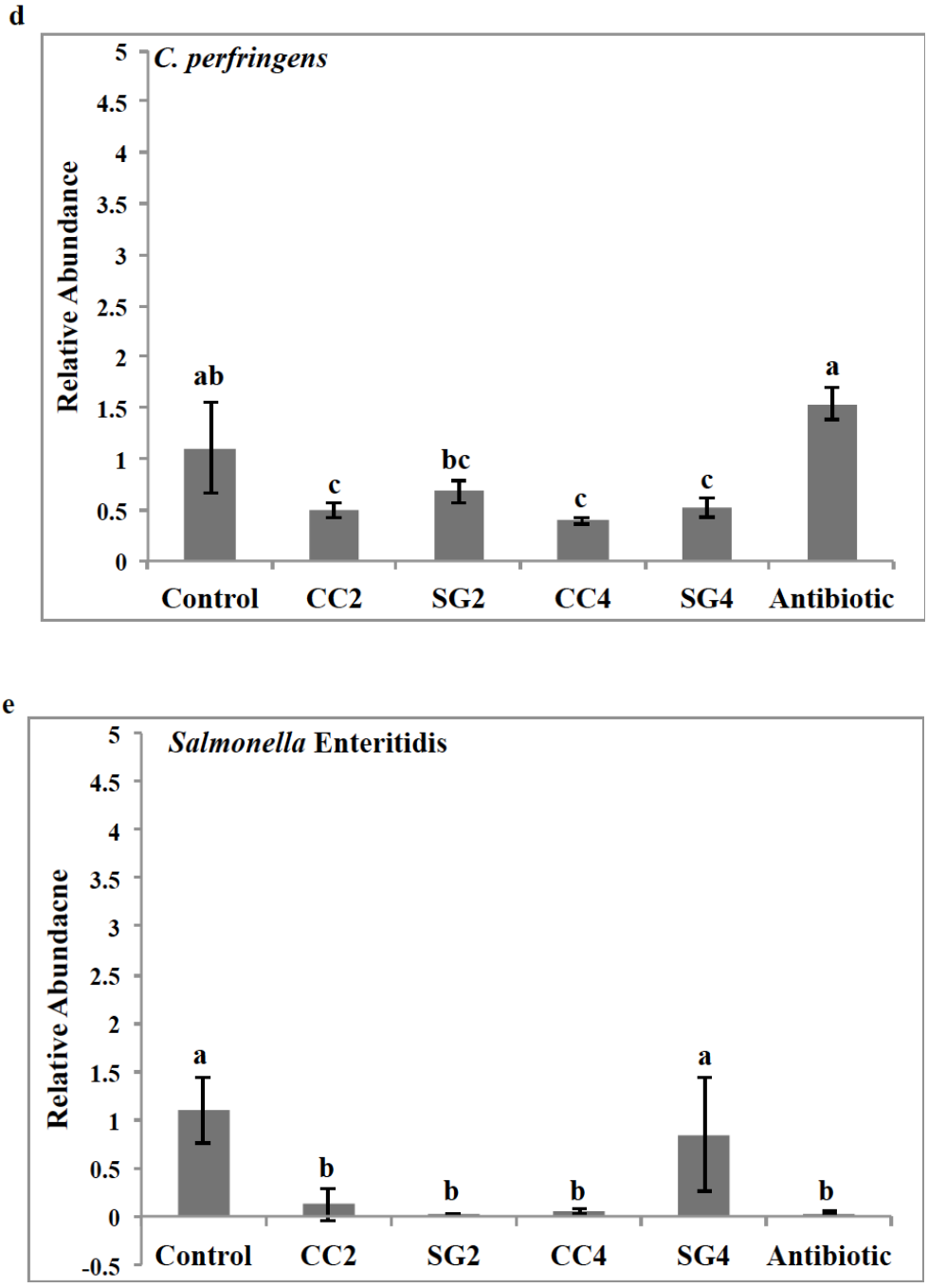

Figure 6. 3. Real time PCR quantification of microbial population in the cecal contents of layer hens.
Relative abundance of *Bifidobacterium longum, Lactobacillus acidophilus, Streptococcus salivarius* and *Clostridium perfringens* in the chicken ileum. Data represent the mean of measurements from two individual chicken ileum contents. Error bars indicate standard error and different letters indicate statistically significant mean values (*P<0.001)*

Table 6. 5. Effect of red seaweed dietary supplements on the concentration of short chain fatty acids (mmol/kg) in the ceca digesta of layer hens infected with *S.* Enteritidis.

	Short chain fatty acids (mmol/kg)				
Diet[1]	**Acetic acid**	**Propionic acid**	**i-Butyric acid**	**n-Butyric acid**	**i-Valeric acid**
CC2	31.04^{ab}	13.46^{b}	0.89^{a}	7.04^{bc}	0.76^{a}
SG2	23.03^{b}	8.49^{b}	0.31^{a}	3.05^{c}	0.29^{a}
CC4	70.58^{a}	32.64^{a}	0.58^{a}	8.37^{bc}	0.40^{a}
SG4	63.54^{a}	25.84^{ab}	0.83^{a}	11.43^{ab}	0.74^{a}
ANTB	44.50^{ab}	17.60^{ab}	0.84^{a}	17.39^{a}	1.06^{a}
C	33.01^{ab}	13.67^{b}	0.50^{a}	5.93^{bc}	0.50^{a}
SEM[2]	17.7	7.72	0.38	3.38	0.34
***P*-value**	0.007	0.003	0.25	0.002	0.05

[1]Values are means for each treatment (Trt) group (CC2: contains 2% of *Chondrus crispus*; SG2: contains 2% of *Sarcodiotheca* variant; CC4: contains 4% of *Chondrus crispus*; SG4: contains 4% of *Sarcodiotheca* variant; C: Control; ANTB: Antibiotic (aureomycin).
[3]Standard errors of the mean (4 replicates per treatment) and different letters indicate statistically significant mean values (*P<0.001)*. Means represent 4 ceca digesta samples per treatment.

6.5 Discussion

In spite of significant progress in food safety attained by implementation of several pathogen control strategies on-farm and in poultry processing units, the incidence of *Salmonella* infection in humans has remained relatively unchanged with an estimated 500 to 2000 deaths each year (WHO Global *Salmonella* survey program) (Betancor *et al.* 2010, Yim *et al.* 2010). In addition to the on-farm intervention strategies, an alternate step to decrease *S.* Enteritidis colonization in birds is critical to reduce the incidence of human illness associated with *S.* Enteritidis (Rajic *et al.* 2007). As *S.* Enteritidis is zoonotic and has shown increasingly high resistance to various antimicrobials, a natural feed additive such as seaweeds could serve as a better alternative.

In the present study, we quantified the effect of dietary inclusion of red seaweeds on the prevalence of *Salmonella* in layer hens. Supplementation of layer feed with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, resulted in a significant reduction of *S.* Enteritidis in the ceca, maintained body weight and egg production and increased the concentration of blood serum immunoglobulin (IgA) and the population of beneficial bacteria in the ceca.

