Effects of Red Seaweed (*Palmaria palmata*) Supplemented Diets Fed to Broiler Chickens Raised under Normal or Stressed Conditions

by

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ABSTRACT

Red seaweed (*Palmaria palmata)* (*P.p.*), was fed to broiler chickens for 33 or 35 days in two trials to compare *P.p.* to inulin and an antibiotic to determine the effective inclusion level of *P.p*. Trial one was conducted in cages to investigate antibiotic, no feed additive and different inclusion levels of *P.p.* or inulin at: 0.6%, 1.2%, 1.8%, 2.4% and 3.0% of the diet. In trial one, birds fed 1.8% *P.p.* had increased beneficial bacteria (e.g., *Lactobacillus*) in the ileum. The second trial was conducted under a combination of stressors on the floor and fed dietary levels of *P.p.* at 0.6%, 1.8% and 3% and 2.5% inulin, antibiotic or no feed additive. The stressed environment suppressed *Lactobacillus* and *Bifidobacterium* in the ileum. Broilers fed 1.8% *P.p.* were heaviour at 33 days and beneficial bacteria in the ileum, serum IgA and ileal villus width, height and surface area were all increased.

LIST OF ABBREVIATIONS USED

Adrenocorticotropic hormone	ACTH
Antibiotic growth promoter	AGP
Ascophyllum nodosum	ANOD
Bacitracin methylene disalicylate	BMD
Body weight	BW
Body weight gain	BWG
Chondrus crispus	CC
de Man, Rogosa and Sharpe Agar	MRS Agar
Deoxyribonucleic acid	DNA
EC	*E. coli*/ coliform
Enzyme-linked immunosorbent assay	ELISA
Feed conversion ratio	FCR
Feed intake	FI
Foot pad dermatitis	FDP
Fructooligosaccharides	FOS
Gastrointestinal Tract	GIT
Glucooligosaccharides	GOS
Gut Associated Lymphoid Tissue	GALT
Heat stress	HS
Hypothalamic-pituitary-adrenal	HPA
Immunoglobulin	Ig
Mannosoligosaccharides	MOS
Necrotic enteritis	NE
Next generation sequencing	NGS
Nucleotides	NS
Operational taxonomic unit	OTU
Palmaria palmata	P. p.

Polymerase chain reaction	PCR
Probiotic mixture	PM
Ribosoaml database project	RDP
Ribosomal ribonucleic acid	rRNA
Sarcodiotheca gaudichaudii	SG
Short chain fatty acid	SCFA
Strepavidin-conjugated horseradish peroxidase	SA-HRP
Stressed room	S
Tetramethylbenzidine	TMB
Unstressed room	US
Volatile fatty acid	VFA
Xylooligosaccharides	XOS

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Chapter 1 INTRODUCTION

Antibiotic growth promoters (AGP) have been commonly used in broiler chicken production to maintain health and improve efficiency. However, due to the development of microbe resistance to antibiotics used to treat human and animal infections, the European Union banned the use of AGP in poultry diets as of Jan 1st, 2006 (Janardhana et al., 2009). Both the United States Food and Drug and Health Administration and Health Canada have implemented the phase out of the use of medically important antibiotics for AGP use in animals (Food and Drug Administration 2012; Health Canada 2015).

This has led researchers to seek alternatives to AGP that improve chicken health, maintain efficiency of production and enhance safety of poultry products. Probiotic and prebiotic feed additives have potential as alternatives for AGP, since they mainly act on gut microbiota and can improve intestinal health (Patterson and Burkholder 2003). Prebiotics are defined as non-digestible ingredients that are selectively fermented and affect the growth and/or activity of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*. Consequently, production of these microbes' increases host health and decreases undesirable bacteria (O'Sullivan et al., 2010). In poultry, oligosaccharides such as inulin, mannanoligosaccharides (MOS), fructooligosaccharides (FOS) and galactooligosaccharides (GOS) have been used widely in poultry to achieve these prebiotic effects (Huyghebaert et al., 2011). Brown seaweeds, such as *Ascophyllum nodosum*, have been recommended as natural replacements for antibiotics (Dierick et al., 2009). Nori (*Porphyra tenera*) and Wakame (*Undaria pinnatifida*) are red and brown seaweeds respectively that alter microbial activity in rats (Gudiel-Urbano and Goñi, 2002). According to Hoebler et al. (2000), 5% of brown seaweed *Laminaria digitata* in a pig diet increased acetic, propionic and butyric acid concentrations in the large intestine. Kulshreshtha et al. (2014) observed that supplementing layer diets with red seaweed (*Chondrus crispus* and *Sarcodiotheca gaudichaudii*) improved performance, egg quality, and overall gut health of laying hens. Each type of seaweed has its characteristic responses and therefore, further research with *Palmaria palmata* needs to be carried out (Evans et al., 2013).

Previous results demonstrate that seaweeds may act as prebiotics and may be a suitable replacement for AGP. Research has not been conducted to evaluate the prebiotic effect of the red seaweed, *Palmaria palmata*. The proposed research will evaluate the effects of dietary inclusion levels of *Palmaria palmata* on intestine microbiota and morphology of broiler chickens. Moreover, the research will demonstrate the effect of of *Palmaria palmata* on beneficial gut bacteria population and determine the optimal dietary inclusion level for growth performance of broiler chicken.

Chapter 2 LITERATURE REVIEW

2.1 Antibiotics

2.1.1 The function of antibiotic growth promoters

For more than half a century, antibiotics have been commonly used as AGP in animal agriculture due to their various effects on poultry health including reducing animal pathogens and improving growth performance (Dibner and Richards 2005). The advantages of using AGP in the poultry industry were first introduced by Moore et al., (1946). Since then, much research has shown that using AGP in poultry diets improve growth parameters, including gain and feed efficiency (Miles et al., 2006). Previous findings have indicated that AGP do not have positive effects on the growth of germ-free animals. AGP primarily acts on the intestinal microbiota as some of the antibiotics are not able to be absorbed (Dibner and Richards 2005; Coates et al., 1963). Gram-positive bacteria, such as *Clostridium*, *Lactobacilli*, *Bifidocbateria*, and *Streptoccus* represent the majority of bacteria in healthy chickens and AGP mostly act on gram-positive bacteria (Engberg et al., 2000; Gaskin et al., 2002; Bjerrum et al., 2006). Furthermore, AGP are able to decrease the load of *Salmonella*, *Escherichia coli* (*E. coli*) and *Campylobacter*, which can be pathogenic bacteria that routinely cause subclinical diseases in mature chickens (Hughes and Heritage, 2004). Moreover, previous studies have shown that using AGP, such as procaine penicillin, in chicken diets, can enhance the growth of broilers, even though the weight and length of the small intestine were reduced (Coates et al., 1963). Antibiotics such as Bacitracin methylene disalicylate (BMD) or virginiamycin can reduce the weight and length of the intestine resulted in improved nutrient use (Miles et al., 2006; Henry et al., 1986).

In order to clarify the mode of action of AGP, four mechanisms have been reviewed to define their beneficial effects on performance:"1) AGP inhibit endemic subclinical infections, thus reducing the metabolic costs of support for the (innate) immune system. 2) AGP reduces growth-depressing metabolites (such as ammonia and bile degradation products) produced by microbes. 3) AGP reduce microbial use of nutrients and 4) AGP enhance the uptake and use of nutrients, because the intestinal walls in AGP-fed animals are thinner" (Niewold 2007). All of these opinions suggest that the properties of an

antibiotic may have a direct or indirect effect on the growth of the animal. The reduction of the intestinal microbial population could be the underlying beneficial action of AGP (Dibner and Richards 2005).

2.1.2 Concerns and problems of AGP

While the use of AGP has resulted in important achievements in animal agriculture, antibiotic resistant bacteria indirectly affect humans (Silbergeld et al., 2008). Consequently, there is a need to seek alternatives to AGP that can maintain or enrich the performance and health of broilers (Smith et al., 2003). Owing to concerns for human health, the European Union has pioneered a ban on all AGP in animal feed as of January 1, 2006. Even though the application of AGP is not completely banned in North America, the United States Food and Drug Administration (USFDA) requested that farmers decrease AGP used in livestock production in April, 2012 (US Food and Drug Administration 2012). Subsequently, Canada phased out AGP in animal agriculture for feed additives; however, AGP is still used at the therapeutic level (Canada Health, 2015).

2.2 Probiotic history, definition and microorganism

The history of the word probiotic roots from the Greek language meaning “for life”. The concept of probiotics was defined initially by Lilly and Stillwell (1965) as “substances secreted by one microorganism which stimulates the growth of another”. Since then, many scientists have revised the definition of a probiotic. Fuller (1989) defined the generally accepted interpretation as “a live microorganism which when administered in adequate amounts can confer beneficial effects on the host’s health.”

The exact mode of action of probiotics by which bacteria confers health benefits are various (Ducatelle et al., 2014). The probable modes of action of probiotics were studied by several researchers (Fuller 1989; Fooks and Gibson 2002). First, it has been proposed that probiotics act by changing the gut microbiota competition for substrate and mucosal adherence. Production of toxic compounds that compete to prevent pathogens from occupying binding sites (Yang et al., 2009), enhancing epithelial cell survival by

producing substantial amounts of volatile fatty acids such as butyrate, propionate and acetate (Gibson and Roberfroid 1995) and improving immune responses that in a way is beneficial to the host (Patterson and Burkholder 2003). This phenomenon, known as competitive exclusion, is the resistance to colonization by pathogenic and other nonindigenous microbes and it is the first important advantage provided by normal microbiota.

Second, the mode of action of probiotics indirectly affects the mucosa barrier function, in a way that is related to the quality of tight junction between intestinal epithelial cells, Paneth cells and mucus cells (Patterson and Burkholder 2003). Paneth cells secretes antimicrobial peptide compounds such as defensins and lysosome. Mucus cells play an important role by forming a protective layer shielding the intestinal cells from direct contact with bacteria (Butel 2014). Therefore, probiotics can indirectly enhance immunity by increasing the mucus layer and improving physiological barrier function, by raising the population of beneficial bacteria, including *Lactobacillus* and *Bifidobacterium* (Baurhoo et al., 2009). Consequently, probiotics, including FOS and MOS, are able to increase immunity (Dunkley et al., 2009), enhance gut maturation and integrity (Stoidis et al., 2010), decrease ammonia production and reduce pH in the gut environment of the host (Stoidis et al., 2011). There are several different kinds of probiotics, described as mono-or mixed cultures of living microorganism, which are able to alter gut microbiota composition in a way beneficial to the host (Fuller, 1991). Probiotics can be arranged or classified as colonizing species like lactic acid bacteria. Primarily *Lactobacillus* and *Bifidobacterium* genera are the common genera, in the human, opportunistic‘non-colonizing’ species like *Bacillus*, *Enterococcus*, have been the most common probiotic genera in livestock species, including poultry (Owens et al., 2008).

2.2.1 Application of probiotic in poultry production

The history of using probiotics in poultry began with Nurmi and Rantala in 1973. They observed that when the birds are raised in the sanitary conditions, the development of normal microbiota was impeded and this caused severe outbreaks of *Salmonella infantis*. In order to test these effects, newly hatched chickens were injected with a 0.5 mL

suspension of gut content obtained from a healthy adult chicken, in order to examine the response of the chicks infected with *Salmonella*. The newly hatched injected chickens were protected against *Salmonella*, due to the competitive exclusion culture which is the same mechanism as that employed by probiotics.

Further studies indicated that this approach can protect birds against *Clostridium perfringens* and *Clostridium botulinim* (Fuller 1989). Subsequent research has highlighted the crucial role of probiotic supplementation in improving health and growth of chickens. Probiotics are able to alter the balance of gut microbiota in a way that improves host health directly through the exclusion of the growth of pathogens due to the release of antimicrobial substances (Ducatelle et al., 2014). Furthermore, probiotics can indirectly affect their host by increasing lactic acid bacteria, which cause a lowering of gut pH (Niers et al., 2005). Other studies have shown that probiotics can decrease mortality by reducing adhering enteric pathogens that colonize the chicken intestine epithelium and mucosa (Timmerman et al., 2006). Moreover, studies on intestinal histomorphology of the chicken have shown that administration of probiotics can improve and alter the microstructure of the gut. Awad et al., (2009) found that a probiotic containing *Lactobacillus* increased villus height and surface area which may increase capacity for greater nutrient absorption. Enhanced absorption area, consequently, increase digestive and absorption function. Chichlowski et al., (2007) observed that *Lactobacilli* and *Bifidobacterium* supplementation as probiotics increased the jejunal villus height and decreased the villus crypt depth compared with the antibiotic salinomycin fed and non-supplemented control chicks.

Using probiotics in the poultry industry is beneficial to the health and growth of birds, but there are challenges in applying probiotics in the GI tract of the host (Patterson and Burkholder 2003). Recent research in the area of probiotic application on poultry performance varied considerably among studies (Huyghebaert et al., 2011). Some probable reasons for these differences are bacteria species type, the probiotics resistance to gastric acid, pancreatic enzymes as well as gut environment variation in terms of microbiota balance (Huyghebaert et al., 2011). Gut environment, which itself is greatly

affected by diet, can positively influence the survival of probiotic microorganisms (Huyghebaert et al., 2011).

2.3 Prebiotics as alternatives to antibiotics

Prebiotics are non-digestible by the host animal. Carbohydrates, such as FOS and MOS, aids the host by encouraging the growth, activity, or both, of one or a limited number of targeted bacteria in the gut (Gibson and Roberfroid, 1995). Gibson and Roberfroid (1995) offered several criteria for an ingredient in human foods to qualify as a prebiotic: "1) be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract; 2) be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated; 3) be able to alter the colonic biota in favor of a healthier composition; and 4) induce luminal or systemic effects that are beneficial to the host health." Galactooligosaccharides (GOS), FOS and MOS have been identified as prebiotics in humans and animals (Gibson and Roberfroid, 1995). These oligosaccharides have been found to selectively stimulate the growth and/or activity of beneficial intestinal bacteria such as *Bifidobacteria* and *Lactobacillus*. Stimulated growth of these organisms confer benefits on host well-being and health by increasing production of lactic acid and short chain fatty acids (SCFA) (Roberfroid, 2007). The use of FOS in poultry has brought positive aspects to gut microbial equilibrium by increasing beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* in the large intestine of broilers (Xu et al., 2003). MOS has been used as a dietary supplement for turkeys to improve gastrointestinal health. MOS accelerated the maturation of the gastrointestinal tract in turkey poults and enhanced the size of the villus and the crypt depth in the ileum (de los Santos et al., 2007). The ease of application has made prebiotics very popular. Although there are different types of prebiotics including carbohydrates, peptides, proteins, and lipids, the oligosaccharides are the main prebiotics of interest because they can be hydrolyzed and fermented by gut bacteria (Gibson and Roberfroid, 1995). It is possible to use prebiotics to improve the intestinal health and animal performance without AGP (Gaggia et al., 2010).

2.3.1 The effects of prebiotics on animal health

Prebiotics can have a number of effects which lead to increased health of the host as well as a decrease in the number of pathogens present in the gut (Roberfroid, 2007). Prebiotics have the greatest effect on the intestinal microbiota concentrations by influencing the SCFA production. These SCFA can be absorbed and contribute to the energy requirement of the host (Dunkley et al., 2009). High fermentation activity and high concentration of SCFA are associated with a lower pH, which limits pathogens such as *Salmonella* and *E. coli* in the gut. This is one of the desirable effects of feeding prebiotics (Barry et al., 2009). Baurhoo et al. (2007) found that 0.1 % inclusion level of MOS in a grower diet from day 29 to day 38, reduced the number of cecal *E. coli* in broilers. Supplementation of diets with 0.4% FOS inhibited the growth of *E. coli* and increased the growth of *Bifidobacteria* and *Lactobacilli* (Xu et al., 2003). Some prebiotics act as receptor mimics reducing the interactions of pathogens with host cell receptors. This is achieved by having the pathogens bind to the prebiotics instead of the host cell surface. Therefore, pathogens pass directly out of the gut attached to the prebiotics. This inhibits the invasion by pathogens (Collins et al., 2009). Another mechanism of pathogen inhibition is the increase of secretory IgA, which is necessary to clear pathogens from the gut (Hosono et al., 2003). Yin et al. (2008) found that IgA levels were increased in pigs supplemented with 0.2% of the prebiotic oligosaccharide galactomannan. In other studies in BalbC mice, 2.5% inclusion level of FOS increased the level of IgA in Peyer's patches (Hosono et al., 2003).

2.3.2 Effects of prebiotics on gut morphology

Prebiotics have a number of effects on gut morphology. Feeding 0.4% FOS to broilers increased ileal villus height, jejunal and ileal microvillus height, villus-height-to-crypt depth ratios at the jejunum and ileum, and decreased crypt depth in the jejunum and ileum on day 49 (Xu et al. 2003). Rehman et al. (2007) reported that adding 1% inulin polysaccharides, generally extracted from chicory roots, to corn-based diets of chicken, resulted in longer jejunal villi. Iji et al., (2001) found that 0.5% MOS supplementation increased the jejunal microvilli length of broilers fed a sorghum-based diet. Villi height

and goblet cell numbers in all intestinal segments increased in broilers fed MOS (Baurhoo et al. 2007).

2.3.3 Improved growth performance with prebiotics

Growth performance in broiler chickens fed the prebiotics FOS and MOS, had lower feed conversion ratios, while weight gain increased (Collins et al. 2009). However, Baurhoo et al. (2007) found that feed intake and feed conversion ratios in broiler diets did not change when supplemented with FOS and MOS. Arata et al. (2011) reported that supplementing broilers with 0.5% Tasco® (sun dried brown seaweed, *Ascophyllum nodosum*) increased the body weight and improved feed to gain ratio compared with the control group (without any feed additives) or diets containing 2.5% inulin. Although some have reported positive effects of prebiotics (Xu et al., 2003) on growth performance, others have shown negative effects (Baurhoo et al., 2009). This inconsistency in the response of the broilers to prebiotics may be due to the effects of different factors such as environment, management, nutrition, type of additive, and dosage as well as bird characteristics (age, species, stage of production) (Yang et al., 2009). Environmental conditions must be considered as an important factor. Orban et al., 1997 observed that more beneficial effects of prebiotics have been observed under suboptimal experimental conditions. For example, broilers fed diets supplemented with MOS or antibiotic (virginiamycin and bacitracin) had no significant effect on broiler performance (BW, FI and FCR) (Baurhoo et al., 2009). The growth performance in broilers in heat stress conditions was improved with prebiotics. According to Sohail et al. (2012), broilers subjected to heat stress, fed MOS had significantly higher body weight gain and lower feed conversion ratio at day 21 and day 42, compared with the birds fed basal diet (without MOS).

2.4 Synbiosis

2.4.1 The concept of synbiotics

Synbiotics can occur by combining probiotics and prebiotics in the diet. The theory, which explains the value of this combination, is that the prebiotic improves the efficacy of action of the probiotic in the gut, thereby increasing its effectiveness (Macfarlane et al., 2006). Combined administration can decrease the time of product fermentation in the gut and increase the chance of strain survival during product storage (Shafi et al., 2014). The exact mode of action concerning synbiosis depends on the adaptability of the probiotic strain to the prebiotic's sugars, in terms of the degree of polymerization and the type of oligomers (Dellaglio et al., 2002). Therefore, before using the combination of probiotic and prebiotic to achieve a synbiotic result for better health and nutrition, it is important to have the best combination of both.

2.4.2 Effect of synbiosis in animal health and growth performance

The application of prebiotic and probiotic synbiosis has shown promising results in animal health and growth. Ghasemi et al. (2010) observed that body weight gain and feed conversion ratio improved with symbiotic Biomin IMBO at 0.1% and 0.15% of broiler diets. A mixture containing fructan-type oligosaccharides and *Bifidobacterium* strains improved the *Bifidobacterium* levels in rats Awad et al., 2009). Biomin ®IMBO, containing a combination of the probiotic strain *Enterococcus faecium* (DSM 3530), a prebiotic (derived from chicory) and immune- modulating substances (derived from sea algae) were shown to be synbiotic in broiler chickens.

Similarly, Li et al. (2009) observed that the combination of probiotic (*Lactobacillus and Bacillus cereus*) and *Astragalus* polysaccharides a popular health-promoting herb in China, improved the health status of birds by increasing the amount of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, by decreasing *E. coli* and with enhancement of humoral and cellular immune function.

2.5 Inulin

Inulin is a mixture of linear polymers and oligomers of fructose, joined by a ß (2—1) glycosidic linkage, often with a terminal glucose unit (Roberfroid, 2005) (Figure 2.1). Although there are several sources of FOS, this prebiotic is generally extracted from chicory root and is found to a lesser extent in onions, garlic, bananas, asparagus, leeks and Jerusalem artichokes (Bosscher, 2009). The configuration of the ß-linkages between fructose monomers in inulin is not hydrolyzed by digestive enzymes and can favorably alter the intestinal microbiota by selectively stimulating the growth and/or activity of indigenous *Bifidobacteria* and *Lactobacilli* (Gibson, 2004; Roberfroid, 2005). Compared diets with no feed additive, Wiseman (2012) observed that feeding inulin at 2.5%, resulted in improved BWG, averaged over three growth periods (starter, grower and finisher periods).

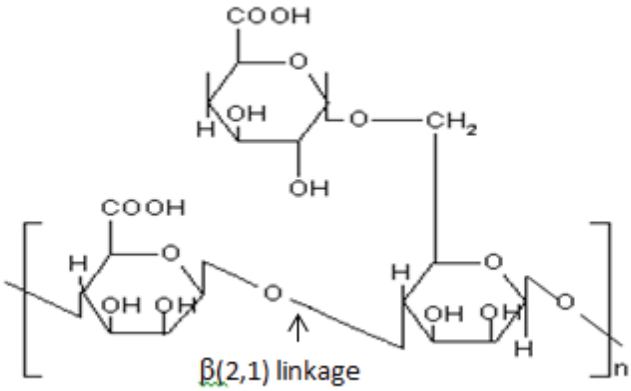


Figure 2.1: Structure of inulin from Chourasia and Jain (2003)

Rebolé et al. (2010) observed that supplementing broilers with 1% inulin increased villi height to crypt depth. Rebolé et al. (2010) observed that inulin increased the number of *Bifidobacterium* and *Lactobacillus* in the ileum and ceca of broilers when fed at 1% and 2%. Rehman et al. (2007) found that feeding 1% inulin to broiler chickens for a duration of five weeks increased the, including an increase in the length of villi in the jejunum. Inulin met all four conditions for classification as a prebiotic and is often considered to be

a reference prebiotic (Roberfroid, 2007). Wiseman (2012) observed that birds given 2.5%, inulin, in comparison with 0.5%, 1.0%, 1.5%, 2.0% and 3.0% levels of inulin performed optimally. These results highlight that the determination of optimal dietary inclusion level of a prebiotic is important.

2.6 Seaweed

Researchers are interested in the biological compounds present in seaweed since aquatic plants have specific functional compounds that are not present in land plants. Research has examined the compounds in seaweed, which inhibit common diseases, such as cancer, inflammation, arthritis, diabetes and hypertension (Wijesinghe and Jeon 2012). The three phyla of seaweed are classified according to their nutrient and chemical make-up. Green algae contain sulphuric acid polysaccharides, sulfated galactans and xylans, while brown algae have alginates and sulfated fucose-containing polymers (FCP, fucoidans), as well as laminarin (Holdt and Kraan 2011). Agars, carrageenans, xylans, sulfated galactans, and porphyrans are found in red algae (Holdt and Kraan 2011). The total dietary fiber of the main seaweed species ranges from 4% to 76% dry weight, with 51-85% being water soluble fibers. *Ascophyllum*, *Porphyra* and *Palmaria* species have the highest content of the total dietary fiber (Holdt and Kraan 2011). Seaweed or macroalgae is a good source of a variety of resistant polysaccharides or other complex carbohydrates, which cannot be hydrolyzed in the upper gastrointestinal tract. Therefore, seaweeds are considered a good source of dietary fiber (Devillé et al. 2004). The structures of resistant polysaccharides (insoluble dietary fiber) in the algal cell wall including cellulose, hemicellulose, and xylans are indigestible by humans, and therefore have high dietary fiber content (Mouristen, 2013). These polysaccharides are thought to be potential prebiotics, as they cannot be digested in the small intestine, but can undergo bacterial fermentation in the large intestine, therefore beneficially affecting the intestinal microbiota (MacArtain et al. 2007). Seaweeds have several types of soluble dietary fiber, such as agar, carrageenan, and alginate, which are not found in land plants (Holdt and Kraan 2011). Polysaccharides, proteins, lipids and polyphenols, with antibacterial, antiviral and antifungal properties are the bioactive substances in red seaweed (Chandini

et al., 2008). In addition, red seaweed contains minerals, vitamins, phlorotannins, carotenoids, amino acids compounds, which can be used in the nutraceutical and pharmaceutical industries (Holdt and Kraan, 2011). Studies using seaweeds as dietary ingredients have shown promising results in animal growth and health (Dierick et al., 2009). Similar to inulin, red seaweed could potentially be a prebiotic.

2.6.1 Effects of seaweed supplementation on health and growth of animals

Many studies have investigated dietary fibre in seaweed as a prebiotic in animal agriculture. Research involving pigs, lambs, cattle and chickens have been conducted using seaweed extracts and polysaccharides of brown and red seaweeds. Kulshreshtha et al. (2014) observed that laying hens fed with 2% red seaweed *Chondrus crispus* (*CC*) and 2% *Sarcodiotheca gaudichaudii* (*SG*) affect feed conversion ratio for egg production. Low level (0.5%) of supplemental (*CC*) and (*SG*) enhanced egg yolk weight and egg weight. Red seaweed supplementation positively affected intestinal histomorphology of the birds. Villus height and villus surface area compared with control birds were increased with supplementation of 2% (*SG*) and (*CC*). Supplementation of layer diets with red seaweeds resulted in enhanced abundance of the beneficial bacteria *Bifidobacterium longum*, *Streptococcus salivarius* (4- to 14-fold) (4- to 15-fold) increased respectively, and reduced the adverse bacteria.

Seaweeds as an animal feed addition, include a large number of studies conducted on brown seaweed, *Ascophyllum nodosum* species. Tasco®, a natural, marine-plant based has positive effect on gut microbiota, growth performance and health of animals (Evans and Critchley 2013). Previous studies indicated that supplementing Tasco®in the diet of animals under stress conditions such as livestock transportation, and increased environment temperature cause a reduction in the effect of the stress among animals to be resistant in stress conditions (Fike et al., 2001). Arata et al. (2011) found a positive effect on the growth of broilers. In comparison with feed containing no additives, Wiseman (2012) observed that feeding birds with 0.5 % Tasco improved BW and BWG. Gardiner et al. (2008) found that feeding pigs with 6% or 9% of *Ascophyllum nodosum* extract decreased ileal coliforms and increased *Bifidobacterium* in the cecal contents. In a study

which used 1.0% or 2.0% of *Ascophyllum nodosum* as a supplemental meal, the health of weanling piglets improved as the *E. coli* in the small intestine decreased and the *Lactobacillus/E. Coli* ratio increased. Similarly, Dierick et al. (2009) reported that supplementation of weanling pig diets with brown seaweed (*Ascophyllum nodosum*) at 1.0%, reduced the *E. coli* in the digestive system. Red (*Chondrus crispus*) and brown (*Undaria pinnatifida*) seaweed supplementations changed the composition and activity of microbiota in rats (Gudiel–Urbano and Goñi, 2002). Hoebler et al., (2000) determined that brown seaweed (*Laminaria digitata*) increased SCFA concentration in the large intestine of pigs, while Turner et al., (2002) observed that supplementation of pigs with 1% brown seaweed (*Ascophyllum nodosum*) increased weight gain.

2.6.1.1 Red seaweed

Red algae (*Rhodophyta*) belongs to a large and ancient group of eukaryotic algae, having 5,000–6,000 species, which are mainly multicellular and marine (Lee, 2008). *Palmaria palmata* is one of the oldest groups of eukaryotic algae and has been consumed by humans for centuries (Mouristen, 2013). The polysaccharides of red seaweed may play an important role as prebiotics, since they cannot be digested in the small intestine and may be fermented by bacteria (Gómez-Ordóñez et al., 2012). Carrageenans are a family of biomolecules extracted from linear polysaccharide chains joined to the sugar unit. The structure of carrageenan allows them to dissolve in water, form highly viscous solutions and stay stable over a wide pH range (Rasmussen and Morrissey 2007). There are three major genres of carrageenans, (kappa, lambda, and iota), which differ in the degree of sulphation and gel-forming abilities. Carrageenans play important roles from a human health perspective and food industry applications. In food uses, they are applied as food additives and have a range of gelling and emulsifying properties. They are usually used in food application as additives to modify the food texture, reduction of fat and salt, enhancement of storage ability, flavor and fiber content (Skoler-Karpoff et al., 2008). Agar originated from a mixture of polysaccharides, agaros and agaropectin, and has a functional structure in the cell of red seaweeds. In this way, fiber can be developed that can create thousands of chains. Agar is insoluble in cold water, but dissolves readily in boiling water (Holdt and Kraan 2011, Mouristen, 2013). The major polysaccharide of

Palmaria palmata, is a water-soluble xylan which has no sulfate ester or methoxyl groups. *Palmaria palmata* xylans are soluble compared to xylans originally from land plant, which are usually insoluble (Morgan et al., 1980).

The use of *Palmaria palmata* as a prebiotic for poultry has not been investigated. Therefore, it is worthwhile to conduct research to evaluate the prebiotic effect of *Palmaria palmata*, using broiler chickens.

2.7 Microbiological studies

In 1880, the agar pour plate method, used to quantify aerobic bacteria, was introduced by Koch to quantify the number of aerobic bacteria in a sample (Gilchrist et al., 1973). The current approach used for counting bacteria colonies (30-300) on agar filled perti dishes was developed by Breed and Dotterer (1916). To perform the pour plate method, an unknown sample is diluted and mixed with buffer then poured into a petri dish containing liquid agar and mixed well prior to solidification (Gilchrist et al. 1973). Typically bacteria such as *E. coli* in the incubator will produce colonies after 18-24 hours of incubation. Depending on the bacteria species only those plates within the range of 30-300 colonies would be counted and those outside of this range would be discarded.

In the past, culture-dependent methods based on the growth media, such as plating, commonly were utilized to demonstrate the gut microbiota of chickens but those methods were not completely successful in highlighting the composition of chicken intestinal microbiota (Choi et al., 2014). These methods have been suitable but they are not able to determine the total bacterial population in the gastrointestinal tract. However, this method can be time-consuming and labor intensive. Modern approaches have overcome these difficulties by determining the sequencing of bacterial genetic material extracted from the community samples (Lan et al., 2004).

2.7.1 Next generation sequencing (NGS)

Preliminary DNA sequencing was the basis of Sanger sequencing technology that has led to important developments in DNA sequencing approaches for over 20 years (Diaz-Sanchez et al., 2013). Even though, the Sanger method was an important technique, it caused problems, such as low throughput of DNA sequencings, time-consuming procedures and high cost (Diaz-Sanchez et al., 2013). The major advance offered by NGS is the ability to produce an enormous volume of data cheaply, in some cases, in excess of one billion short reads per instrument run. There are various NGS platforms, which have differing protocols, based on template preparation, sequencing, imaging, and genome alignment and assembly methods (Metzker 2010). These can be done at more reasonable costs than former methods (Metzker 2010). In 2005, Roche/454 technology was devolved (Voelkerding et al., 2009). As a basis for this approach, the genomic DNA of a few hundred base pairs is mechanically trimmed and bonded to microbeads in a 1:1 ratio. Next, they are captured in micelles of an emulsion. Template amplification is conducted on those micelles in order to acquire adequate light signal strength for reliable recognition in the sequencing-by-synthesis reaction steps. After denaturation with PCR amplification cycles, the microbeads are moved into fibre-optic slides where the four DNA nucleotides are added; it is converted to a light signal through the firefly enzyme luciferase (Wicker et al., 2006). The fiber-optic slides are excellent light guides and the other sides of them face a charge-couple device camera. The camera is able to detect the light from the DNA to determine the number of nucleotides incorporated within the original DNA (Ansorge 2009). Owing to the large numbers of reads, it is essential to determine sequencing error. Furthermore, primers resulting in an incomplete extension of DNA, lead to inaccurate estimations of operational taxonomic units (OTUs). The new developments in sequencing, such as AmpicationNoise and Chimera, can control the sequencing errors (Quince et al., 2011). The data was merged into the final file and revealed a number of significant changes in the ilea of chickens treated with *Palmaria palmata*, inulin and antibiotic, in the presence or absence of environmental stress (Pourabedin et al., 2015).