To replicate the conditions of *S.* Enteritidis infection in commercial poultry farms, half of the total number of the birds/ treatment (8/16 birds per treatment) were challenged with *S.* Enteritidis and those remaining were expected to develop infection by horizontal transmission of bacteria. Surprisingly, *S.* Enteritidis was not recovered from any of the non-challenged hens in the trial. This could be either due to a lower level of bacteria shed into the environment or due to reduced colonization of *S.* Enteritidis in the challenged hens. The possible mechanisms of horizontal transmission of *S.* Enteritidis in birds could be either by contaminated feed, water supply, feathers and feces or due to aerosolized *S.* Enteritidis released into the environment (Bailey *et al.* 1990; Baskerville *et al.* 1992). In the present study, *S.* Enteritidis colonized and persisted in the cecum of challenged hens (Figure 6.1), however the lower level of bacteria shed into the environment could likely have been be responsible for the absence of *S.* Enteritidis in the cecal and fecal samples isolated from non-challenged birds. Asakura *et al.* (2001) demonstrated that housing birds at higher stock density and in unsanitary conditions increases their susceptibility to *S.* Enteritidis infection. Henceforth, in our study, the lowered stress level in the room (1 bird/cage), regular change of feed troughs and frequent cleaning of fecal trays post-inoculation might have lowered the bacterial shedding into the environment.

In the present study, addition of red seaweeds in the feed did not affect feed intake, body weight and egg production of the birds until week3 of the trial. However, the feed intake, body weight and egg production in the control birds declined significantly from week 4 until the end of trial (Table 6.1). The drop in growth parameters and egg production coincided with the time that the birds were inoculated with *S.* Enteritidis. Thus, it can be inferred that *S.* Enteritidis challenge depressed the growth parameters and egg production rate in control birds. This could have been due to the anorexic response of birds to infection. Additionally in control birds, the available energy would most likely be reallocated towards immune development to combat *S.* Enteritidis infection. This would have caused inefficient nutrient utilization for growth and egg production, thus resulting in detrimental effects on the health (body weight) and productivity (egg production) of birds (Cox *et al.* 2010). However, the aureomycin and seaweed dietary supplementation maintained feed intake, egg production and reduced body weight loss due to *S.* Enteritidis

infection (Table 6.1). This could have been due to the immune enhancing effect of red seaweeds. The role of oligosaccharide-rich diet, like seaweeds, in animal feed has been well researched. Dietary inclusion of seaweeds has been shown to improve growth performance and gut microbiota of birds by enhancing the immune system (Strand *et al.* 1998). Likewise in the present study, supplementation of layer feed with two red seaweeds (CC and SG) maintained feed intake and body weight most likely by improving immune status, thus resulting in sufficient nutrient availability for advancing growth. Additionally, the egg production rate in week 1 was significantly lower in CC4 and aureomycin supplemented birds (Table 6.1). The lower egg production in week 1 could have been due to the birds' response to the transportation during the start of the trial. A drop in egg production of birds indicates a particular way of coping with stress related to transport (Braastad & Katle, 1989).

Upon consumption of *S.* Enteritidis contaminated water or feed by hens, *S.* Enteritidis enters the esophagus and colonizes intestinal cells, passes through the mesenteric lymph nodes, and is carried by macrophages to spread in the organs (Okamura *et al.* 2001). In our study, *S.* Enteritidis fecal shedding was significantly reduced in seaweed (CC) supplemented birds than the control (Table 6.2). Several reports have indicated that cell mediated immunity is responsible for the clearance of *S.* Enteritidis from the tissue, while humoral immunity is critical for the reduction of intestinal colonization (Babu *et al.* 2004; Van Immerseel *et al.* 2005). Previously, sulphated polysaccharides obtained from the edible fungus *Agrocybe chaxingu* significantly augmented the level of both cellular and humoral immunity in broiler chicks (Zhang *et al.* 2013). Moreover, sulphated polysaccharides from marine sources have been shown to enhance the activity of neutrophils, macrophages and function as activators of humoral immune response (Kuznetsova *et al.* 2002). Similarly, in the present study, sulphated polysaccharides of red seaweed possibly induced the immune response of the birds resulting in the reduced fecal shedding of *S.* Enteritidis. The primary site for *S.* Enteritidis colonization is the cecum and then it spreads to the spleen and liver by lymphatic routes. The number of *S.* Enteritidis positive samples was highest in the ceca, followed by the spleen and lowest in the reproductive tract, which was consistent with the previous

reports (Gantois *et al.* 2008, Cho *et al.* 2010). *Chondrus crispus* supplemented feed was effective in reducing *S.* Enteritidis numbers in the feces and cecal content. This could have been due to the ability of seaweed to block the initial attachment of bacteria to the epithelial cells by targeting the motility and virulence of *S.* Enteritidis (Chapter 3). Furthermore, none of the egg yolk samples were positive for *S.* Enteritidis in the trial. This is in agreement with the previous research by Garicia *et al.* (2011), where no *Salmonella* spp. were detected in the egg content of *S.* Enteritidis infected birds. This could have been due to the protective effect of the egg's complex system of antimicrobial components (Garcia *et al.* 2011).

Although the exact mechanism (s) of action of dietary supplements of seaweed on the pathogen or the health of chicken is unclear, it has been suggested that the pathogen inhibitory effect can be correlated with the metabolic characteristics of health enhancing bacteria and their immunomodulation activity. Probiotic bacteria such as *Lactobacillus* have been shown to suppress the growth of pathogens by mechanisms such as competitive exclusion, secretion of SCFA, and antimicrobial peptides as well by priming the host immune system (Torok *et al.* 2008). The population of probiotics such as *Lactobacillus* can be altered by dietary inclusion of prebiotics, which selectively enhance their growth. Interestingly in the present study, a negative correlation was observed between the relative abundance of *Lactobacillus acidophilus* and *Salmonella* Enteritidis in birds supplemented with CC (4%) (Figure 6.2), suggesting *Lactobacillus acidophilus* as a suppressor of *Salmonella* Enteritidis (Gibson *et al.* 2005). Moreover, this could have been due to the effect of dietary *C. crispus* on epithelial cell turnover, thus altering the growth in favour of the beneficial bacteria (Chapter 5). Previously, it has been documented that the host microbiota composition is dependent on the dietary carbohydrate intake (Rinttila & Apajalahti 2013). Supplementing polysaccharide rich seaweeds, CC and SG altered the cecal microbiota of layer hens after *S.* Enteritidis infection. Significant changes in the abundance of genera belong to the phyla *Firmicutes* and *Bacteroidetes* were observed with *C. crispus* (4%) supplementation, the treatment group that showed maximum protection against colonization of SE. This indicates that dietary supplementation of *C. crispus* to layer hens altered their microbiota

composition that imparted resistance against *Salmonella*. An increase in *Bacteroidetes* was observed with CC dietary inclusion. In chickens ceca, genus *Bacteroidetes* are one of the most dominant anaerobic genera that play an important role in the breakdown of complex molecules such as polysaccharides into simpler compounds to provide energy (Reeves *et al.* 1997). The increase in the abundance of *Bacteriodtees* could be due to the bioavailability of undigested fermentable polysaccharides as a result of *C. crispus* supplementation. Thus the selectivity of fermentation substrate could most likely be the reason for the shift in the cecal micro biota with the *C. crispus* supplement. Short-chain fatty acids are fermentation end products of gut microbes, which proliferates enterocytes and also have inhibitory effects on the growth of pathogens such as *Salmonella* (Hinton *et al.* 1990). Previously, dietary inclusion of seaweeds has been shown to increase the cecal concentration of acetate and propionate (Gomez-Ordonez *et al.* 2012). Intestinal fatty acid propionate controls *Salmonella* invasion through post-translational modification of HilD, which is a major regulator of *Salmonella* pathogenicity island 1 (SPI-1) (Hung *et al.* 2013). Thus, increase in propionate concentration in birds fed on seaweed (CC) (Table 6.5) would have repressed the SPI-1 regulator (HilD), leading to decreased expression of effector proteins, eventually resulting in decreased *S.* Enteritidis penetration into the cells.

Probiotic bacteria such as *Lactobacillus* can modulate immune response by stimulating the production of immunoglobulin such as IgA. Immunoglobulin prevents the pathogen colonization by blocking their attachment to the epithelial cell receptors (Rocha *et al.* 2012). Therefore in present study, an increase in the production of IgA could be correlated to the reduced colonization of *S.* Enteritidis. Moreover, higher levels of *Lactobacillus* could have resulted in the higher IgA production.

In conclusion, dietary supplementation of layer fed with the red seaweeds reduced the negative effect on layer growth and egg production caused by *Salmonella* Enteritidis. Dietary inclusion of *Chondrus crispus* (CC) inhibited colonization of *Salmonella* in the feces and ceca. This could be by promoting the growth of *Lactobacillus* and increasing the concentration of short chain fatty acids. Moreover, the higher level of IgA in birds supplemented with CC indicates a direct role of seaweed on the maturation of the humoral immune system. Since chickens are major reservoir of *S.* Enteritidis, innovative

on-farm strategies such as seaweed feed supplements for reducing *S.* Enteritidis colonization in birds can serve as an effective alternative to controlling human infections. Additionally, producers including organic farmers, without concerns for toxicity, would likey accept a natural feed additive like seaweed.

CHAPTER 7. DISCUSSION

S. Enteritidis is a food borne pathogen carried by contaminated poultry products to the human food chain. As chickens do not show visible symptoms of the disease, *S.* Enteritidis can easily spread through a layer flock and persist in the avian tissue for an extended periods of time (Guard-Petter, 2001). The ability of *S.* Enteritidis to establish persistent infection in the albumen within the laid egg and avian tissues is responsible for its invasion into the human food chain (Revolledo *et al.* 2009, Yim *et al.* 2010). The contamination of poultry by *S.* Enteritidis can result in lack of consumer confidence in the products, causing a significant economic loss to the poultry industry. Moreover, human infection by *S.* Enteritidis can increase the burden on the health care administration. The diminishing activity of available antibiotics as therapeutics highlights the need to find natural alternatives to limit the growth of pathogens. This thesis explored the potential of red seaweeds as an antimicrobial agent against *S.* Enteritidis and their use as an alternative to antibiotics in poultry.

This thesis documents that red seaweeds (CC and SG) effectively reduced the growth of *S.* Enteritidis *in vitro* and *in vivo* in both *C. elegans* and layer hens. A detailed analysis further revealed that water extract but not organic extract of CC and SG reduced biofilm formation, motility and down-regulated the expression of genes encoding virulence factors of *S.* Enteritidis. The survival capabilities of *S.* Enteritidis are facilitated by cell surface appendages (flagella) and virulence factors involved in biofilm formation, motility and quorum sensing (Bogomolnaya *et al.* 2014). *Salmonella* adhere and survive on biotic and abiotic surfaces for a prolonged period by forming biofilms (Prouty *et al.* 2002). Since biofilms interfere with the action of several antimicrobial agents, eradication of biofilm forming bacteria is more difficult. In the present study, the bioactivity of SWE (CC and SG) could have been due to the polysaccharides present in the water extract. Previously, polysaccharides derived from bacteria and eukaryotes have been shown to be lectin inhibitors. Lectins are adhesive proteins that facilitate attachment of bacteria to host cells. These surface proteins play a major role in biofilm formation by binding to the glycan substrate present on the surface of the target cell (Esko & Sharon,

2009). The lectin mediated bacterial attachment facilitates the secretion of bacterial virulence factors resulting in the host cell destruction, which is co-regulated with the lectins involved in quorum sensing signals (Gilboa-Garber *et al.* 1997). Polysaccharides inhibit lectin-dependent adhesion by competing for the sugar binding domain of lectins (Zinger-Yosovich & Gilboa-Garber, 2009). Similarly in the present study, polysaccharides in CC and SG water extracts could have competitively blocked the binding sites of lectins to the target cell surface receptors, thus inhibiting lectin-dependent attachment of a pathogen and biofilm formation. Moreover, seaweed polysaccharides (water extracts) did not show bactericidal activity at anti-biofilm concentration. This property extends their use as anti-biofilm agents to industrial and medicinal applications by reducing the emergence of resistance development by natural selection or selective pressure. The reduction of biofilm formation could also be associated with anti-quorum sensing compounds present in the water extract. Previously, brominated furanones from red algae *Delisea pulchra* have been shown to mimic acyl-homoserine lactones structure and function as anti-quorum sensing and anti-biofilm compounds (Janssens *et al.* 2008). Likewise, CC and SG water extracts are likely to contain furanone-like derivates that inhibit quorum sensing and reduce biofilm formation. Further research will help to identify specific red seaweed compounds responsible for repressing quorum sensing and biofilm formation. Recent reports have indicated that in the USA, annually 1.7 million hospital-acquired infections are associated with microorganisms with biofilms, incurring an additional cost of $11 billion in healthcare sectors (Romling *et al.* 2014). Such hospital-acquired infections have led to more than half a million deaths annually. Thus, it is evident that compounds that impair bacterial motility and biofilm formation are of pharmaceutical and economic interest in treating diseases caused by drug resistant bacteria.

Natural antimicrobial compounds isolated from seaweeds such as halogenated and brominated metabolites, micosporine-like amino acids and sulphated polysaccharides have shown antimicrobial properties (Vairappan *et al.* 2008), but their *in vivo* affect is not often examined. Conducting poultry trials to validate the *in vitro* activity poses difficulties with experimental cost, time and space. Hence, prior to the animal study, a

model organism, *C elegans* was used to validate the antimicrobial activity of the two identified red seaweed (CC and SG) species. Several model organism including mice and rodents are commonly used to validate bioactivity *in vivo*. However, *C. elegans* is anatomically simple, genetically tractable nematode and can be easily grown and replicated in the laboratory (Sifri *et al.* 2005). SWE (CC and SG) significantly increased the survival of *C. elegans* infected with *S.* Enteritidis and reduced the accumulation of *S.* Enteritidis in *C. elegans* gut. It is speculated that the decrease in *S.* Enteritidis colonization could be partially due to the decrease in the ability of bacteria to attach to the surface of the intestinal epithelium of *C. elegans*. It is also likely that the significant reduction in expression of virulence-associated genes in *S.* Enteritidis enhanced the survival of *C. elegans*. CC and SG induced immune response related genes, which contributed to the survival of *S.* Enteritidis infected *C. elegans* (Chapter 3). To my knowledge, this is the first report of a direct antimicrobial effect of red seaweed on the enteric pathogen *S.* Enteritidis and the antimicrobial activity was demonstrated in a live model organism *C. elegans.*