2.8 Antibodies production in the chicken

Immunoglobulins (Ig) are antibody glycoprotein molecules that consist of four peptide chains; two of the chains are heavy and two light, and by disulfide bonds. The place where antigens tie to the Igs is termed “Fab fragment” and the other portion, typically distinguished by phagocytic cells; such as neutrophils and macrophages, is called a Fc fragment. In mammals Igs are identified by IgM, IgA, IgG, IgD, and IgE, which are defined by their five different heavy chains μ, α, γ, δ, and ε, respectively. IgA and IgG are the most common Igs in the gastrointestinal tract. Chicken serum includes three classes of Ig, recognized as the homologs of mammalian IgA and IgG (Härtle et al., 2014).

2.8.1 IgA

The main antibody in the intestinal tract is IgA, or the mucosal antibody. Secretory IgA is the first line of protection for the host against many pathogens in the gut lumen. The secretion level of IgA, in chicken bile, saliva, intestinal fluid and tears, versus serum ratio for IgA is much higher than that in IgG (Lebacq-Verheyden et al., 1974)

The defensive mechanism of IgA includes protecting the intestinal epithelium from enteric pathogen and toxins (Lewis et al., 2010). It can therefore alter the microbial populations and prevent pathogenic microflora overgrowth by targeting the immune system (Lewis et al. 2010). In another study, Prez-Carbajal et al., (2010) observed chicken IgA means range from 0.051±9.8 to 0.140±25.2 mg/ml at day 14 increased to 0.325-0.369 mg/ml after disease challenge at day 28. The average concentration of IgA in chicken serum is about 0.33 mg/ml and represents less than 4% of the total immunoglobulins, of which chicken IgA is predominant in chicken bile and intestinal secretions (Lebacq-Verheyden, 1974).

IgA is made in a specific immune tissue called “Peyer’s patch and secreted into the gut lumen, where it protects the apical surface of the brush border (Lewis et al., 2010). The preliminary shape of secretory IgA is polymeric, while the serum type is a monometric form. In serum, monomeric IgA constitute 7%–15% of the serum antibody. Most IgA is secreted as a dimer within mucosal fluids (Manz et al., 2005). It has been shown that

administration of probiotics and prebiotics significantly increased immunization with antigens or immunogens in chicken IgA titers at both the serum and the mucosal levels (Haghighi et al., 2006; Agunos et al., 2007). Previous studies have shown that feeding birds with 1% oligosaccharides extracted from palm kernel increased IgA levels from 0.539±52.85, mg/ml, compared to no feed additive groups 0.263±52.85, mg/ml. Intestinal pathogens are the main targets of IgA. Various antigens, such as food antigens, commensal bacteria, and pathogenic bacteria, adversely influence the epithelial layer in the gastrointestinal tract. Kim et al., (2011) observed that supplementation of broiler feed with (0.25% and 0.5%) FOS (0.586±0.45 and 0.575±0.45 mg/ml) and (0.25% and 0.5%) MOS (0.591±0.45 and 0.599±0.45 mg/ml) did not significantly alter the concentration of IgA, compared to the no feed additive group (0.586±0.45 mg/ml). Therefore, IgA secretion play an important role in protecting the gastrointestinal tract by preventing the adherence of pathogenic bacteria (Ohashi et al., 2006)

2.8.2 IgG

Even though the IgA is the prominent immunoglobulin formed in the gastrointestinal tract, IgG is the main serum antibody and has a role in the mucosal immune response. IgG is the main antibody active in infections and the predominant form in sera (Prez-Carbajal et al., 2010). IgG is extremely opsonic, supporting phagocytosis by leukocytes. Antibodies of the IgG subclasses are most abundant, making up about 75% of total serum antibody levels in animals and mainly synthesized by the plasma cells of spleen and lymph nodes (Manz et al., 2005). Immunity within the mucosa is due to the effect of IgA and makes up a crucial component of humoral immunity, giving immunoprotection to the digestive tract system. IgG binds to the antigens and helps to remove bacteria. IgG compared to IgA and IgM has longer life and can shuttle between serum and endothelium surfaces (Manz et al., 2005). Janardhana et al., (2009) observed that FOS administration in broiler chickens and IgG antibody levels in the plasma were enhanced (Härtle et al., 2014).

2.8.3 IgM

Chicken IgM is the first antibody made by lymphocytes after primary immunization; there is a short lag phase before other immunoglobulin (e.g. IgA) is produced. IgM is expressed on the surface of most chicken B cells. During the course of an immune response, IgM is produced during the first week post-infection (Rathnapraba, 2007). Salim et al. (2013) observed that supplementation of direct-fed microbes in the bird's diet increased the IgA level. Similarly, Janardhana et al. (2009) reported that FOS increased IgM, IgG and B cells in birds.

2.9 Mucosal immune system in chicken intestine

The immune system in the chicken intestine is the site of interaction between microbes and feed ingredients and functions to defend against invasion by pathogens in the intestine (Forstner and Forstner 1994.). Routinely, pathogens enter from the environment to the animal mucosal surface of the gastrointestinal, respiratory and reproductive tracts. Thus, the mucosal immune system plays an important role to block the entry of pathogens via the mucosal tissues. The mucosal immune system of the gut, which is largest immune organ in poultry, is highly developed and recognized as gut-associated lymphoid tissue (GALT). It involves several lines of defense. GALT consists of Peyer's patches, cecal tonsils, the bursa of fabricius, Meckel's diverticulum and different lymphoid aggregates (Befus et al., 1980; Muir et al., 2000). Phagocytic M cells, which cover Peyer's patches, produced by lymphoid aggregates, attack foreign antigens and transport them from the lumen to follicular environments. The composition of the diet play an important role on intestine histomorphology by altering the structure of villi, crypts, and of mucosa (Awad et al., 2009).

2.10 Heat stress

A number of studies have been conducted on the effect of heat stress (HS) on broilers (Sohail et al., 2012). Heat stress, a combination of temperature and relative humidity has several serious and economic effects on poultry production and is a crucial environmental problem. HS can adversely affect broilers in different ways, such as endocrine disorders, decreasing metabolic rate, reducing feed consumption, decreasing body weight gain, increasing feed conversion ratio and intestinal microbial imbalance (Sohail et al., 2012). As a case in point, in broilers, HS can disrupt the stable balance of intestinal microbiota (Lan et al., 2004, Sohail et al., 2010, 2011), which elevates the growth of *Enterobacteriaceae* and *Staphylococcus* in the intestine (Suzuki et al., 1983). Thus, it can increase mortality and decrease feed consumption, which causes reduced body weight gain in broilers (Suzuki et al., 1983).

It is well documented that the optimal environmental temperature for the growth of birds on litter should be near 21 °C, which causes an improvement in the feed consumption and body weight gain (Recce and Deaton, 1971). The normal body temperature of fowl is in the range of 41 to 42°C and by increasing the environmental temperature, their body temperature will consequently be increased (Donkoh, 1989). Yahav et al., (1995) observed that broilers had maximal growth performance and feed intake at 60% and 56% relative humidity between the ages of 4 and 8 weeks.

High humidity and ambient temperatures cause secretion of corticosteroids (Beard and Mitchell, 1987), which have catabolic roles in the metabolic mechanism in poultry and therefore, substantially decrease animal growth performance (Lan et al., 2004; Sohail et al., 2010, 2011). HS enhances the concentration of adrenal corticosterone, and is associated with a decline in circulating thyroid hormone (T_3 and T_4) levels in plasma (Garriga et al., 2006). In poultry, heat stress conditions result in greater levels of cortisol and adrenocorticotropic hormone (ACTH) or corticotropin-releasing hormone being circulated. This results in higher activity of hypothalamic-pituitary-adrenal (HPA), which leads to cardiovascular diseases, gastrointestinal lesions, and modified immune responses (Siegel, 1995).

Many studies have been conducted to determine whether prebiotics may improve the intestinal microbiota, by creating some elements that are absorbed from the gut and changed in the hypothalamic-pituitary-thyroid and HPA axes. Sohail et al., (2012) observed that feeding 0.5% MOS to birds increased body weight gain and decreased FCR, compared with a negative control, which was held at the normal ambient temperature. Furthermore, birds which were supplemented by a 1% probiotic mixture, in a heat stress environment, had greater villus width and surface area, in comparison with control without any feed additive.

2.11 Litter

Using the same litter to raise more than one flock is commonly practiced in the United States of America poultry industry as this method of use of bedding material is considered economical and effective (Nagaraj et al., 2007). Nevertheless, bedding type and quality can have an important effect on health and performance of birds. Increased moisture in the litter caused an increase in the incidence of footpad dermatitis (Martland 1985; Bilgili et al., 2009). Concentrations of ammonia (NH_3) generated by microbial breakdown of excreta in the used litter material, decreased bird growth performance and endangered health (Shepherd and Fairchild 2010; Martins et al., 2013). Stress, disease and diet are the important factors which can affect the microbial communities of the GIT of healthy adult birds and destabilize bacterial colonization of the GIT (Sohail et al., 2012).

2.12 Microbiota species of interest

The intestinal tract of chickens consist of a complex and dynamic microbial community which affect the health and growth of the host animal. This microbial community condition may change with various factors, such as pathogen invasion, diet, environmental stress and antibiotic application (Ducatelle et al., 2014, Sohail et al., 2012). Environmental stress can have adverse effects on young animals by changing the

composition and activity of the gut microbiota (Dale and Fuller 1979). As a case in point, Suzuki et al., (1983) observed that bacterial population composition in the chicken intestine was affected under heat stress resulting in supression of BWG and FCR. Therefore, the balance of microflora population between beneficial and adverse bacteria plays an essential role in the health of birds. Beneficial bacteria such *Bifidobacterium* and *Lactobacillus* in the gut exert positive effects on many of the host's systems, including improved metabolism, health and growth (Amit-Romach et al. 2004). These play an important role in the development of the intestinal immune system and prevent pathogen adhesion to host cells (Gabriel et al. 2006). Contrarily, adverse or undesirable bacteria can endanger the health and growth of birds (Gabriel et al. 2006).

2.12.1 Beneficial bacteria

Indigenous *Lactobacillus* and *Bifidobacterium,* in the gastrointestinal tract of chickens, are beneficial species of interest. They positively influence the health of the birds by competition with pathogens, thus reducing the proliferation of pathogenic species in the intestine (Gibson and Roberfroid 1995). *Lactobacillus* is a gram positive, strictly fermentative, mostly facultative genus (Fooks and Gibson 2002). *Bifidobacterium* is a gram positive and non- spore forming rod-shaped organism (Fooks and Gibson 2002). *Lactobacillus* species make up most of the dominant species of microbiota in the chicken gastrointestinal tract, especially in the ileum and duodenum (Lu et al., 2003). Beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium,* improve host health and decrease pathogen growth by the production of SCFA, which not only provides energy for the chickens, but also indirectly benefits them by lowering cecal pH and increasing mineral absorption. Their fermentation creates lactic, acetic and butyric acids, which play an important role in reducing the pH in the gut (Pourabedin et al., 2015).

Butyrate is an important energy source for cecal epithelial cells and inhibits inflammatory responses by acting on proinflammatory cytokines (Eeckhaut et al., 2011). Chichlowski et al. (2007) observed that after feeding chickens with the probiotics *Bifidobacterium thermophilum* and *Enterococcus faecium,* changes in intestinal structure resulted.

Increased jejunal villus height and decreased crypt depth occurred, compared to diets fed with Salinomycin and the control. Additionally, using *Lactobacillus* as a probiotic in poultry production improved growth, overall body weight and feed conversion ratio, compared with control groups without any antimicrobials (Kim et al., 2011). Furthermore, dietary *Lactobacillus* addition improved digestive health by blocking the binding sites in the intestinal wall for *Salmonella* adhesion (Gusils et al., 1999). Ghareeb at al. (2012) found that administration of *Lactobacillus* reduced the colonization of *Campylobacter jejuni* in broiler chickens**.**

2.12.2 Adverse bacteria

Clostridium perfringens is a gram positive, spore forming, toxigenic anaerobic bacteria found in soil, dust, feces, feed, poultry litter, and intestinal contents (McDonel 1980). Necrotic enteritis (NE) usually occurs in broiler chickens 2 to 6 weeks after hatching, is described as having a sudden onset of diarrhea and mucosal necrosis originating from the overgrowth of *C. perfringens* in the small intestine (Fukata et al., 1991). Altering the population distribution of the regular intestinal microbiota may raise the normal number of *C. perfringens* in the intestine resulting in the development of clinical NE or mortality (Kondo 1988). *C. perfringens* exhibits various toxic effects, categorized into Types A to E. Type A is the most prevalent toxinotype in the environment. The toxin causes an important effect on human and animal health (Van Immerseel et al., 2004). *Clostriduim perfringens* type A, and to a lesser extent type C can cause infection in poultry (Van Immerseel et al., 2004).

The addition of prebiotics, such as MOS, to chicken feed mixed with competitive exclusion cultures, caused a decreased mortality and reduced subclinical effects of *C. perfringens* on feed efficiency, similar to birds receiving the AGP, bacitracin methylene disalicylate (Hofacre et al., 2003). Similarly, Fukata et al. (1991), observed that supplementation of chicks, by supplying probiotic cultures of *Lactobacillus acidophilus* or *Streptococcus faecalis* in their feed, decreased the pathogenic effects of *C.*

perfringens. Therefore, to address the demand for a decrease in the use of AGP in the poultry industry, it is necessary to find a suitable replacement, which would limit the emergence of NE and provide better nutrition, without antibiotics (McDevitt et al., 2006).
E. coli are gram-negative bacilli which are facultative anaerobes, showing both fermentive and respiratory metabolism and they develop individually or in pairs in liquid media. *E. coli* is one of the most serious concerns, causing mortality and disease and therefore, large economic losses in the poultry industry (Ron 2006). Environmental conditions, such as poor litter quality, dust, ammonia and high temperature, can increase the risk of infection by *E. coli* (Barnes and Gross 1997*)*. Therefore, it is critical to control stress by protecting birds from environmental challenges, which impact the effective functioning of the immune system. Huff et al., (2006) observed that feeding birds yeast β-1, 3/1, 6-glucan in an experiment where birds were exposed to a pathogenic *E. coli* strain, lessened the decrease in growth performance (Huff et al., 2006)
Salmonella are facultatively anaerobic, gram-negative rod-shaped bacteria that belong to the family of *Enterobacteriaceae.* Infections with *S. enterica, E. coli* and *Campylobacter* are the most usual causes of food poisoning in the world. *Salmonella* are able to colonize without symptoms in bird (Dunkley et al., 2009). *Salmonella* under disease conditions, causes an infection in humans after consumption of contaminated poultry meat, water or eggs, causing gastroenteropathy which involves fever, diarrhea and abdominal pain (Davies and Wray 1996).