The novel finding of this thesis also includes the identification of a pure seaweed component floridoside, which was identified to improve the efficacy of an antibiotic (tetracycline) against *S.* Enteritidis (Chapter 4). Discovery of antibiotics in the early 20th century is considered as one of the wonder discoveries in the history of Science. However, the rise of antibiotic resistance bacteria in humans, farm animals and agriculture has been a challenge for modern medicine. The genetic capabilities of microbes have benefited from the overuse of antibiotics by humans. Microbes have utilized their innate genetic resistance and lateral gene transfer to acquire resistance to several antibiotics used in clinical and agricultural practices. This indicates that since the time antibiotics were first employed, their successful use was compromised by the development of tolerance or resistance. Thus in the era of diminishing activity of available antibiotics, an additive effect such as using combined therapies could enhances the lifeline of existing antibiotics (Hussin & El-Sayed, 2011, Singh *et al.* 2013). Here, SWE (CC and SG) and the pure component floridoside was shown to enhance the activity of an antibiotic (Tetracycline). Floridoside was able to inhibit the growth of *S.* Enteritidis in combination with an

otherwise ineffective dose of tetracycline. This finding is beneficial as there is a lack in the discovery of new antibiotics. A recent report has shown that there are only 7 new drugs in the pipeline (phase 2 or phase 3 trials) for the treatment of infections caused by Gram-negative bacteria (IDSA). More striking is that these drugs in the development pipeline might not be approved by the FDA and are not guaranteed to work against resistant human pathogens (Boucher *et al.* 2013). Hence, the current finding where to revive the ineffective doses of antibiotics were made effective using natural compounds such as floridoside could be a suitable alternative.

Recently teixobactin has been the only new antibiotic to be discovered in 30 years. It was isolated from a soil bacterium and was shown to exhibit cell wall inhibitory effects against Gram-positive pathogens. Although, this discovery has offered hope to solve the antibiotic crisis, the drug still faces plenty of hurdles on its way to hospitals and drug stores (Ling *et al.* 2015). Human trials for teixobactin are two to three years away, and it would take an estimated minimum $100 million to develop this drug over the span of 4-6 years (interview by co-discoverer) (Grant, 2015). Currently, due to lesser financial incentives, pharmaceutical companies have limited their research on the development of new antibiotics. In this scenario, an alternative strategy to increase the efficacy of existing antibiotics could save the cost of production and development of new antibiotics. Thus the implementation of combined therapy, i.e., the use of compounds such as floridoside, might improve existing antibiotic performance.

Floridoside (2-*O*-α-D-galactopyranosylglycerol) is a neutral heteroside found in red algae as a photosynthetic product and has been previously reported as an anti-quorum sensing compound (Park *et al.* 2007). Interestingly, in *Pseudomonas aeruginosa* quorum sensing has been shown to regulate efflux pumps (mediators of antibiotic resitance) (Maseda *et al.* 2004). Relatedly, in the present study, floridoside might have inhibited quorum sensing in *Salmonella*, which could have repressed efflux-related gene expression. Interference with efflux activity would have resulted in the accumulation of tetracycline within the cell eventually leading to cell death. Thus, this finding shows that seaweed compounds can be used as an effective alternative to increase the useful life of overused antibiotics (Chapter 4).

In vitro laboratory assays have been used for decades to predict *in vivo* effect. Still the rate of successful translation from *in vitro* data sets to *in vivo* or commercial trials is low. Previously, *in vitro* studies on the pathogenicity of avian pneumovirus in oviduct organ cultures have failed to be replicated in chicken oviducts (Khehra & Jones, 1999). Hence, to validate the bioactivity of red seaweeds in poultry, layer hen trials were conducted. Prior to the infection study, a feeding trial was conducted to evaluate any negative effect of seaweeds on feed intake, growth, digestibility and performance of layer hens (Chapter 5). This study helped to access the effect of seaweed dietary inclusion on the health of the bird in the absence of any abiotic or biotic stress condition. Dietary inclusion of red seaweeds improved the performance of layers, improved histomorphology and increased relative cecal weight (Chapter 5). Red seaweeds contain sulphate esters of polysaccharide, which are absent in terrestrial plants. Since the intestinal microbiota lack genes encoding enzymes for the degradation of these carbohydrates, initially studies in 2001 showed that the seaweed polysaccharides were poorly fermented by colonic microbiota in rats (Goni *et al.* 2001). However, bacterial adaptation by horizontal gene transfer over time brought about a change in the fermentation of these polysaccharides. For example in humans, bacteria of the gut microbiota have evolved to catabolize red seaweed glycan with carbohydrase active enzymes obtained by horizontal gene transfer (Hehemann *et al.* 2012). Similarly in chickens, the abundance of transferable genetic elements was evident in metagenomic analysis of the chicken cecal microbiome. Approximately 25% of the assembled contigs showed similarity with the mobile genetic elements indicating an evolution in the microbiome due to horizontal gene transfer (Qu *et al.* 2008). These studies indicate that horizontal gene transfer would have mediated a way for enzymes from algae-associated marine bacteria to reach intestinal bacteria resulting in the optimum fermentation of seaweed polysaccharide. In the present study the improved histomorphology and better performance of layer hens could have been due to an increase in the population of beneficial bacteria and a reduction of pathogenic bacteria in the gut (Chapter 5). Moreover, the fermentation products of beneficial microbes i.e., short chain fatty acids (SCFA) were higher in red seaweed treatments. This indicates the presence of red seaweeds in the lower GIT and its ability to be readily fermented by gut microflora

(Chapter 5). These results satisfy the basic criteria for the red seaweeds to qualify as prebiotics to improve health of layer hens. Thus, this part of the study validates that cultivated strains of *C. crispus* and *S. gaudichaudii* could be used as prebiotics for layer hens. Further research to measure the amount of undigested polysaccharide in the digesta of lower GIT and to determine the gut microbiome using next generation sequencing will strengthen the evidence towards establishment of red seaweeds as prebiotics.

Despite the significant progress in several pathogen control strategies, the incidence of *Salmonella* Enteritidis in poultry and its subsequent transmission to the human food chain has continued to be a food safety issue (Rajic *et al.* 2007). Though several antimicrobials can be used as therapeutics against such zoonotic pathogens, an increasingly high resistance towards such antimicrobials point out the need to find natural feed alternatives. Among others, seaweeds could be potential candidates as they are rich in antimicrobial compounds such as sulphated polysaccharides, halogenated metabolites, and phlorotannins (Ventura *et al.* 1994). In chickens, the microbial flora maintains overall health through the effects on gut morphology, and immune responses. The gut microflora competitively protects against colonization of intestinal pathogens such as *Salmonella*. Since red seaweeds (CC and SG) enhanced the beneficial microflora in layer hens (Chapter 5) and enhanced the survival of *S.* Enteritidis infected worms (Chapter 3), they were tested for their ability to protect layer hens from *Salmonella* infection. Dietary inclusion of red seaweeds (CC and SG) in layer feed significantly reduced *S.* Enteritidis colony count in the ceca, maintained body weight and egg production and increased the relative abundance of *Lactobacillus* in the ceca (Chapter 6). Previous studies have reported that SPI-1 virulence factors are essential to colonize the ceca in the chickens. *Salmonella* mutants with knocked out virulence-related genes had reduced ability to colonize chicken epithelial cells (Dieye *et al.* 2009). In the present study, since CC and SG water extracts repressed the expression of these SPI-1 associated virulence genes (*in vitro*), one possible mechanism by which red seaweeds reduced the colonization of *S.* Enteritidis in the ceca of layer hens could be by attenuating virulence factors of *S.* Enteritidis. Moreover, these virulence factors of *S.* Enteritidis are known to be essential for colonization of the intestinal tract in *C. elegans* (Tenor *et al.* 2004). Since CC and SG

water extracts also reduced the colony count of *S.* Enteritidis in the intestine of *C. elegans*, the mode of action of red seaweed to reduce *Salmonella* colonization in the model organism (*C. elegans*) can be correlated to the layer hens. Hence, this study has also shown that *C. elegans* model can be used to provide preliminary insight to study pathogenesis in chickens.