2.12.3 Size of bacteria populations

Choi et al., 2014 studied the microbial communities of the crop, gizzard, duodenum, jejunum, ileum, cecum, and large intestine. They observed that phylum *Firmicutes* was the most dominant component in most regions of the gut (Pourabedin et al., 2015). The phyla *Proteobacteria* and *Actinobacteria* were more abundant in the upper GIT, including crop, gizzard, duodenum, jejunum, and ileum, than in cecum and large intestine, whereas *Bacteroidetes* tended to be abundant in the latter segments (Choi et al., 2014). At the lower phylogenetic levels, the members of *Lactobacillales* (51.8 ± 34.5%)

were abundant in the upper GIT, whereas *Clostridiales* (55.9 ± 31.4%) was abundant in the cecum and large intestine (Choi et al., 2014).

Based on traditional microbial profiling methods, the dominant species in the crop and small intestine (duodenum, jejunum, and ileum) of chicken is *Lactobacilli* while in the cecum the dominant species is *Clostridia*. *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla form the majority of the GIT microbiome (Pourabedin et al., 2015)

2.13 Focus of the literature

Prebiotics are ingredients being explored as feed additives with the potential to replace antibiotics. They have proven to have a great effect in enhancing gut health, nutrient absorption, and growth in livestock (Collins et al. 2009). Prebiotics selectively increase beneficial populations such as *Biofidobacterium* and *Lactobacillus* in order to increase beneficial fermentation products including SCFA (Gibson and Roberforid 1995). Tasco®, a candidate prebiotic made from sun-dried brown seaweed (*Ascophyllum nodosum* (ANOD)) has been found to have positive effects on the growth of broilers (Arata et al. 2011). In other studies, brown seaweed has been observed to have antioxidant, antimicrobial, and immunomodulatory actions and has resulted in increased growth and improved gut health in pigs (Dierick et al. 2009). In rats, feeding red seaweed (*Porphyra ternera*) or brown seaweed (*Undaria pinnatifida*) altered the composition and metabolic activity of intestinal microflora (Gudiel-Urbano and Goñi, 2002). These results show red and brown seaweed to have the potential to act as prebiotics. The effects of *Palmaria palmata* on gut microbe populations are not known. It has not yet been determined if the *Palmaria palmata* meets the criteria as a prebiotic. To evaluate *Palmaria palmata* function as a prebiotic, further research must be carried out. Further research is required, to determine the prebiotic effect of *Palmaria palmata* and the optimal levels at which it is effective in broiler diets. It is well documented that the efficacy of prebiotic may differ due to factors such as environment, management, nutrition, type of additive and dosage and when stressors are present (Yang et al., 2009). A number of studies have been conducted on the effect of stressor such as heat stress and dirty litter, as they are present in most commercial setting (Bailey et al., 1991; Orban et al., 1997).

Environmental stress may be the result of using dirty litter, elevated humidity, high temperature and ammonia. The evaluation of microbial composition and intestinal morphology of broiler gastrointestinal tract by NGS and culture-dependent methods, can determine whether changes have occurred to beneficial bacteria. The traditional culture-based technique was limited by the number of cultivable bacterial species present. Therefore, next generation sequencing techniques should be applied to investigate the complex microbial populations of poultry.

In order to examine whether microbial changes influence intestinal morphological parameters, ileum segments should be examined for changes in villus width, height, and surface area, crypt depth and mucosal thickness. Intestinal morphological structure can be improved by a well-balanced bacterial community. Prebiotics positively affect intestinal morphological structure by fermenting in the gut and producing SCFA (mainly acetate, propionate and butyrate), which are energy sources for epithelial cells. SCFA can indirectly benefit the host by decreasing cecal pH, enhancing mineral absorption and preventing growth of pathogens. Thus, the effects of *Palmaria palmata* on the growth performance and immune function of the gut by changing the IgA can be investigated. Prebiotics enhance the immunity of broiler chickens and hence, the level of IgA is an indicator of the birds' response to prebiotic supplementation. The secretion of IgA can be affected by prebiotics and plays an essential role in the mucosal immune system by inhibiting pathogenic bacteria and neutralizing biologically active antigens (Rezaei et al., 2015). The mechanism by which *Palmaria palmata* contributes to the health and growth of animals is not yet understood and therefore, further research should be conducted.

Chapter 3 EFFECTS OF RED SEAWEED (*PALMARIA PALMATA*) SUPPLEMENTED DIETS FED TO BROILER CHICKENS

3.1 Abstract

This study investigated the effects of dietary supplementation with inulin or red seaweed (*Palmaria palamata*) on the growth performance, the small intestinal microbiota, and the immune response of broilers. Seven hundred and twenty, 1-d-old, Ross 308 female broilers were randomly assigned to 12 dietary treatment groups: inulin and *Palmaria palmata*, at different levels: 0.6%, 1.2%, 1.8%, 2.4% and 3%. The 0% of inulin or *Palmaria palmata* contained no feed additive or antibiotic. The randomized complete block design had dietary treatment as the main factor and room was as a block. All treatments were equally represented in two rooms. Each treatment was fed to 4 replicate cages of 15 birds per cage, for 5 wk. Twenty four birds initially, and 3 birds per cage at 7, 14, 24 and 35 days of age, were randomly selected and euthanized by cervical dislocation, for microbiological, histological and immunological analysis sampling. On d 24, cecal content pH decreased ($P < 0.05$) for inulin at 1.2% (5.42 ± 0.05) and red seaweed at 1.8 % (5.53 ± 0.05), compared to the no feed additive (5.87 ± 0.05). No differences were found among the control and the supplemented groups in overall weight gain, feed intake and feed conversion ratio ($P>0.05$). No differences were observed in total bacterial counts and intestinal morphology among treatments ($P>0.05$). There was a significant increase in the relative abundance of beneficial bacteria (e.g., *Lactobacillus* genus) in the ileum. The overall microbial diversity was not affected by treatments with red seaweed, inulin, antibiotic and no feed additive.

Keywords: prebiotic, inulin, red seaweed, NGS, histology, poultry

3.2 Introduction

Many studies in animal agriculture have been conducted to find alternatives to AGP (Pourabedin et al., 2015). Prebiotics are one of the most promising alternatives to AGP. Prebiotics beneficially affect the host animal's health by providing a substrate for the growth and establishment of beneficial bacteria within the gut (Gibson and Roberfroid 1995). Supplementation with prebiotics improved the immune function in the gut measured by increasing IgA (Agunos et al., 2007). Compounds such as complex polysaccharides contained in seaweeds may have prebiotic effects, which could potentially improve intestinal health and animal performance in the absence of AGP (Kulshreshtha et al., 2014). Previous studies have indicated that seaweeds such as Tasco®, *Chondrus crispus* and *Sarcodiotheca gaudichaudii* contain components which may act like prebiotics in broiler and layers (Wiseman 2012 and Kulshreshtha et al., 2014). Inulin is a reference prebiotic in human studies (Bosscher 2009; Gibson and Roberfroid 1995). The exact effects of *Palmaria palmata* are relatively unknown.

Therefore, comparing *Palmaria palmata* with inulin provides a reference point, as there are no scientific studies comparing inulin and *Palmaria palmata* on broiler performance. The present study was designed to determine if *Palmaria palmata* contains constituents that may have prebiotic effects in broiler chickens causing improved growth performance, changing in gut microbiota and change intestinal histological status of broilers.

3.3 Objectives

1) To determine the optimal dietary inclusion level of *Palamria palmata* growth performance of broilers under normal environmental conditions.
2) To determine the effect of dietary inclusion of *Palmaria palmata* on the health of broilers by examining small intestine morphology, bacteria, ceca pH and IgA under normal environmental conditions.

3.4 Hypothesis

1) Growth performance of the broiler chickens fed diets with the optimal levels of *Palmaria palmata* will be greater than those fed inulin or antibiotic and no feed additive.
2) *Palmaria palmata* will demonstrate the characteristics of a prebiotic, specifically by improving the health of broilers in terms of intestinal morphology and microbiota, ceca pH and IgA.

3.5 Materials and methods

In this trial, red seaweed was compared, level by level, with a commercial inulin product Oliggo- Fiber™ DS2 inulin provided by Cargill Inc. (Wayzata, MN). A positive control group included an antibiotic (Bacitracin Methylene Disalicylate (BMD)) commercially used in broiler production and a negative control group where no feed additives were included. This study investigated the use of *Palmaria palmata* fed to broilers in cages.

3.5.1 Management and housing

Seven hundred and twenty, one-day-old, female, broiler chickens (Ross 308) were obtained from the Faculty of Agriculture, Dalhousie University Hatchery in Truro, NS. The birds were vaccinated by injection with 0.05 mL of Marek's vaccine on the day of hatch. Birds were placed randomly in cages in two identical controlled environment rooms in the Atlantic Poultry Research Centre (APRC) with the same conditions set for temperature, humidity, lighting schedule and birds density. Chicks were randomly distributed to 48 cages (60cm x 48cm) per room, at a stocking rate of 15 birds per cage (1.92 kg/m^2). Three broilers were euthanized during sampling on days 7, 14 and 24 and

35 and stocking rate was 6 per cage at day 35. The initial temperature of the rooms at bird arrived was 32ºC and the temperature at the end of trial (day 35) was 21ºC. Lighting duration and intensity was set, according to the schedule described in Appendix A. Feed from troughs at the front of the cages and water from two nipple drinkers per cage were supplied *ad libitum* to the birds, throughout the trial. . Birds were introduced to water by dipping beaks in the nipple drinkers.The care of birds was conducted in accordance with Canadian Council on Animal Care guidelines (CCAC 2009). Feed was measured into the troughs as needed and uneaten feed was weighed on each weigh day. At 0, 7, 14, 24 and 35 days of age, the birds were group weighed per pen and feed was weighed back and recorded. Feed provided was weighed and recorded each day at feeding. When mortalities occurred, it was recorded, the dead birds was weighed, and the feed of that cage weighed back (Avian Pathology Lab, Nova Scotia). The cause of mortality was determined from necropsy by a veterinary pathologist.

3.5.2 Diets and seaweed preparation

3.5.2.1 Seaweed preparation

Dried, samples of cultivated *Palmaria palmata* were provided by Acadian Seaplants Limited, Nova Scotia, Canada. Freshly harvested biomass was dried in a batch fluidized bed drier, at 50º C until a moisture content of 12% was reached then stored for future use. This biomass was then ground to a powder that passed through a screen mesh size, 0.4 mm using a micro Wiley mill, standard model 3 (Arthur H. Thomas Co., Philadelphia, PA). The crude protein content of *Palmaria palmata*was reported to be 19.0 % on dry matter basis (Lourenco et al., 2002). Grounded seaweed was stored at room temperature until in incorporated into diets.

3.5.2.2 Diet preparation

Diets were formulated to be isocaloric and isonitrogenous within the starter (day 0-14), grower (day 15-24) and finisher (day 25-35) phases. Diets were fed in mash form throughout the trial (Appendix D). Diet composition for starter diet treatments including *Palmaria palmata* diets (Table 3.1) and inulin diets (Table 3.2) were fed until at day 15. Grower diets (Table 3.3 and Table 3.4) were fed until day 25.

Table 3.1. Diet formulations used in the starter period (day 0-14) containing levels *Palmaria palmata*, antibiotic or no feed additive.

			Palmaria palmata				
	No feed additive	Antibiotic	0.6%	1.2%	1.8%	2.4%	3%
Ingredient (% as fed)							
Corn	43.26	43.25	42.38	41.51	40.64	39.77	38.90
Soybean Meal	38.95	38.95	38.87	38.77	38.64	38.58	38.94
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Tallow-grease	3.64	3.64	4.02	4.41	4.79	5.17	5.56
Limestone, ground	1.67	1.67	1.67	1.66	1.66	1.66	1.66
Mono-dicalcium phosphorus	0.83	0.83	0.83	0.84	0.84	0.85	0.85
Methionine premix[1]	0.63	0.63	0.64	0.64	0.64	0.65	0.65
Vit/min/px[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.45	0.42	0.47	0.38	0.36	0.34	0.32
Lysine-HCL	0.05	0.06	0.05	0.05	0.05	0.05	0.04
BMD[3]	-	0.004	-	-	-	-	-
Palmaria palmata[4]	-	-	0.60	1.20	1.80	2.40	3.00
Total	100	100	100	100	100	100	100
Calculated analysis							
Metabolizable energy (kcal/kg)	3050	3050	3050	3050	3050	3050	3050
Protein (%)	23.0	23.0	23.0	23.0	23.0	23.0	23.0
Calcium (%)	1.05	1.05	1.05	1.05	1.05	1.05	1.05
Lysine (%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Methionine (%)	0.70	0.70	0.70	0.70	0.70	0.70	0.70
Methionine+Cysteine (%)	1.07	1.07	1.07	1.07	1.07	1.07	1.07
Sodium (%)	0.19	0.19	0.19	0.19	0.19	0.19	0.19
Available P (%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Determined analysis							
Protein (%)	23.3	23.2	23.3	23.0	22.6	22.0	23.0
Total calcium (%)	0.78	0.74	0.75	0.78	0.80	0.83	0.83
Total phosphorus (%)	0.80	0.77	0.74	0.76	0.75	0.77	0.75
Sodium (%)	0.20	0.18	0.20	0.19	0.22	0.22	0.27
Crude fat (%)	6.66	6.75	7.05	6.92	7.50	8.18	8.43

[1] The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vitamin/min/px contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3] BMD: medicated bacitracin methylene disalicylate premix at 0.04% level

[4] *Palmaria palmata* provided by Acadian Seaplants Ltd. (Dartmouth, NS)

Table 3.2. Diet formulations used in the starter period (day 0-14) containing levels of inulin.

	Inulin				
	0.6%	1.2%	1.8%	2.4%	3%
Ingredient (% as fed)					
Corn	41.98	40.72	39.62	38.20	36.94
Soybean Meal	39.18	39.40	39.47	39.85	40.05
Wheat	10.00	10.00	10.00	10.00	10.00
Tallow-grease	4.09	4.53	4.98	5.42	5.86
Limestone, ground	1.66	1.66	1.66	1.66	1.65
Mono-dicalcium phosphorus	0.84	0.84	0.86	0.86	0.87
Methionine Premix[1]	0.63	0.64	0.64	0.64	0.64
Vit/min/px[2]	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.42	0.43	0.43	0.43	0.45
Lysine	0.05	0.04	0.04	0. 04	0.04
Inulin[3]	0.60	1.20	1.80	2.40	3.00
Total	100	100	100	100	100
Calculated analysis					
Metabolizable energy (kcal/kg)	3050	3050	3050	3050	3050
Protein (%)	23.0	23.0	23.0	23.0	23.0
Calcium (%)	1.05	1.05	1.05	1.05	1.05
Lysine (%)	1.43	1.43	1.43	1.43	1.43
Methionine (%)	0.69	0.70	0.70	0.70	0.70
Methionine +Cysteine (%)	1.07	1.07	1.07	1.07	1.07
Sodium (%)	0.19	0.19	0.19	0.19	0.19
Available P (%)	0.50	0.50	0.50	0.50	0.50
Determined analysis					
Protein (%)	23.1	23.1	23.4	23.0	22.6
Total calcium (%)	0.80	0.65	0.76	0.96	0.71
Total phosphorus (%)	0.73	0.68	0.70	0.62	0.75
Sodium (%)	0.22	0.18	0.23	0.22	0.19
Crude fat (%)	6.90	7.25	7.52	8.71	8.45

[1] The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vitamin/min/px contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3]Oliggo-Fiber DS2 inulin (Cargill Inc., Wayzata, MN)

Table 3.3. Diet formulations used in the grower period (day 15-24) containing levels of *Palmaria palmata,* antibiotic or no feed additive

			Palmaria palmata				
	No feed additive	Antibiotic	0.6%	1.2%	1.8%	2.4%	3%
Ingredient (% as fed)							
Corn	50.86	50.76	49.98	49.11	48.24	47.37	46.56
Soybean Meal	31.19	31.21	31.10	31.01	30.91	30.82	30.70
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Tallow-grease	4.21	4.25	4.59	4.98	5.36	5.74	6.10
Limestone, ground	1.43	1.43	1.43	1.43	1.42	1.42	1.42
Mono-dicalcium phosphorus	0.71	0.71	0.71	0.72	0.72	0.73	0.73
Methionine Premix[1]	0.59	0.59	0.59	0.59	0.59	0.60	0.60
Vitamin/min/ px[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.40	0.40	0.38	0.36	0.34	0.32	0.29
Lysine	0.12	0.12	0.11	0.11	0.11	0.11	0.10
BMD[3]	-	0.04	-	-	-	-	-
Palmaria palmata[4]	-	-	0.60	1.20	1.80	2.40	3.00
Total	100	100	100	100	100	100	100
Calculated analysis							
Metabolizable energy (kcal/kg)	3150	3150	3150	3150	3150	3150	3150
Protein (%)	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Calcium (%)	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Lysine (%)	1.24	1.24	1.24	1.24	1.24	1.24	1.24
Methionine (%)	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Methionine+Cysteine (%)	0.95	0.95	0.95	0.95	0.95	0.95	0.95
Sodium (%)	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Available P (%)	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Determined analysis							
Protein (%)	19.8	18.9	19.2	19.3	19.7	19.04	19.03
Total calcium (%)	0.71	0.70	0.68	0.68	0.71	0.71	0.63
Total phosphorus (%)	0.64	0.64	0.73	0.68	0.70	0.68	0.68
Sodium (%)	0.20	0.18	0.14	0.18	0.16	0.23	0.22
Sodium	7.14		8.05	7.68	7.60	8.14	8.34

[1] The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vitamin/min/px contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3] BMD: medicated bacitracin methylene disalicylate premix at 0.04% level

[4] *Palmaria palmata* provided by Acadian Seaplants Ltd. (Dartmouth, NS)

Table 3.4. Diet formulations used in the grower period (day 15-24) containing levels of inulin.