Interestingly dietary inclusion of CC also increased the concentration of serum immunoglobulin IgA in layer hens (Chapter 6). Previously it has been shown that chicken immunoglobulin prevented pathogen colonization by blocking their attachment to the epithelial cell receptors (Rocha *et al.* 2012). Thus in the present study, the reduced colonization of S. Enteritidis could also have been due to higher levels of IgA. An immunomodulating effect of red seaweed was also observed in *C. elegans* infected with SE, where CC the water extract induced the expression of immune-related genes in infected worms. Therefore, another possible means by which red seaweeds reduced the colonization of *S.* Enteritidis could have been by enhancing the host immune system. Additionally, this correlation further verifies that the laboratory model with *C. elegans* could be utilized for preselecting prebiotics such as red seaweeds to study *Salmonella* pathogenesis in poultry.

To summarize, the work in this thesis highlights the antimicrobial activity of red seaweed *in vitro* and its successful translation into *in vivo* animal trials. In layer hens, since red seaweed responses were comparable to antibiotic (aureomycin) action, seaweeds can be used as an organic feed alternative to antibiotics.

Further Directions

This thesis explored the application of red seaweeds in poultry feed to control a disease-causing pathogen (*Salmonella* Enteritidis) and as a prebiotic source for layer hens. Further research can be carried out to verify the proposed mechanisms of action of red seaweeds on the growth of *S.* Enteritidis in chickens. Detailed experiments involving challenge of layer hens with labelled wild type and *Salmonella* SPI-1 (*hilA*, *invF*) mutants would allow real time observation (using flow cytometry) of how the dissemination of *S.* Enteritidis is restricted by red seaweeds in the chickens. This would also help to identify

the effect of red seaweed on the specific cell type that *S.* Enteritidis is residing in within the layer hens. Furthermore, infected phagocytic cells from the challenged hens in different treatments can be isolated and specific cytokines and chemokine profile could help us determine the effect of red seaweed on immune cells in layer hens.

The seaweed component, floridoside was efficient in enhancing the potency of tetracycline against *S.* Enteritidis. The proposed mechanism of action is the interference of floridoside with the efflux activity resulting in the accumulation of tetracycline within the cell. Further experiments to quantify tetracycline and efflux pumps in the bacterial cell will help to prove the proposed mode of action.

As seaweeds potentiated the activity of antibiotics *in vitro,* it would be worthwhile to try various combinations of antibiotics and seaweeds at different dose levels in chickens. A feed additive that could lower the doses of antibiotics could be extremely useful in decreasing the consumption of antibiotics in commercial poultry farms.

Contribution to Science

The chapters in this thesis represent a novel and comprehensive study on the use of red seaweeds as an alternative to antibiotics in poultry production.

In the model organism *C. elegans* was used to screen and validate the immunomodulatory and antimicrobial activity of red seaweeds (CC and SG) against the poultry pathogen *Salmonella* Enteritidis (SE). This study is first of its kind where red seaweeds showed similar effect against *S.* Enteritidis colonization in both *C. elegans* and layer hens. This validates that *C. elegans* can be used as a model to screen compounds against infectious diseases in chickens. In addition, antibiotic potentiation by red seaweed water extract and a pure component (floridoside) against *S.* Enteritidis was identified. A sub lethal concentration of tetracycline in combination with floridoside exhibited antimicrobial activity that was comparable to full strength tetracycline. Further, prebiotic activity of red seaweeds (CC and SG) enhanced the overall health of layer hens. Dietary

inclusion of red seaweeds improved performance, egg quality and gut microflora of layer hens, which can be translated to commercial poultry operations.

This study has generated the following publications, and manuscripts:

Kulshreshtha G, Rathgeber B, Stratton G, Thomas N, Evans F, Critchley A, Hafting J, and Prithiviraj B. Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, affects performance, egg quality, and gut microbiota of layer hens. *Poultry Science* 93, no. 12 (2014): 2991-3001.

Kulshreshtha G, Rathgeber B, Stratton G, Stone S, Thomas N, Evans F, Critchley A, Hafting J, and Prithiviraj B. Red seaweeds, *Sarcodiotheca gaudichaudii and Chondrus crispus* down regulate virulence factors of *Salmonella* Enteritidis and induce immune response in *Caenorhabditis elegans*. (Manuscript submitted to Frontiers in Microbiology)

Kulshreshtha G, Rathgeber B, Stratton G, Stone S, Thomas N, Evans F, Critchley A, Hafting J, and Prithiviraj B. Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii,* reduce *Salmonella* Enteritidis in layer hens. (Manuscript in preparation for Poultry Science)

This study has generated the following patent:

Kulshreshtha G, and Prithiviraj B. *In vitro* combinatory antimicrobial effect of red seaweeds and pure compound with tetracycline against *Salmonella* Enteritidis (Submitted for Report of Invention)

Other related publications:

The extraction of bioactive compounds from seaweeds can be enhanced using enzyme-assisted hydrolysis. Previous research has shown that enzymatic hydrolysis increases the availability of bioactives present in seaweeds. Moreover, enzyme assisted extraction is a solvent free, eco-friendly and cost effective extraction method. Hence, the efficacy of enzyme-assisted extraction to improve yields of bioactive compounds from *Chondrus crispus* was evaluated. Red seaweeds are rich in sulphated polysaccharides, which have been demonstrated to inhibit the establishment and growth of enveloped viruses, such as

herpes simplex virus type 1 (HSV-1) and 2 (HSV-2). Prof. Nathalie Bourgougnon's lab at University of Brittany is well known for their HSV-1 infection studies using a cell culture system. The conditions for enzyme-assisted extractions were optimized for red seaweed and the bioactive compunds were tested against HSV-1 virus using Vero cell lines. This study was published in Marine Drugs.

Kulshreshtha G, Burlot AS, Marty C, Critchley A, Hafting J, Bedoux G, Bourgougnon N, and Prithiviraj P. Enzyme-assisted extraction of bioactive material from *Chondrus crispus* and *Codium fragile* and its effect on *Herpes simplex* Virus (HSV-1)." *Marine Drugs*13, no. 1 (2015): 558-580.