	Inulin				
	0.6%	1.2%	1.8%	2.4%	3%
Ingredient (% as fed)					
Corn	49.59	48.3	47.07	45.80	44.59
Soybean Meal	31.41	31.6	31.84	32.06	32.26
Wheat	10.00	10.0	10.00	10.00	10.00
Tallow-grease	4.66	5.10	5.55	5.99	6.41
Limestone, ground	1.43	1.40	1.42	1.42	1.41
Mono-dicalcium phosphorus	0.72	1.20	0.73	0.74	0.74
Methionine Premix[1]	0.59	0.70	0.59	0.59	0.59
Vitamin/min/ px[2]	0.50	0.6	0.50	0.50	0.50
Iodized salt	0.40	0.5	0.40	0.40	0.41
Lysine	0.11	0.40	0.10	0.09	0.09
Inulin[3]	0.60	1.20	1.80	2.40	3.00
Total	100	100	100	100	100
Calculated analysis					
Metabolizable energy (kcal/kg)	3150	3150	3150	3150	3150
Protein (%)	20.0	20.0	20.0	20.0	20.0
Calcium (%)	0.90	0.90	0.90	0.90	0.90
Lysine (%)	1.24	1.24	1.24	1.24	1.24
Methionine (%)	0.63	0.63	0.63	0.63	0.63
Methionine+Cysteine (%)	0.95	0.95	0.95	0.95	0.95
Sodium (%)	0.18	0.18	0.18	0.18	0.18
Available P (%)	0.45	0.45	0.45	0.45	0.45
Determined analysis					
Protein (%)	19.00	20.7	20.3	19.4	18.8
Total calcium (%)	0.64	0.66	0.55	0.61	0.61
Total phosphorus (%)	0.70	0.68	0.63	0.66	0.64
Sodium	0.17	0.18	0.15	0.17	0.17
Crude fat (%)	6.82	8.22	7.84	8.76	9.00

1 The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

2Vitamin/mineral premix contains the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

3 Oliggo-Fiber DS2 inulin (Cargill Inc., Wayzata, MN)

Diets were changed at day 25 to finisher diets containing *Palmaria palmata* (Table 3.5) and inulin (Table 3.6). All diets met or exceeded the National Research Council (NRC, 1994) nutrient requirements for birds at each phase of growth. A corn-based diet was formulated with soybean meal and wheat as other main ingredients. Either *Palmaria palmata* or inulin were fed at: 0.6%, 1.2%, 1.8%, 2.4% and 3.0 % of the diet. Antibiotic (BMD) containing at 0.04% level and a no feed additive diets constituted two other test treatments. Diets were made in the feed mill at the Agricultural Campus of Dalhousie University All diets were mixed in a Hobart bowl type mixer (model L.800, The Hobart Manufacturing Co. Ltd, Don Mills, Ontario, Canada). Diets were fed in mash form.

Table 3.5. Diet formulations used in the finisher period (day 24-35) containing levels of *Palmaria palmata*, antibiotic or no feed additive.

			Palmaria palmata				
	No feed additive	Positive control	0.6%	1.2%	1.8%	2.4%	3%
Ingredient (% as fed)							
Corn	56.90	56.80	56.10	55.29	54.49	53.68	52.90
Soybean Meal	25.84	25.85	25.73	25.36	25.53	25.42	25.30
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Tallow-grease	3.67	3.70	4.00	4.33	4.65	4.98	5.3
Limestone, Ground	1.40	1.40	1.39	1.39	1.80	1.39	1.4
Mono-dicalcium Phosphorus	0.64	0.60	0.64	0.65	0.65	0.65	0.70
Methionine Premix[1]	0.53	0.53	0.53	0.54	0.54	0.54	0.50
Vit/min/px[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.40	0.40	0.38	0.36	0.33	0.31	0.29
Lysine	0.13	0.12	0.13	0.12	0.12	0.12	0.10
BMD[3]	-	0.04	-	-	-	-	-
Palmaria palmata[4]	-	-	0.60	1.20	1.80	2.40	3.00
Total	100	100	100	100	100	100	100
Calculated analysis							
Metabolizable energy (kcal/kg)	3200	3200	3200	3200	3200	3200	3200
Protein (%)	18.0	18.0	18.0	18.0	18.0	18.0	18.0
Calcium (%)	0.85	0.90	0.85	0.85	0.85	0.85	0.85
Lysine (%)	1.09	1.09	1.09	1.09	1.09	1.09	1.09
Methionine (%)	0.58	0.58	0.58	0.58	0.58	0.58	0.58
Methionin+Cysteine (%)	0.86	0.86	0.86	0.86	0.86	0.86	0.86
Sodium (%)	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Available P (%)	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Determined analysis							
Protein (%)	18.6	18.33	19.1	20.1	18.8	22.6	18.8
Total Calcium (%)	0.82	0.71	0.68	0.81	0.78	0.97	0.63
Total phosphorus (%)	0.46	0.47	0.46	0.79	0.80	0.50	0.44
Sodium (%)	0.17	0.15	0.17	0.18	0.21	0.25	0.14
Crude fat (%)	6.44	3.84	6.66	6.77	7.24	8.63	7.58

[1] The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vit/min/ px contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3] BMD: Medicated Bacitracin Methylene Disalicylate Premix at 0.04%

[4] *Palmaria palmata* provided by Acadian Seaplants Ltd. (Dartmouth, NS)

Table 3.6. Trial 1 diet formulations used in the finisher period (Day 24-35) containing levels of inulin.

	Inulin				
	0.6%	1.2%	1.8%	2.4%	3%
Ingredient (% as fed)					
Corn	55.72	54.53	53.35	52.16	50.98
Soybean Meal	26.04	26.25	26.45	26.66	26.86
Wheat	10.00	10.0	10.00	10.00	10.00
Tallow-grease	4.05	4.43	4.81	5.19	5.57
Limestone, ground	1.39	1.39	1.39	1.38	1.38
Mono-dicalcium phosphorus	0.64	0.65	0.66	0.66	0.67
Methionine Premix[1]	0.53	0.53	0.53	0.53	0.53
Vit/min/px[2]	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.40	0.40	0.40	0.40	0.40
Lysine	0.12	0.12	0.11	0.11	0.10
Inulin[4]	0.60	1.20	1.80	2.40	3.00
Total	100	100	100	100	100
Calculated analysis					
Metabolizable energy (kcal/kg)	3200	3200	3200	3200	3200
Protein (%)	18.0	18.0	18.0	18.0	18.0
Calcium (%)	0.85	0.85	0.85	0.85	0.85
Lysine (%)	1.09	1.09	1.09	1.09	1.09
Methionine (%)	0.58	0.58	0.58	0.58	0.57
Methionine+Cysteine (%)	0.86	0.86	0.86	0.86	0.86
Sodium (%)	0.18	0.18	0.18	0.18	0.18
Available P (%)	0.42	0.42	0.42	0.42	0.42
Determined analysis					
Protein (%)	19.2	20.9	19.3	19.4	19.2
Total Calcium (%)	0.77	0.72	0.78	0.74	0.78
Total phosphorus (%)	0.52	0.51	0.51	0.46	0.46
Sodium (%)	0.19	0.19	0.15	0.18	0.19
Crude fat (%)	6.05	6.5	7.26	7.73	8.10

[1] The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vit/min/ px contained the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3] Oliggo-Fiber DS2 inulin (Cargill Inc., Wayzata, MN)

3.5.3 Sample collection

Initially 24 birds were randomly selected from the whole flock for sample collection. Three birds per cage were randomly selected to be euthanized by cervical dislocation at days 7, 14, 24 and at 35 days of age. Each bird was weighed and the entire ileum was removed from the Meckel's diverticulum (identified as the start of ileum) to the ileocaecal junction. Two pieces, 0.5-1.0 cm long each including digesta were, cut from the midpoint of ileum for NGS and histology analysis. The rest of ileal digesta was harvested, placed in sterile plastic bags for conventional microbiology analysis. For NGS analysis ileal digesta samples were, immediately placed on ice, then frozen at -80°C within two hours of collection for subsequence analysis. For histology analysis, each sample was placed in 10% buffered formalin for storage with subsequent preparation of paraffin embedded sections. One caecum was removed and then the pH of the caecum was measured with a VWR 8000 Series bench-model pH meter (Fisher Scientific, Ottawa, ON) using the procedure by Catala-Gregori et al. (2008).

At the end of experiment (day 35), the three birds randomly selected were euthanized using an electric knife. Five ml of blood were collected from the neck into a sterile syringe and kept in vacutainer tubes containing sodium heparin. Plasma were obtained from these blood samples by centrifuging for 15 min at 1000 × g at room temperature and stored at −15°C until measurement of IgA were done ELISA kits according to the manufacturer's instructions (Kim et al., 2011).

3.5.4 Chemical analysis

Two grams of diet was weighed into aluminium dishes in triplicate using an analytical balance (Mettler-Toledo GmbH, Laboratory & Weighing Technologies, Greifensee, Switzerland). To determine the dry matter content, the samples were dried to constant weight in a drying oven (Iso temp 300 series, Fisher Scientific Company, Ottawa, Ontario, Canada) at 100°C, for approximately 3 h. After 3 h, the dry matter content was calculated as described by the method 934.01 (AOAC 2005).

Nitrogen content (%) of diets was determined using a Leco Nitrogen Determinator (Leco FP-528, Leco Corporation, St. Joseph, Michigan, USA), by combusting the

samples with pure oxygen (900°C) and injecting continuous helium into the system, as described in Method 990.03 of AOAC (2005). EDTA was used for the calibration. The crude protein content (%) was calculated (N% x 6.25). Mineral content of test meal ingredients was determined by the AOAC Method 968.08 (AOAC 2005). Mineral content of feed was calculated by the AOAC method 968.08. In duplicate, 1 g of feed content was ashed in a well-glazed porcelain dish overnight, in a muffle furnace, at 550°C and then moistened with distilled water. Ash was transferred into a 200 mL beaker. By diluting 500 mL of concentrated HCl (Trace metal grade concentrated HCl, Fisher Scientific Company, Ottawa, Ontario) in 1500 mL distilled water, 1:3 diluted hydrochloric acid (HCl) was prepared. A mixture of forty mL of diluted HCl and a few drops of concentrated nitric acid (Trace metal grade concentrated HNO3, Fisher Scientific Compay, Ottawa, Ontario) were added. In order to solubilize the minerals in the sample, the mixture was placed on a hot plate covered with a watch glass and boiled for 10 minutes. The mixture was filtered (using Whatman #42 ashless filter paper) into 250 mL flasks and made up to a volume of 250 mL with distilled water, then cooled. In order to avoid losses during the filtering process, beakers, watch glasses and filter papers were rinsed several times with distilled water. Then, a sample was poured into scintillation vials and sent to the Nova Scotia Department of Agricultur lab for mineral analysis. For the mineral analysis, Inductively Coupled Argon Plasma analyzer (ICAP) (Varian Vista Pro Axial ICP-169 OES, Agilent Technologies Inc., USA) was used.

Crude fat content of test diets was determined by extracting the samples with anhydrous ether, as described by Method 920.39 (AOAC, 2005), with an ANKOMXT15 Extractor (model XT, Ankom Technology, Macedon, New York, USA).

3.5.5 Measurement of growth performance

At 0, 7, 14, 24 and 35 days of age, the birds were group weighed per cage and feed remaining in the trough was weighed back and recorded using a balance equipped with live weight capability (Mettler PM 34-K Delta Range, Mississauga, ON, Canada. Mortality was recorded and the dead birds were sent to the veterinary pathologist for necropsy (Animal Health Laboratory, Truro, Canada). Feed intake, body weight, body

weight gain and feed conversion ratio were measured. The percent mortality was measured or calculated at each stage of growth. Causes of mortality were summarized.

3.5.6 Microbiota analysis

In order to determine whether different treatments affected ileal microbiota, a culturing technique was used (Gilchrist et al., 1973). On the day of culturing, intestinal ileal samples content were removed from the freezer at -80°C and placed at room temperature for one hour. Sample were homogenized in filtered stomacher bags with buffer peptone water (BPW, Oxoid CM 0509) at a 1:10 ratio for 100 seconds (Mix 2, AES Laboratories, Bruz, France).

Six dilutions were prepared (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) by adding BPW for analysis of ileal samples. All samples were plated in duplicate for each dilution. The *Clostridium perfringens* counts were determined by plating 1 ml of dilutions 10^{-1} and 10^{-2} using the duplicate pour plated method (Bolder et al., 1999) (Figure 3.1). The dilutions were prepared using *Perfringens* Agar Base (Oxoid CM 587) supplemented with 400 mg D-cycloserine per liter (Oxoid CM 0587, Hampshire, England) and were incubated anaerobically in a Bactron Anaerobic Chamber (model IV, Sheldon Manufacturing Inc., Cornelius, Oregon). After incubation under anaerobic conditions at 37°C for 48 hours, typical black colonies were enumerated (Figure 3.2).

The dilutions 10^{-5} and 10^{-6} were plated for enumerating *Bifidobacterium* using de Man, Rogosa and Sharpe (MRS) agar with 0.02% NaN_3 and 0.05% $_L$-cystine hydrochloride monohydrate by the poured plate method Kim et al., (2011). Plates were incubated anaerobically in a Bactron Anaerobic Chamber at 37°C for 48 hours under anaerobic conditions and typical white colonies were enumerated.