Note: These data have not been included in the thesis

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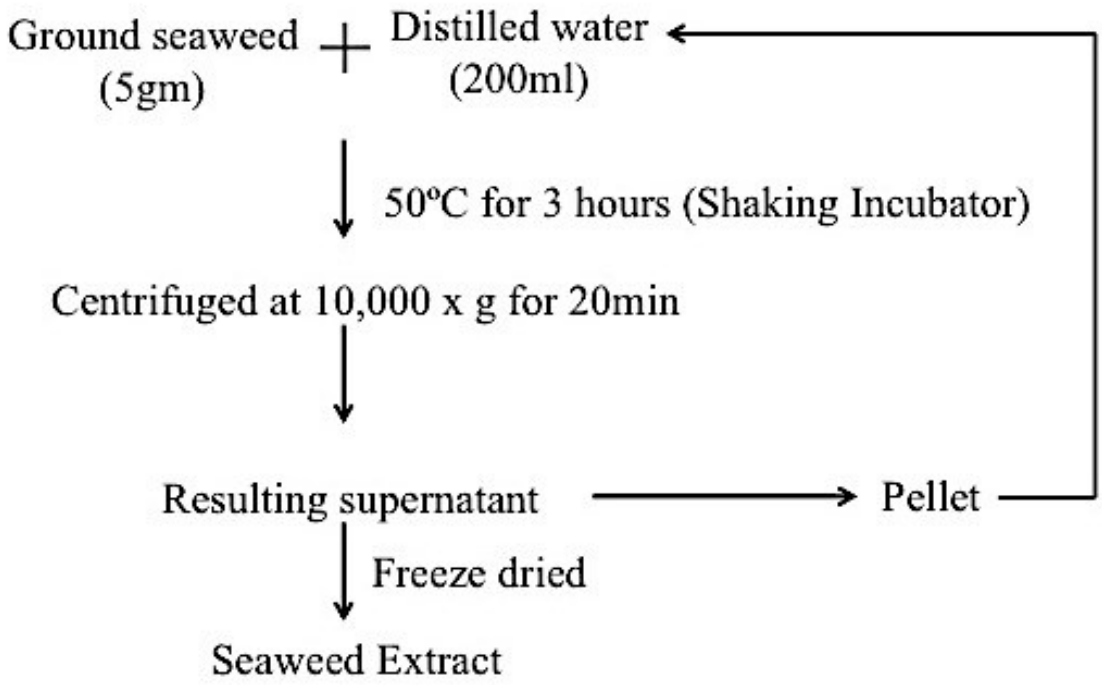
Appendix A

Scheme S1: The procedure for the extraction of red seaweed water extract



Scheme S2: Extraction process of floridoside from crude seaweed extract (Protocol provided by NRC, Halifax)

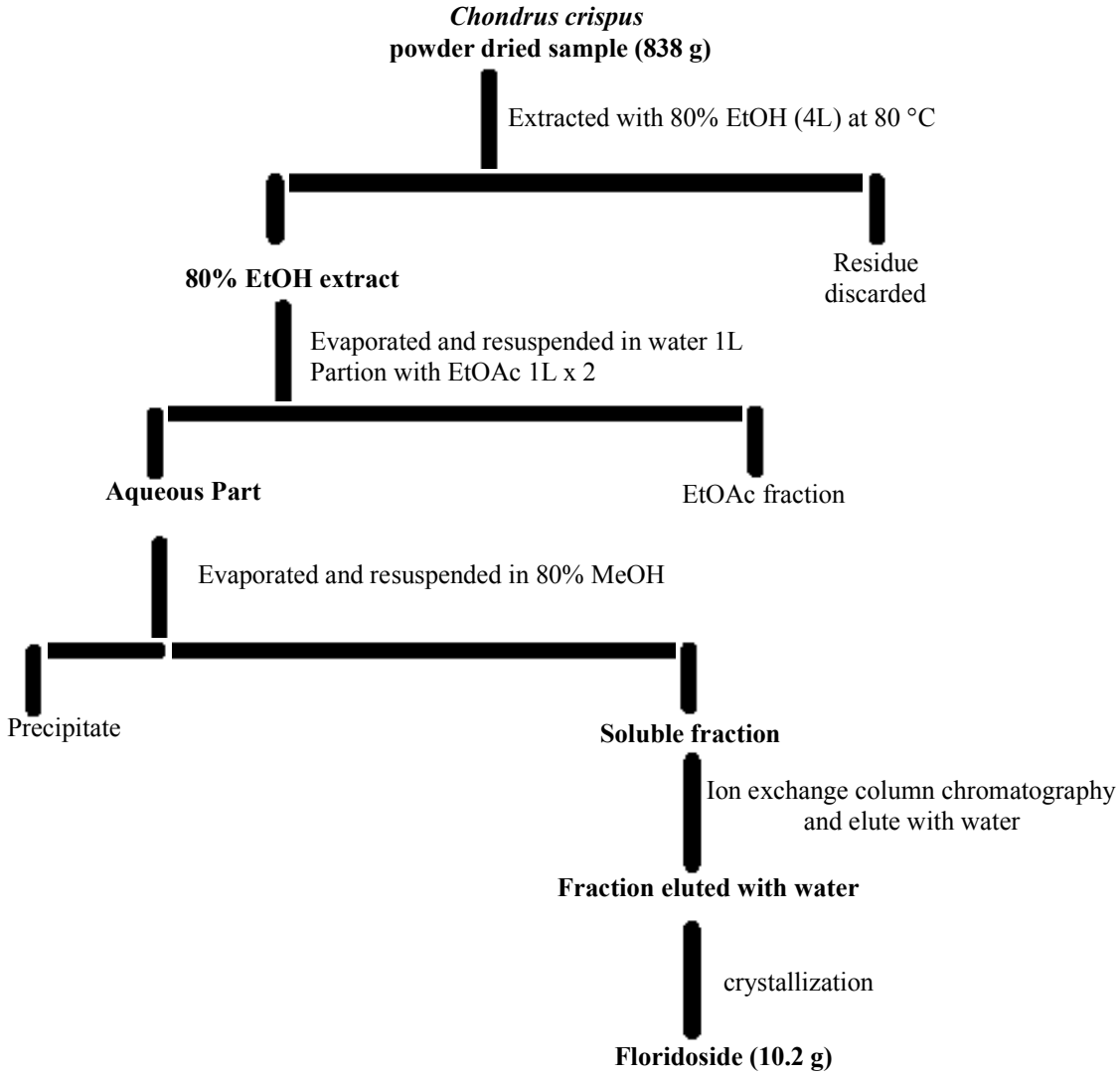


Table S1: The genes and primer sequences of *Salmonella* Enteritidis used for amplification by RT-qPCR.

Gene	**Primer Sequence (5′ → 3′)**
16S rRNA	*GCGGCAGGCCTAACACAT* *GCAAGAGGCCCGAACGTC*
tufA	*TGTTCCGCAAACTGCTGGACG* *ATGGTGCCCGGCTTAGCCAGTA*
sipA	*CCAACGCAATGGCGAGTCAC* *GCCGTCTCCGTTTGATGCGT*
fliD	*TCACCACCAAAATTGCCACC* *CCTTGTAACGGGCAACGGT*
invF	*TTTGCGAGCAGGCCGTTGTC* *GCGCCATCGATAAATGCCAGT*
hilA	*GGTTTAATCGTCCGGTCGTAGTG* *CCTGATCCTGCATCTGAAAAGG*
sdiA	*GCCGCCCAGCGTTTCGGATT* *AAAAGCCCAGCGCCCGGTTC*

Table S2: The immune responsive genes and primer sequences of *C. elegans* used in RT-qPCR.