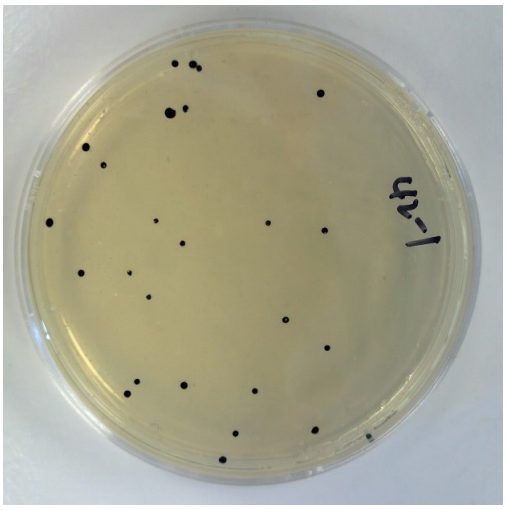


Figure 3.1. The culture of total *Clostridium perfringens* bacteria on Petrifilm™ count plates

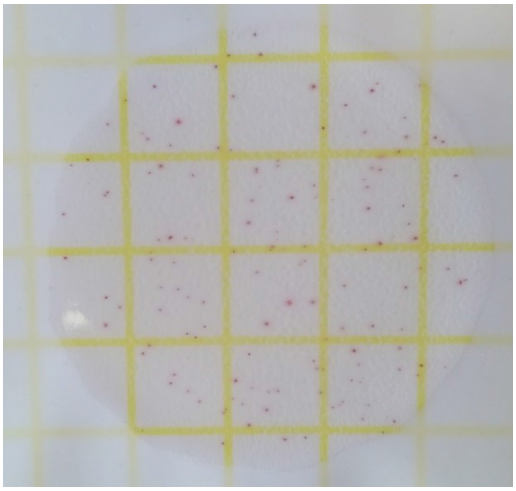


Figure 3.2. The culture of total anaerobic on petrifilm^{TM} count plates

For counting the aerobic and anaerobic bacteria populations, 1 ml from dilutions 10^{-5}, 10^{-6} and 10^{-7} were plated on aerobic Petrifilm^{TM} plates (3M, St. Paul, MN) in quadruplicate. In order to count aerobic bacteria populations, half of the Petrifilm^{TM} plates were incubated in a standard incubator (Geneq, Inc., Montreal, Quebec) at 37°C for 24 hours, while the other half were incubated in a Bactron Anaerobic Chamber at 37°C for 24 hours. All red colonies were counted as aerobes or anaerobes depending on the incubators (AOAC 2005), (Figures 3.3).

For counting *E. coli* and total coliforms, 1 ml from dilutions 10^{-2}, 10^{-3} and10^{-4} were plated on *E. coli*/Coliform count (EC) plates (3M, St. Paul, MN) then incubated aerobically at 37°C for 24. Blue colonies with gas were considered a coliform on the petrifilm. All plates were re-incubated for another 24 hours and blue colonies closely associated with entrapped gas were counted as *E. coli* (Figure 3.4).

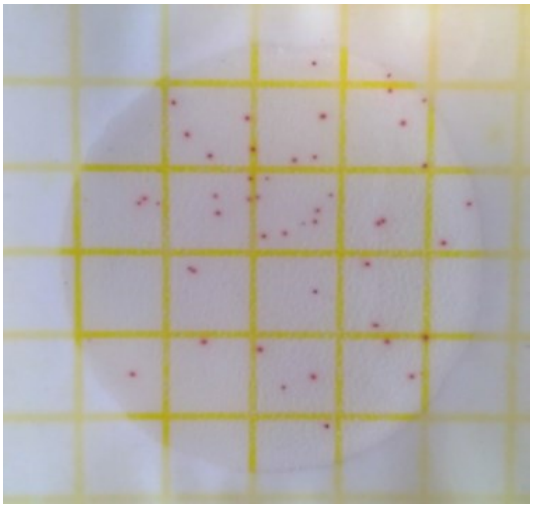


Figure 3.3. The culture of total aerobic bacteria on Petrifilm™ count plates.

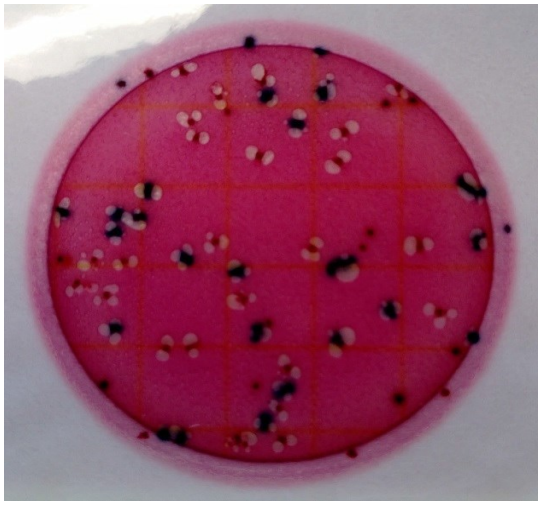


Figure 3.4. The culture of *E. coli* on petri dish.

3.5.7 Intestinal morphology

Intestinal histology was determined according to the method of Awad et al., 2009. Sample sections (0.5-1.0 cm) were removed from the middle of the ileum and rinsed in deionized water. Then, each sample was placed in 10% buffered formalin for storage in a labelled plastic jar. For subsequent histological analysis, intestinal samples were placed in a cassette. Prepared cassettes were placed in the Tissue-Tek® TEC ™ (Sakura Finetek USA, Inc., Torrance, CA), then dehydrated in a series of alcohol solutions ranging from 70% to 100%. After dehydration, the tissue slices were treated with xylene to clear the tissues and fixed in paraffin wax. After the fixing process, three ileum cross-sections were cut at 0.5 μm thickness using a microtome (Leica RM2255, Nussloch, Germany) then placed on glass slides at 35°C and stained by the Tissue Tissue-Tek® TEC ™ with hematoxylin and eosin (Drury and Wallington 1980). For intestinal histomorphology

measurements analysis, the clearest cross-sections of the three slices on a slide were selected for measurements. The slides then were scanned onto the computer using a Nikon Super CoolScan 400ED (Nikon Inc., Japan). Measurements were then taken using SigmaScan Pro 5 (SPSS Inc., Chicago, IL).

Villi height (A) was measured from the top of the villus to the start of the crypt (Figure 3.5). Crypt depth (E) was measured from the bottom end of the villi to the start of the mucosa (Figure 3.5). Villi width (B) was defined as the width of the villus at its midpoint (Figure 3.5). Apparent surface area (D) was measured via the imaging software from the sum of calibrated pixel units within the defined region of each villi. Mucosal depth (C) was defined as the length from the end crypt end to serosa (Figure 3.5). For each cross-section, ten measurements were taken (Figure 3.5).

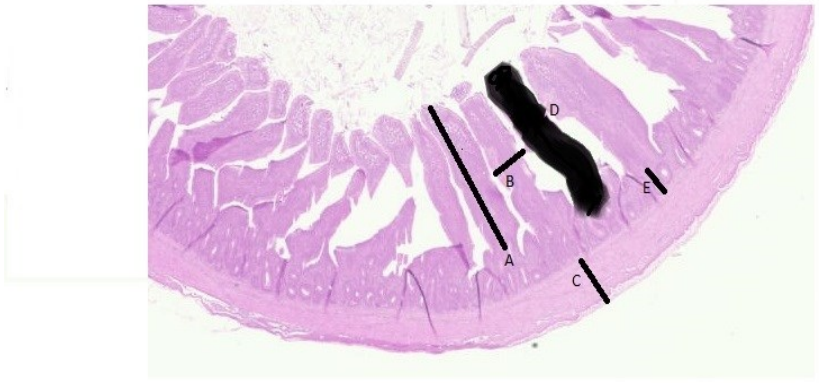


Figure 3.5 histology measurements of the cross-sections A: villus height, B: villus mid width C: mucosal depth, D: villus surface area, E: crypt depth

3.5.8 Analysis of serum immunoglobulin (ELISA)

Serum IgA concentrations were determined in diluted samples by a sandwich ELISA kit (Bethyl Labratories Inc., Montgonery, TX) using 96-well microtiter plates. In order to determine the dilution sample, at first, three dilution samples were prepared (1:100, 1:1000 and 1:10000) in 1X wash buffer provided in the kit using the serial dilution technique. Dilution 1:1000 was within the dynamic range of the standard curve. Then, 100 µl of diluted sample (1:1000) in the 2X wash buffer provided in the kit was added to the 96-well microtiter plate. This was done in order to capture chicken IgA present in the test sample with anti-chicken IgA antibody pre-inoculated on the surface of the microtiter plate. To clean the plates of unbound proteins and molecules after sample binding, the plates were washed four times with 1X wash buffer provided in the kit. Then, 100 µl of chicken IgA detection antibody was added to each plate to bind the IgA. The plate was covered and incubated at room temperature for 1h. After washing the plates four times with 1X wash buffer, 100 µl of a streptavidin-conjugated horseradish peroxidase (SA-HRP) was added to the plate. The plate was covered and incubated at room temperature for 30 minutes. The plate was again washed with 1X wash buffer four times, then 100 µl of TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate solution was added to each plate in order to make a catalyst reaction with SA-HRP. The plate was kept in a dark room at room temperature for 30 min resulting in a blue product. One hundred µl of stop solution (dilute sulfuric acid) was added to each plate and the absorbance was measured on a plate reader (Model Synergy HT, Bio-Tek Instrument, Inc., Winooski, USA) at 450 nm. A calibration curve was made using standard solutions and the IgA concentration was calculated by using the absorbance into the standard curve in accordance ELISA kit data sheet. IgA was measured as µg/ml.

3.5.9 Extraction of DNA from digesta for bacteria detection

On the day of extracting DNA, intestinal ileal digesta samples were removed from the freezer at -80°C and placed at room temperature. Before the samples were completely thawed, they were transferred to the stomacher. Then 10 ml of buffer ASL (QIAGEN Mat. No. 1014755) was added to the stomacher bags. After squeezing and mixing the

digesta in the stomacher bags, they were inserted into the stomacher (Mix 2, AES Laboratories, Bruz, France) and paddle-blended for 3 min. All of the sample lysate was transferred with a pipette into 1.5 ml tube, each sample was vortexed for 20 sec, heated for 5 min at 70°C then heated for 3 additional min at 95°C followed by vortexing. Samples were centrifuged for 5 min at *3000 x g.* One and half ml of the supernatant of each sample was pipetted into a new 2 ml microcentrifuge tube. The pellets were discarded.

Half of the inhibitEX tablet was added to each sample, and vortexed immediately and continuously until the sample was thoroughly suspended. The suspension stood for one min at room temperature in order for the inhibitors to be absorbed into the inhibitEX matrix. The samples were then centrifuged at *16,300 x g* for 3 min to ensure that all inhibitors were bound to the inhibitEX matrix. All of the supernatants were pipetted into new 1.5 ml microcentrfiuge tubes, the residual pellet was discarded. The samples were again centrifuged at *16,300 x g* for 3 min. Following that, 200 µl of supernatants was pipetted off to a new microcentrifuge tube. Then, 10µl of RNAse was pipetted into each sample. The samples were then kept for 10 min at room temperature and then centrifuged for 3 min at *16,300 x g.* Fifteen µl of proteinase K (QIAGEN Mat. 1014023) was added to each of the samples. Next, 200 µl of buffer AL (QIAGEN Mat.1014600) was added and samples were vortexed for 15 seconds. The samples were incubated at 70°C for 10 min. Next, 200 µl ethanol (96-100%) was added to the lysate and then mixed well by vortexing. Next sample were centrifuged for 15 sec at *16,300 x g* to eliminate drops from the inside of the tube lid. A new QIAamp spin column was labeled and positioned in a 2 ml collection tube. Following that, 630 µl of lysate was carefully applied to the QIAamp spin column without moistening the rim. After closing the caps, tubes were centrifuged at *16,300 x g* for 1 min. The QIAamp spin column was placed in a new 2 ml tubes and the tube containing the filtrate was discarded. Then 500 µl of buffer AW1 (QIAGEN Mat. 1014790) was added into the QIAamp spin column. Next the caps were closed and centrifuged at *16,300 x g* for one minute and finally QIAamp spin column was placed in a new 2 ml tube and the tube containing the filtrate was discarded. The QIAamp spin column was then carefully opened and 500 µl of buffer AW2 (QIAGEN Mat. 1014592) was added, centrifuged at *16,300 x* g for 3 min and the filtrate was removed. The

collection tube of filtrate was discarded and QIAamp spin column was transferred into a new labeled 2 ml microcentrifuge tube. The QIAamp spin columns were then opened and 100 µl AE buffer directly pipetted onto the QIAamp membrane. The caps were closed and were incubated for 1 min at room temperature and then centrifuged at *16, 300 x g* for 1 min to elute DNA. Finally, the eluted DNA was transferred into a new 1.5 ml tube and the tube was labeled and stored at -20°C. This method was based on the manufacturer's instructions (QIAGEN, Toronto, ON).

3.5.9.1 PCR amplification and sequencing

Before sending the samples for next generation sequencing, the quality of DNA in each pooled sample was checked through imaging by gel electrophoresis. PCR was performed on pooled samples using a master mix. A mixture was made with 18.5 µl of the master mix (GoTaq® Hot Start Polymerase: 5X Colorless GoTaq® Flexi buffer, $MgCl_2$ Solution, PCR Nucleotid Mix, upstream primer, downstream primer, GoTaq® Hot Start Polymerase) and 1.5 µl of each sample was added. PCR reactions were performed by initial reaction at 95°C for 5 min and then 95°C for 30s, 60°C for 20s, 72°C for 45 s, then cycled at 9°C for 34 more times and a final incubation at 9°C for 5 min. The samples were sent to the Genome Quebec Innovation Center (McGill University and Genome Quebec, Quebec, Canada) for next generation sequencing analysis using a MiSeq sequencer.

3.5.9.2 Sequence analysis and quality control

The 16SRNA amplicon sequencing performed at the Genome Quebec Innovation Center generated a total of 22,811,878 raw reads using the MiSeq sequencer. Reads were first scanned for contaminants (e.g. Illumina, 454 or PacBio adapter sequences) and PhiX reads. Reads were assembled using the FLASH software. The trimmed assembled/single-end reads were filtered for quality. All reads having an average quality score lower than 30 or more than 10 nucleotides (Ns) and 10 Ns below quality 20 were discarded. These reads were referred to as filtered reads from now on. Filtered reads were then clustered

with our in-house clustering algorithm. Briefly, reads were clustered at 100% identity and then clustered/denoised at 99% identity. Clusters having abundance lower than 3 were discarded. Remaining clusters were then scanned for chimeras with UCHIME denovo and UCHIME reference and clustered at 97% to form the final clusters/OTUs. OTUs were then analyzed for taxonomic distribution using a combination of in-house programs and scripts from the Qiime (qiime.org) software suite. OTUs were classified with the RDP classifier using an in-house training set containing the complete Greengenes database supplemented with eukaryotic sequences from the Silva databases and a customized set of mitochondria and chloroplasts 16S sequences. ITS2 database consist of the UNITE ITS database (ITS1-ITS2) region. The RDP classifier gives a score (0.00 to 1.00) to each taxonomic depth of each OTUs. Each taxonomic depth having a score ≥0.5 were kept to reconstruct the final lineage. For PacBio data type, clusters/OTUs were blasted against nucleotide. Using taxonomic lineages obtained from step 9, a raw OTU table was generated. From that raw OTU table, an OTU table containing both bacterial and archeal organisms was generated. From this latter OTU table, one more OTU table was generated: one rarefied to 1000 reads per sample. Additional OTU tables in which OTUs having abundance less than 1 in all samples were discarded. A summary of read counts throughout the different steps of the pipeline were then generated. The data files were merged into the final file (Quince et al., 2011). In this study, rRNA amplicon sequencing was performed at the Genome Quebec Innovation Center, using a MiSeq sequencer; when the different steps of the pipeline were completed, a summary of the read counts was generated

3.5.10 Statistical analysis

The trial was a randomized complete block design with 12 dietary treatments as the main factor and room as the blocking factor. Cage was used as the experimental unit. Data were analyzed using ANOVA in SAS 9.3 (SAS Institute Inc., Cary, NC) ($P \leq 0.05$). Growth data including feed intake, body weight gain and feed conversion ratio was analyzed as repeated measures with time as a factor. Data on physiological variables were analyzed as a measurement at a single time point. Any significant main or interaction

effects (α=0.05) were analyzed using Tukey-Kramer to differentiate the means (Gbur et al., 2012). The chi-squared test was used, to analyse the difference in mortality.

3.5.10.1 Statistical analysis of growth performance data

The feed intake (FI), body weight (BW), body weight gain (BWG) and feed conversion ratio (FCR) were analyzed as repeated measures when time was a factor.

Statistical model for trial 1 repeated measures analysis was:

$$\mathbf{Y_{ijklm}=\mu+Treatment_i+Level_j+Treatment*Level_{ij}+Day_k+\ Treatment*Day_{ik}+ Level*Day_{jk}+Treatment*Level*Day_{ijk}\ +Room_l+\varepsilon_{\ ijklm}}$$

Where:

Y_{ijkl} was the response of the variable (BW, BWG, FI and FCR).

μ was the overall mean response of that parameter.

$Treatment_i$ was the effect of inclusion level of treatment (i=1-12).

$Level_j$ was the effect of inclusion level for the j^{th} level of supplement (j=1-7).

Day_k was the effect of the k^{th} level of day (k=1-4).

$Room_i$ was the effect of the l^{th} level of the block (room) (l=1-2).

ε_{iijkl} was the effect of the uncontrollable factors for the the i^{th} level of treatment, j^{th} level of inclusion level, k^{th} level of day, l^{th} level of the block (room).

Statistical Model for Trial 1 Single Measurement Analysis was:

$$\mathbf{Y_{ijkl}=\mu+Treatment_i+Level_j+Treatment*Level_{ij}+Room_k+\ \varepsilon_{\ ijkl}}$$

The statistical model for single measurement analysis is likewise displayed above where all terms are the same, with the exception that day is no longer a factor.

3.5.10.2 Statistical analysis of microbiology and analysis data

Before analyzing the data for microbiology analysis, they were transformed to $\log_{10}$. Y was the response variable for total aerobes, anaerobes, coliforms, *E. coli*, *Clostridium perfringens* and *Bifidobacterium*. The statistical model for single measurement analysis was the same as previously described.

3.5.10.3 Statistical analysis of histomorphology and IgA analysis

The intestinal measurements evaluated were villus height, width, surface area and mucosal depth and crypt depth. IgA were analyzed. The statistical model for single measurement analysis was the same as previously described.

3.5.10.4 Statistical analysis for NGS data

For NGS analysis, the most abundant sequences were merged by selecting those classifications where the taxa had ≥0.5% representation in at least one sample. The data files were merged into the final file (Quince et al., 2011). The relative abundance data of bacteria were run as randomized block design using SAS. The statistical model analysis was the same as previously described.

3.6 Results and discussion

3.6.1 Analyzed nutrient composition of diets

For the majority of the treatments, the calculated CP (23%) of the starter diet was similar to the analyzed CP. However, the analyzed CP of the starter diet was lower for 2.4% *Palmaria palmata* (22%) in comparison with other treatments (Table 3.1 and Table 3.2). The reason for this discrepancy was unknown. The samples were reanalyzed to determine whether diets are meet or exceed the NRC (1994) requirement for birds. Reanalyzed failed to correct the problem as the scond analysis confirmed the values from the first.

The values determined for analyzed grower CP of 1.2% and 1.8% inulin (20.7% and 20.3%) were higher (Table 3.3 and Table 3.4) than the calculated CP (20%) in all treatments. The analyzed CP for all treatments was higher than calculated CP in finisher phase (18%) (Table 3.5 and Table 3.6). The total analyzed calcium was lower than the calculated calcium among all treatments for the starter (1.05%), grower (0.90%) and finisher (0.85%) periods. The reason is unknown. Therefore, samples were reanalyzed. The reanalyzed results indicated that the total calcium was found to be lower than NRC (1994) requirements. However, this calcium deficiency did not cause any disease or mortality in the flock related to the lack of calcium.

3.6.2 Growth performance

Room, the block used in this trial, was not found to be significant for any parameter ($P \geq 0.10$). Therefore, data was reanalyzed with the block removed. There were no significant changes observed among dietary treatments on BW (Table 3.7), BWG (Table 3.8), feed intake (FI) (Table 3.9) and FCR (table 3.10), at any point in the experiment. This trial was conducted in a well-controlled and, clean environment. There was no interaction effect between day and treatment for all days. Previous studies have reported inconsistent results on the effects of prebiotics on broiler growth performance (Baurhoo et al., 2009). Some researchers have found positive effects of prebiotics on growth performance (Xu et al., 2003) and health of birds (Pourabedin et al., 2015), while other researchers have not (Houshmand et al., 2012). This variation in the prebiotic

effectiveness may be attributed in part to several factors such as environment, management, nutrition, type of additive, and dosage as well as bird characteristics such as age, species, stage of production (Yang et al., 2009).

Table 3.7. Body weight (g $bird^{-1}d^{-1}$) of broiler chickens during the starter (day 0-14), grower (day 15-24), and finisher (day 25-35) experimental periods with *Palmaria palmata*, inulin, antibiotic and no feed additive.

	Days Posthatch		
	0-14	15-24	25-35
Diet and level of treatment (%)			
Palmaria palmata (%)			
0.6	416±33	1114±33	2228±33
1.2	423±33	1136±33	2206±33
1.8	414±33	1107±33	2300±33
2.4	419±33	1114±33	2147±33
3.0	422±33	1132±33	2125±33
Inulin (%)			
0.6	372±33	1044±33	2154±33
1.2	429±33	1172±33	2218±33
1.8	419±33	1123±33	2250±33
2.4	377±33	1092±33	2121±33
3.0	415±33	1143±33	2246±33
No feed additive	408±33	1129±33	2184±33
Antibiotic (BMD)[1]	418±33	1125±33	2163±33
Day mean	411±9[c]	1120±9[b]	2195±9[a]
ANOVA			
Treatment	0.14		
Day	<.0001		
Treatment*Day	0.28		

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.8. Body weight gain (g bird^{-1}d g bird^{-1}) of broiler chickens during the starter (day 0-14), grower (day 15-24), and finisher (day 25-35) experimental periods with *Palmaria palmata*, inulin, antibiotic and no feed additive.

	Days Posthatch		
	0-14	15-24	25-35
Diet and level of supplement (%)			
Palmaria palmata (%)			
0.6	26.2±3	71.5±3	93.0±3
1.2	24.0±3	71.5±3	95.5±3
1.8	27.0±3	72.5±3	95.2±3
2.4	26.2±3	71.0±3	101.7±3
3.0	27.0±3	70.2±3	90.7±3
Inulin (%)			
0.6	23.2±3	73.0±3	92.2±3
1.2	26.5±3	69.0±3	99.5±3
1.8	27.5±3	68.7±3	102.2±3
2.4	27.0±3	70.2±3	100.5±3
3.0	26.7±3	69.7±3	93.2±3
No feed additive	26.0±3	74.0±3	105.5±3
Antibiotic (BMD)[1]	26.7±3	68.7±3	99.0±3
Day mean	26.1^{c}±0.8	70.8^{b}±0.8	97.2^{a}±0.8
ANOVA	P-value		
Treatment	0.33		
Day	<. 0001		
Treatment*Day	0.56		

$^{a-c}$ means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.9. Feed intake (g $bird^{-1}$ d^{-1}) of broiler during the starter (day 0-14), grower (day 15-24), and finisher (day 25-35) periods by birds fed *Palmaria palmata*, inulin, antibiotic and no feed additive.

	Days Posthatch		
	0-14	15-24	25-35
Diets and level of treatments (%)			
Palmaria palmata (%)			
0.6	36.2±3	107.5±3	170.2±3
1.2	37.5±3	109.0±3	178.0±3
1.8	36.2±3	105.0±3	165.7±3
2.4	36.5±3	107.2±3	163.7±3
3.0	36.7±3	98.7±3	168.5±3
Inulin (%)			
0.6	35.0±3	102.5±3	170.2±3
1.2	34.0±3	110.5±3	166.0±3
1.8	36.0±3	110.2±3	166.7±3
2.4	34.0±3	107.5±3	172.5±3
3.0	36.7±3	102.2±3	177.7±3
No feed additive	36.0±3	109.7±3	178.2±3
Antibiotic (BMD)[1]	37.0±3	109.0±3	164.0±3
Day mean	35.5±0.9[c]	106.5±0.9[b]	170.3±0.9[a]
ANOVA	**P-value**		
Treatment	0.28		
Day	<. 0001		
Treatment*Day	0.31		

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.10. Feed conversion ratio of broiler chickens during the starter (day 0-14), grower (day 15-24), and finisher (day 25-35) experimental periods with red seaweed, inulin, antibiotic and no feed additive

	Days Posthatch		
	0-14	15-24	25-35
Supplement Inclusion Level (%)			
Palmaria palmata (%)			
0.6	1.36±0.05	1.52±0.05	1.74±0.05
1.2	1.37±0.05	1.53±0.05	1.83±0.05
1.8	1.30±0.05	1.54±0.05	1.55±0.05
2.4	1.21±0.05	1.54±0.05	1.75±0.05
3.0	1.37±0.05	1.40±0.05	1.81±0.05
Inulin (%)			
0.6	1.52±0.05	1.53±0.05	1.70±0.05
1.2	1.31±0.05	1.50±0.05	1.72±0.05
1.8	1.29±0.05	1.57±0.05	1.72±0.05
2.4	1.56±0.05	1.50±0.05	1.84±0.05
3.0	1.28±0.05	1.40±0.05	1.77±0.05
No feed additive	1.47±0.05	1.54±0.05	1.76±0.05
Antibiotic (BMD)[1]	1.30±0.05	1.54±0.05	1.73±0.05
Day mean	1.37±0.02[c]	1.50±0.02[b]	1.76±0.02[a]
ANOVA	**P-value**		
Treatment	0.62		
Day	<. 0001		
Treatment*Day	0.43		

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Alzueta et al. (2010), found that supplementation of broiler feed with (0.5% to 2%) inulin in maize-soybean meal based diet did not improve the growth performance of broiler chickens. In addition, Biggs et al. (2007) observed that feeding the birds with 0.2% or 0.8% of oligosaccharides did not significantly improve growth performance of broilers. These results are in agreement with Baurhoo et al. (2009), who observed that feeding birds with mannanoligosaccharides or antibiotics had no effects on the growth variables, under sanitary conditions. However, Wiseman (2012) observed that feeding Tasco® at 0.5% improved growth performance variables even under optimal growth conditions.

Similarly, Kulshreshtha el. (2014) observed that feeding red seaweed (*CC* and *SG*) did not affect feed intake in layer hens. Furthermore, supplementation of brown seaweed in

laying hens diets (*Macrocystis pyrifera*, *Sargassum sinicola*) and a green seaweed (*Enteromorpha sp.)* did not affect FI. The present findings, demonstrated that feed additives were not needed for growth maximization under the conditions of this study. Inconsistent results have been reported in some literature on the effects of prebiotics on broiler growth in cage performance (Santos et al., 2008). Santos et al. (2008) observed broiler grown on the litter in pens had better feed conversion ratio lower cecal *Salmonella* levels, and increased villi height, villi SA, villi height to crypt depth ratio, and increased mucosal depth in a study looking at growth of birds from day 0 to 42 in comparison with birds raised in nonlitter cage-based housing. However, Wiseman (2012) reported that birds grown and supplemented with Tasco® in cages had improved growth performance compared to a no feed additive.

3.6.3 Bird mortality

Red seaweed had no effect on mortality rate of broiler chickens throughout the trial ($p>0.05$). Mortality rates were found to be extraordinary low in this trial. The mortality for the trial was 2.8% in the starter phase, 0.25% in the grower phase and 0.0% in the finisher phase (Table 3.11). Mortality for the starter phase was caused by septicemia, varus-valgus deformities of the leg, and ascites, with some dehydration occurring in the early growth period; these were not treatment related. Since the data did not satisfy the normality assumption, the mortality data was analyzed, using chi-square. The results indicated that there was no significant difference among treatments, for mortality.

Table 3.11. Effect of *Palmaria palmata,* inulin, antibiotic and no feed additive on mortality (%) during starter (0-14), grower (15-24) and finisher (25-33) periods.

	Phase/day			
	Starter 0-14 d	Grower 15-24 d	Finisher 25-35 d	Total (%) 0-35 d
Treatment				
Palmaria palmata (%)				
0.6	0	0	0	0.0
1.2	2	0	0	0.2
1.8	0	0	0	0.0
2.4	1	0	0	0.1
3.0	1	0	0	0.1
Inulin (%)				
0.6	4	1	0	0.5
1.2	2	0	0	0.2
1.8	2	0	0	0.2
2.4	3	0	0	0.3
3.0	1	0	0	0.1
No feed additive	3	0	0	0.3
Antibiotic (BMD)[1]	1	1	0	0.2
Time totals	20	2	0	3.05
	P-value			
Mortality	0.42			

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

3.6.4 Intestinal microbiota

There was no block effect (P>0.05) on bacterial numbers for samples collected during all sampling days. At 7, 14, 24 and 35 of age, the bacterial populations examined were: culturable aerobic (Table 3.12), anaerobic (Table 3.13), coliforms (Table 3.14), *E. coli* (Table 3.15), *Lactobacillus* (Table 3.16), *Bifidobacteria* (Table 3.17) and *Clostridium perfringens* (Table 3.18). Feeding of *P.p.* at different inclusion levels had no effect on total culturable aerobic, anaerobic, coliforms, *E. coli, Lactobacillus*, *Bifidobacteria* and *Clostridium perfringens*. There was an increasing trend in the count of aerobic bacteria during days 7 (7.00±0.07), 14 (7.92±0.07), 24 (7.95±0.07) and 35 (7.82±0.07) (log10 cfu g^{-1}) (Table 3.12).

Table 3.12. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive to the feed on total aerobes (log_{10} cfu g^{-1}) in the ileum of broiler chickens on days 7, 14, 24 and 35.

Age (day)	7	14	24	35
Diet and level of treatment (%)				
Palmaria palmata (%)				
0.6	8.14±0.16	7.82±0.19	7.62±0.10	8.25±0.27
1.2	8.24±0.16	7.50±0.19	7.58±0.10	8.16±0.27
1.8	7.91±0.16	7.82±0.19	7.56±0.10	8.06±0.27
2.4	8.01±0.16	7.88±0.19	7.55±0.10	7.71±0.27
3	8.09±0.16	7.56±0.19	7.57±0.10	8.00±0.27
Inulin (%)				
0.6	8.00±0.16	7.83±0.19	7.65±0.10	8.16±0.27
1.2	7.86±0.16	8.34±0.19	7.70±0.10	8.33±0.27
1.8	7.96±0.16	7.74±0.19	7.55±0.10	8.27±0.27
2.4	8.31±0.16	8.22±0.19	7.43±0.10	8.21±0.27
3	8.22±0.16	7.92±0.19	7.33±0.10	8.55±0.27
No feed additive	8.30±0.16	7.47±0.19	7.61±0.10	7.99±0.27
Antibiotic (BMD)[1]	8.03±0.16	7.58±0.19	7.41±0.10	7.32±0.27
Day mean	7.00±0.07[c]	7.92±0.07[b]	7.95±0.07[b]	7.82±0.07[a]
ANOVA	P-value			
Treatment	0.37			
Age	<.0001			
Treatment* Day	0.95			

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.13. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive to the feed on total anaerobes (log_{10} cfu g^{-1}) in the ileum of broiler chickens on days 7, 14, 24 and 35.

Age (day)	7	14	24	35
Diet and level of treatment (%)				
Palmaria palmata %				
0.6	6.77±0.2	7.53±0.2	7.64±0.2	7.67±0.2
1.2	6.93±0.2	8.01±0.2	7.98±0.2	7.92±0.2
1.8	6.73±0.2	7.77±0.2	7.90±0.2	8.02±0.2
2.4	6.98±0.2	7.96±0.2	8.00±0.2	8.01±0.2
3	6.55±0.2	8.00±0.2	8.10±0.2	7.68±0.2
Inulin%				
0.6	7.22±0.2	7.60±0.2	7.70±0.2	7.97±0.2
1.2	6.83±0.2	7.90±0.2	8.00±0.2	7.66±0.2
1.8	7.66±0.2	8.08±0.2	7.69±0.2	8.15±0.2
2.4	7.17±0.2	8.24±0.2	8.35±0.2	7.58±0.2
3	7.23±0.2	7.74±0.2	7.85±0.2	7.97±0.2
No feed additive	6.82±0.2	8.08±0.2	8.18±0.2	7.92±0.2
Antibiotic (BMD)[1]	7.05±0.2	8.20±0.2	8.07±0.2	7.30±0.2
Day mean	6.59±0.03[c]	7.42±0.03[b]	7.54±0.03[b]	7.74±0.03[a]
ANOVA	P-value			
Treatment	0.35			
Day	<.0001			
Treatment*Day	0.54			

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.14. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive to the feed on total coliforms (log_{10} cfu g^{-1}) in the ileum of broiler chickens on days 7, 14, 24 and 35.