Gene	**Primer Sequence (5′ → 3′)**
f49f1.6	TGCACTACTACATCCTGCCTATTC CCGGACATGTGATCATTGAG
spp-1	TGAACATCGGAACTCTTTGC TCAGCTCTTCCTCACACTCG
f38d1.3	CTGGGCCGGTATTAATTTGT GTCTTCTTCGTCACGCACAT
abf-1	TGCCTTCTCCTTGTTCTCCT ATCCTCTGCATTACCGGAAC
ama-1	CTGACCCAAAGAACACGGTGA TCCAATTCGATCCGAAGAAGC

Table S3: SWE (CC and SG) protect *C. elegans* against *S.* Enteritidis infection[1]

Treatment	**SWE added over the media**		**SWE added into the media**	
	Pretreatment of *Salmonella Enteritidis* with SWE Survival % (Mean, STDEV)	**Pretreatment of *C. elegans* with SWE Survival % (Mean, STDEV)**	**Pretreatment of *Salmonella Enteritidis* with SWE Survival % (Mean, STDEV)**	**Pretreatment of *C. elegans* with SWE Survival % (Mean, STDEV)**
***Salmonella* Enteritidis**	7.71+4.86^{f}	5.63+5.52^{f}	8.68+5.48^{e}	5.15+2.88^{f}
CC200	22.81+3.58^{e}	18.06+5.08^{e}	16.82+5.31^{de}	15.77+6.23^{e}
CC400	25.63+2.65^{de}	22.71+3.49^{de}	18.65+5.54^{d}	22.34+4.13^{de}
CC800	39.51+4.82^{c}	34.37+5.49^{c}	32.7+6.63^{c}	33.81+6.11^{bc}
SG200	30.96+5.1^{d}	28.61+4.41^{cd}	19.17+2.92^{d}	22.15+6.33^{de}
SG400	43.65+5.34^{c}	45.55+5.87^{b}	35.85+7.43^{bc}	31+5.72^{cd}
SG800	53.01+2.48^{b}	52.10+4.65^{b}	43.95+3.71^{b}	41.29+7.4^{b}
HK-*E.Coli*	82.64+1.63^{a}	83.24+1.29^{a}	82.81+1.59^{a}	85.04+2.63^{a}
HK-*S.*Enteritidis	84.17+1.82^{a}	79.97+1.46^{a}	82.27+1.66^{a}	84.77+1.98^{a}
Pooled STDev	3.85	4.44	4.89	5.16
P- value	<0.0001	<0.0001	<0.0001	<0.0001

[1]Values represent Mean ± Standard deviation from three experiments with six biological replicates on day 13 post infection. Different letters indicate statistically significant mean values (*P<0.05)*

Three concentrations of SWE (200, 400 and 800 µg/mL) were used with. Worms were grown with seaweed water extract as food supplements and were exposed to *S.* Enteritidis. SG, *Sarcodiotheca* treatment; CC, *Chondrus crispus* treatment; HK- *S.* Enteritidis, Heat killed *S.* Enteritidis; HK- *E. coli*, Heat killed *E. coli.*

Table S4:The efflux pump related genes and primer sequences used in RT-qPCR

Gene	**Primer Sequence (5′ → 3′)**
ramA	*CGTCATGCGGGGTATTCCAAGTG* *CGCGCCGCCAGTTTTAGC*
marA	*ATCCGCAGCCGTAAAATGAC* *TGGTTCAGCGGCAGCATATA*
acrB	*TTTTGCAGGGCGCGGTCAGAATAC* *TGCGGTGCCCAGCTCAACGAT*

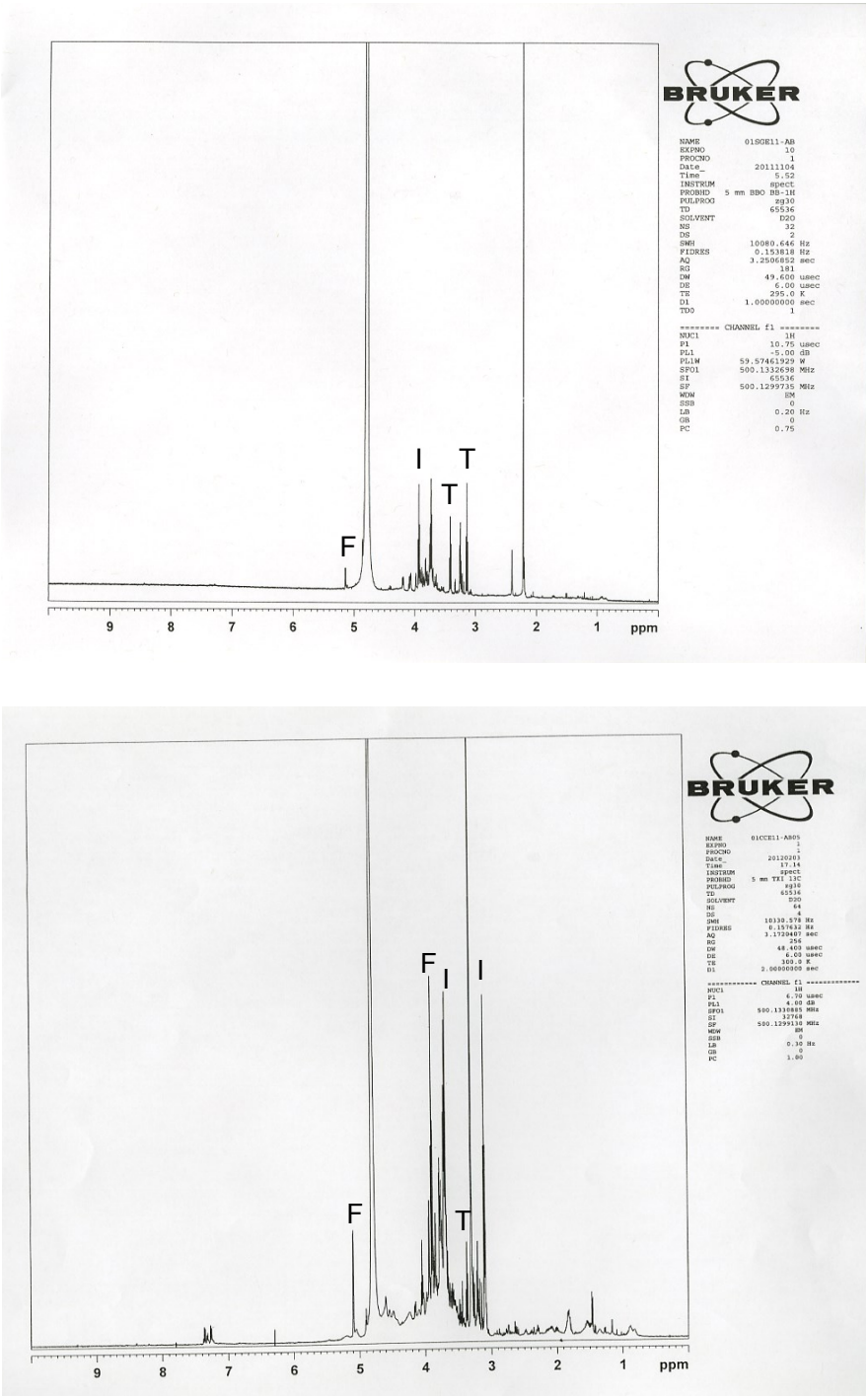


Figure S1: ^{1}H NMR of a) *Sarcodiotheca gaudichaudii* b) *Chondrus crispus*. F, Floridoside; I, Isethionic acid; T, Taurine.

Table S5: Experimental diet formulations and nutrient composition of diets

Ingredients % as feed	Seaweed meal (%)[1]							
	C	INU2	CC0.5	CC1	CC2	SG0.5	SG1	SG2
Corn	53.53	55.54	58.91	58.34	57.21	58.94	58.38	57.29
Soybean meal	19.53	20.09	19.28	19.16	18.90	19.25	19.10	18.78
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
SG Meal	-	-	-	-	-	0.50	1.00	2.00
CC Meal	-	-	0.50	1.00	2.00	-	-	-
Ground Limestone	4.86	4.91	4.91	4.91	4.91	4.91	4.91	4.91
Barley	5.00	-	-	-	-	-	-	-
Mono- Dicalcium Phosphorus	0.19	-	-	-	-	-	-	-
Oyster shell	2.43	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Shell mix	2.43	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Poultry fat	1.0	1.58	0.56	0.80	1.30	0.56	0.81	1.30
Inulin	-	2.00	-	-	-	-	-	-
MCL4[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Biophytase	-	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Methionine Premix[3]	0.135	0.14	0.15	0.15	0.17	0.15	0.15	0.17
Iodized salt	0.30	0.30	0.24	0.19	0.07	0.25	0.20	0.10
Total	100	100	100	100	100	100	100	100
Nutrient content, Calculated values								
Metabolizable energy (kcal-kg^{-1})	2820.56	2820.56	2820.56	2820.56	2820.56	2820.56	2820.56	2820.56
Protein (%)	15.22	15.22	15.22	15.22	15.22	15.22	15.22	15.22
Crude fiber (%)	2.28	2.28	2.28	2.28	2.28	2.28	2.28	2.28
Calcium (%)	3.91	3.91	3.91	3.91	3.91	3.91	3.91	3.91
Non-Phytate phosphorus (%)	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Lysine (%)	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Methionine + cysteine (%)	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58

[1]Treatment group (C: control; IN: inulin; SG0.5: contains 0.5% of *Sarcodiotheca* variant; SG1: contains 1% of *Sarcodiotheca* variant; SG2: contains 2% of *Sarcodiotheca* variant; CC0.5: contains 0.5% of *Chondrus crispus*; CC1: contains 1% of *Chondrus crispus*; CC2: contains 2% of *Chondrus crispus*.

[2]Providing per kg of diet: retinol, 7,800 IU/kg; cholecalciferol, 2,500 IU/kg ; DL-alpha-tocopherol acetate, 20 IU/kg;; thiamine, 1.94 mg/kg; riboflavin, 7.6 mg/kg; pantothenic acid, 7.2 mg/kg; niacin, 30.7 mg/kg; pyridoxine, 3.96 mg/kg; choline chloride, 641mg/kg ; vitamin K, 2.97 mg/kg; biotin 0.16 mg/kg; cyanocobalamin, 12 mg/kg; antioxidant, 1 mg; manganese, 70.2 mg/kg; zinc, 66 mg; iron, 33 mg; copper sulphate, 25 mg/kg; iodine, 0_9 mg; selenium, 0.15 mg/kg ethoxyquin, 50 mg/kg; folic acid, 0.66 mg/kg.

[3]Methionine premix is composed of 50% wheat middlings and 50% DL methionine.

Table S6: Species-specific primers of bacteria used in this study

Gene	**Primer Sequence (5′ → 3′)**
All bacteria	*TCCTACGGGAGGCAGCAGT* *GGACTACCAGGGTATCTAATCCTGTT*
Bifidobacterium longum	*TTCCAGTTGATCGCATGGTC* *TCSCGCTTGCTCCCCGAT*
Lactobacillus acidophilus	*GAA AGA GCC CAA ACC AAG TGA TT* *CTT CCC AGA TAA TTC AAC TAT CGC TTA*
Streptococcus salivarius	*TTATTTGAAAGGGGCAATTGCT* *GTGAACTTTCCACTCTCACAC*

Table S7: Dietary compositions of experimental diets containing seaweeds

Ingredients % as feed	Seaweed meal (%)[1]					
	C	ANTB	CC2	CC4	SG2	SG4
Corn	50.61	50.59	48.46	46.11	48.31	46.02
Soybean meal	24.13	24.13	23.68	23.26	23.72	23.31
Wheat	10.00	10.00	10.00	10.00	10.00	10.00
SG Meal	-	-	-	-	2.0	4.0
CC Meal	-	-	2.0	4.0		-
Ground Limestone	5.04	5.04	5.04	5.04	5.04	5.04
Mono- Dicalcium Phosphorus	1.17	1.17	1.16	1.16	1.17	1.16
Oyster shell	2.52	2.52	2.52	2.52	2.52	2.52
Shell mix	2.52	2.52	2.52	2.52	2.52	2.52
Poultry fat	2.72	2.72	3.53	4.42	3.58	4.45
Antibiotic	-	0.004	-	-	-	-
MCL4[2]	0.50	0.50	0.50	0.50	0.50	0.50
Methionine Premix[3]	0.45	0.46	0.46	0.47	0.46	0.47
Iodized salt	0.34	0.34	0.11	0.00	0.18	0.01
Total	100	100	100	100	100	100
Nutrient content, Calculated values						
Metabolizable energy (kcal-kg^{-1})	2850.03	2850.03	2850.03	2850.03	2850.03	2850.03
Protein (%)	15.59	15.59	15.59	15.59	15.59	15.59
Crude fiber (%)	2.28	2.28	2.28	2.28	2.28	2.28
Calcium (%)	4.17	4.17	4.17	4.17	4.17	4.17
Non-Phytate phosphorus (%)	0.35	0.35	0.35	0.35	0.35	0.35
Lysine (%)	0.82	0.82	0.82	0.82	0.82	0.82
Methionine + cysteine (%)	0.68	0.68	0.68	0.68	0.68	0.68

[1]Treatment group, CC2: contains 2% of *Chondrus crispus* ; SG2: contains 2% of *Sarcodiotheca* variant; CC4: contains 4% of *Chondrus crispus*; SG4: contains 4% of *Sarcodiotheca* variant; C: Control; ANTB: Antibiotics (aureomycin)

[2]Providing per kg of diet: retinol, 7,800 IU/kg; cholecalciferol, 2,500 IU/kg ; DL-alpha-tocopherol acetate, 20 IU/kg;; thiamine, 1.94 mg/kg; riboflavin, 7.6 mg/kg; pantothenic acid, 7.2 mg/kg; niacin, 30.7 mg/kg; pyridoxine, 3.96 mg/kg; choline chloride, 641mg/kg ; vitamin K, 2.97 mg/kg; biotin 0.16 mg/kg; cyanocobalamin, 12 mg/kg; antioxidant, 1 mg; manganese, 70.2 mg/kg; zinc, 66 mg; iron, 33 mg; copper sulphate, 25 mg/kg; iodine, 0_9 mg; selenium, 0.15 mg/kg ethoxyquin, 50 mg/kg; folic acid, 0.66 mg/kg.

[3]Methionine premix is composed of 50% wheat middlings and 50% DL methionine.

Appendix B

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07/10/2015

Dear Garima Kulshreshtha

RE: Garima Kulshreshtha et al. Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, affects performance, egg quality, and gut microbiota of layer hens *Poultry Science (December 2014) 93 (12): 2991-3001 first published online October 28, 2014 doi:10.3382/ps.2014-04200*

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