Age (day)	7	14	24	35
Diet and level of treatment (%)				
Palmaria palmata (%)				
0.6	1.84±0.4	1.67±0.4	2.08±0.4	2.07±0.4
1.2	2.04±0.4	2.10±0.4	2.43±0.4	2.92±0.4
1.8	2.32±0.4	2.02±0.4	2.36±0.4	2.71±0.4
2.4	1.66±0.4	2.71±0.4	3.03±0.4	2.22±0.4
3	2.19±0.4	1.92±0.4	2.14±0.4	2.13±0.4
Inulin (%)				
0.6	1.65±0.4	2.07±0.4	2.77±0.4	2.47±0.4
1.2	1.87±0.4	1.87±0.4	2.32±0.4	1.92±0.4
1.8	2.05±0.4	2.10±0.4	2.74±0.4	2.88±0.4
2.4	1.82±0.4	2.03±0.4	2.41±0.4	2.47±0.4
3	1.87±0.4	2.26±0.4	2.80±0.4	2.97±0.4
No feed additive	2.42±0.4	2.85±0.4	3.22±0.4	2.55±0.4
Antibiotic (BMD)[1]	5.43±0.40	2.22±0.4	2.75±0.4	2.44±0.4
Day mean	5.84±0.09[a]	5.40±0.09[b]	5.46±0.09[b]	5.15±0.09[b]
ANOVA	P-value			
Treatment	0.35			
Day	0.005			
Treatment* Day	0.99			

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.15. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive to the feed on total *E. coli* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on days 7, 14, 24 and 35.

Age	day 7	day 14	day 24	day 35
Diet and level of supplement (%)				
Palmaria palmata (%)				
0.6	5.43±0.2	5.18±0.25	5.27±0.2	5.50±0.2
1.2	5.64±0.2	5.46±0.25	5.52±0.2	5.55±0.2
1.8	6.02±0.2	5.13±0.25	5.20±0.2	5.51±0.2
2.4	6.82±0.2	4.94±0.25	5.00±0.2	5.51±0.2
3	5.02±0.2	4.99±0.25	5.07±0.2	5.52±0.2
Inulin (%)				
0.6	5.80±0.2	4.85±0.25	4.92±0.2	5.50±0.2
1.2	5.79±0.2	5.45±0.25	5.51±0.2	5.53±0.2
1.8	5.81±0.2	4.87±0.25	4.94±0.2	5.57±0.2
2.4	6.20±0.2	4.47±0.25	4.57±0.2	5.57±0.2
3	5.94±0.2	5.46±0.25	5.52±0.2	5.54±0.2
No feed additive	6.24±0.2	5.11±0.25	5.18±0.2	5.68±0.2
Antibiotic (BMD)[1]	5.78±0.2	5.02±0.25	5.11±0.2	5.27±0.2
Day mean	5.87±0.08[a]	5.08±0.08[c]	5.15±0.08[c]	5.52±0.08[b]
ANOVA	P-value			
Treatment	0.19			
Day	<.0001			
Treatment* Day	0.39			

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.16. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive to the feed on total *Lactobacillus* bacteria (log_{10} cfu g^{-1}) in the ileum of broiler chickens on days 7, 14, 24 and 35.

Age (day)	7	14	24	35
Diet and level of treatment (%)				
Palmaria palmata (%)				
0.6	6.59±0.1	7.50±0.1	7.71±0.1	7.69±0.1
1.2	6.75±0.1	7.44±0.1	7.55±0.1	7.64±0.1
1.8	6.45±0.1	7.51±0.1	7.51±0.1	7.93±0.1
2.4	6.54±0.1	7.41±0.1	7.47±0.1	7.74±0.1
3	6.70±0.1	7.36±0.1	7.46±0.1	7.73±0.1
Inulin (%)				
0.6	6.59±0.1	7.42±0.1	7.52±0.1	7.92±0.1
1.2	6.62±0.1	7.33±0.1	7.47±0.1	7.68±0.1
1.8	6.47±0.1	7.46±0.1	7.62±0.1	7.59±0.1
2.4	6.66±0.1	7.45±0.1	7.59±0.1	7.68±0.1
3	6.62±0.1	7.39±0.1	7.40±0.1	7.63±0.1
No feed additive	6.45±0.1	7.39±0.1	7.53±0.1	7.83±0.1
Antibiotic (BMD)[1]	6.60±0.1	7.43±0.1	7.65±0.1	7.82±0.1
Day mean	6.59±0.03[c]	7.42±0.03[b]	7.54±0.03[b]	7.74±0.03[a]
ANOVA	P-value			
Treatment	0.96			
Day	<.0001			
Treatment*Day	0.97			

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.17. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive to the feed on total *Bifidobacterium* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on days 7, 14, 24 and 35.

Age (day)	7	14	24	35
Diet and level of supplement (%)				
Palmaria palmata (%)				
0.6	6.60±0.07	7.23±0.07	7.28±0.07	7.76±0.07
1.2	6.84±0.07	7.18±0.07	7.23±0.07	7.80±0.07
1.8	6.50±0.07	7.22±0.07	7.27±0.07	7.82±0.07
2.4	6.50±0.07	7.24±0.07	7.28±0.07	7.60±0.07
3	6.69±0.07	7.21±0.07	7.26±0.07	7.59±0.07
Inulin (%)				
0.6	6.59±0.07	7.26±0.07	7.30±0.07	7.85±0.07
1.2	6.60±0.07	7.23±0.07	7.27±0.07	7.86±0.07
1.8	6.36±0.07	7.22±0.07	7.27±0.07	7.74±0.07
2.4	6.75±0.07	7.25±0.07	7.30±0.07	7.60±0.07
3	6.62±0.07	7.22±0.07	7.26±0.07	7.76±0.07
No feed additive	6.45±0.07	7.19±0.07	7.24±0.07	7.72±0.07
Antibiotic (BMD)[1]	6.60±0.07	7.21±0.07	7.26±0.07	7.78±0.07
Day mean	6.93±0.02[c]	7.22±0.02[b]	7.27±0.02[b]	7.74±0.02[a]
ANOVA	P-value			
Treatment	0.33			
Day	<.0001			
Treatment*Day	0.08			

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.18. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive to the feed on total *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on days 7, 14, 24 and 35.

Age (day)	7	14	24	35
Diet and level of supplement (%)				
Palmaria palmata (%)				
0.6	1.84±0.4	1.67±0.4	2.08±0.4	2.07±0.4
1.2	2.04±0.4	2.10±0.4	2.43±0.4	2.92±0.4
1.8	2.32±0.4	2.02±0.4	2.36±0.4	2.71±0.4
2.4	1.66±0.4	2.71±0.4	3.03±0.4	2.22±0.4
3	2.19±0.4	1.92±0.4	2.14±0.4	2.13±0.4
Inulin (%)				
0.6	1.65±0.4	2.07±0.4	2.77±0.4	2.47±0.4
1.2	1.87±0.4	1.87±0.4	2.32±0.4	1.92±0.4
1.8	2.05±0.4	2.10±0.4	2.74±0.4	2.88±0.4
2.4	1.82±0.4	2.03±0.4	2.41±0.4	2.47±0.4
3	1.87±0.4	2.26±0.4	2.80±0.4	2.97±0.4
No feed additive	2.42±0.4	2.85±0.4	3.22±0.4	2.55±0.4
Antibiotic (BMD)[1]	2.39±0.4	2.22±0.4	2.75±0.4	2.44±0.4
Day mean	2.01±0.12[b]	2.15±0.12[ab]	2.59±0.12[a]	2.48±0.12[ab]
ANOVA	P-value			
Treatment	0.35			
Day	0.005			
Treatment*Day	0.99			

[a-c] means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

There was no effect of dietary treatment or bird age on level of total anaerobic bacteria in the small intestine. There was an increasing trend for anaerobic bacteria during days 7

(6.59±0.03), 14 (7.42±0.03), 24 (7.54±0.03) and a decrease at day 35 (7.74±0.03) (log10 cfu g^{-1}) (Table 3.13). The number of coliform has a decreasing trend during days 7, 14, 24 and 35. At day 7, the number of coliforms was 5.84±0.09 (log10 cfu g^{-1}) compared to days 14 (5.40±0.09), 24 (5.46±0.09) and 35(5.15±0.09) (Table 3.14). The number of E. *coli* was 5.87±0.08 at day 7 (log10 cfu g^{-1}) and decrease at days 14 (5.08±0.08) and 24 (5.15±0.08) and then increased at day 35 (5.52±0.08) (Table 3.15). The number of *Lactobacillus* had increasing trend during days 7, 14, 24 and 35. The number of *Lactobacillus* was 6.59±0.03 (log10 cfu g^{-1}) and increased at days 14 (7.42±0.03), 24 (7.54±0.03) and 35 (7.74±0.03) (Table 3.16). The proportion of *Bifidobacterium* had increasing trend during days 7, 14, 24 and 35. The number of *Bifidobacterium* at day 7 was 6.93±0.02 (log10 cfu g^{-1}) and then increased and was stable at days 14 (7.22±0.02), 24 (7.27±0.02) and increased at day 35 (7.74±0.02) (Table 3.17). The number of *Clostridium perfringens* was 2.01±0.12 (log10 cfu g^{-1}) at day 7 had an increasing trend during days 14 (2.15±0.12), 24 (2.59±0.12) and then decreased at day 35 (2.48±0.12) (Table 3.18).

These results are in agreement with Jin et al. (1998), who observed that feeding broilers with diets containing *Lactobacillus* did not alter the number/populations of total aerobes, total anaerobes, *Lactobacilli* or *Streptococci* in the small intestines and ceca of the basal diets (control birds) compared to those of treated groups. Pourabedin et al. (2015) reported that supplementation of the birds with MOS or virginaminaycin did not affect the population of *E. coli* in the cecal content of chickens. Contrary, Kulshreshtha et al. (2014) observed that the relative abundance of probiotic bacteria such as *Bifidobacterium longum* and *Streptococcus salivarius* were greater in birds fed 1% CC, 1% SG, and 2% SG. In addition, Baurhoo et al. (2007) observed that supplementation of birds with 2% Alcell lignin and challenged with *E. coli* were found to have decreased *E. coli* levels. It is well documented that the development of microbiota species in the chicken gut is closely affected by different husbandry practices (Bojesen et al., 2010). In the current study, the birds were raised under optimal environmental conditions; hence, the bacteria in the intestinal tract may not have been the same, either in type and number, in comparison to samples from birds raised under stressful environmental conditions.

3.6.5 Cecal pH

The pH of cecal contents was significantly different among treatments at 24 and 35 day of age (Table 3.19) which can indicate SCFA production in response to prebiotic supplementation (Immerseel et al. 2004). On day 24, feeding 1.2% inulin resulted in significantly lower pH compared to *Palmaria palmata*, antibiotic and no feed additive. Short chain volatile fatty acids (SCFA), which are produced by the fermentation of breakdown products of NSPs, cause a decreased in pH. These products provide energy for chicken, prevents growth of pathogens and enhances mineral absorption (Pourabedin et al., 2015). Among *Palmaria palmata*, groups, 1.8% *Palmaria palmata*, had the lowest pH on day 24 (Table 3.19). On day 35, birds supplemented with *Palmaria palmata*, inulin and antibiotic were significantly different, compared to the no feed additive (Table 3.19). This decrease in pH would indicate a beneficial increase in fermentation of the prebiotic (Dhama et al., 2008). As was obvious from the microbiota results, the increase in bacterial fermentation caused by the supplements was not enough to increase growth of the birds. A decrease in intestinal pH with prebiotics in the diet may result in pathogen inhibition associated with high fermentation activity and high concentration of SCFA in the gut (Barry et al., 2009). Furthermore, Rebole et al. (2010) observed that supplementation of birds with inulin and enzyme complex, individually or in combination in broilers had no effect on pH of cecal contents.

Table 3.19. Effect of *Palmaria palmata,* inulin, antibiotic and no feed additive on cecal pH on days 14, 24 and 35.

	Days Posthatch		
	14	24	35
Dietary Supplement			
Palmaria palmata (%)			
0.6	5.70±0.2	5.74±0.05^{ab}	6.19±0.03^{a}
1.2	5.80±0.2	5.66±0.05^{abc}	6.17±0.03^{a}
1.8	5.55±0.2	5.53±0.05^{bc}	6.18±0.03^{a}
2.4	5.51±0.2	5.75±0.05^{ab}	6.15±0.03^{a}
3.0	5.57±0.2	5.71±0.05^{ab}	6.14±0.03^{a}
Inulin (%)			
0.6	5.72±0.2	5.74±0.05^{ab}	6.19±0.03^{a}
1.2	5.70±0.2	5.42±0.05^{c}	6.12±0.03^{a}
1.8	5.36±0.2	5.60±0.05^{bc}	6.15±0.03^{a}
2.4	5.54±0.2	5.54±0.05^{bc}	6.14±0.03^{a}
3.0	5.65±0.2	5.74±0.05^{ab}	6.05±0.03^{a}
No feed additive	5.85±0.2	5.87±0.05^{a}	6.44±0.03^{b}
Antibiotic (BMD)[1]	5.85±0.2	5.86±0.05^{a}	6.17±0.03^{a}
P-value	0.67	0.001	0.0001

[a-c] means±SEM with different letters within an experimental period are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

3.6.6 Histomorphological analysis

There was no effect of treatment level on ileal crypt depth, villi height, villi apparent area, villus width or mucosal depth on days 7 (Table 3.20), 14 (Table 3.21), 24 (Table 3.22) and 35 (Table 3.23) ($P>0.05$).

Table 3.20. Day 7 ileal intestinal histomorphology measurements of broiler chickens with dietary supplementation of *Palmaria palmate*, inulin, antibiotic and no feed additive.

	Villus Height (μm)	Villus Width (μm)	Crypt Depth (μm)	Villus Surface Area (mm^2)	Mucosal Depth (μm)
Treatment					
Palmaria palmata (%)					
0.6	343.5±5.3	125.0±3.4	120.2±6.3	0.057±0.003	123.0±6.1
1.2	349.5±5.3	133.2±3.4	126.7±6.3	0.053±0.003	121.9±6.1
1.8	337.2±5.3	126.0±3.4	116.7±6.3	0.060±0.003	132.2±6.1
2.4	334.5±5.3	136.0±3.4	127.7±6.3	0.057±0.003	129.8±6.1
3.0	346.5±5.3	133.0±3.4	120.0±6.3	0.058±0.003	127.0±6.1
Inulin (%)					
0.6	327.2±5.3	126.0±3.4	118.7±6.3	0.056±0.003	127.2±6.1
1.2	340.0±5.3	130.5±3.4	125.5±6.3	0.050±0.003	128.3±6.1
1.8	339.2±5.3	122.2±3.4	115.2±6.3	0.051±0.003	131.2±6.1
2.4	345.0±5.3	124.5±3.4	118.7±6.3	0.059±0.003	117.0±6.1
3.0	346.5±5.3	127.0±3.4	121.5±6.3	0.066±0.003	115.7±6.1
No feed additive	310.0±5.3	122.2±3.4	113±6.3	0.051±0.003	130.0±6.1
Antibiotic (BMD)[1]	335.2±5.3	129.5±3.4	125.5±6.3	0.050±0.003	120.7±6.1
P-value	0.55	0.97	0.33	0.72	0.63

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.21. Day 14 ileal intestinal histomorphology measurements of broiler chickens with dietary supplementation of *Palmaria palmata*, inulin, antibiotic and no feed additive.

	Villus height (μm)	Villus width (μm)	Crypt depth (μm)	Villus surface area (mm^2)	Mucosal depth (μm)
Treatments					
Palmaria palmata (%)					
0.6	616.9±33.9	150.6±8.6	135.9±7.9	0.101±0.007	169.1±9.3
1.2	603.2±33.9	143.7±8.6	137.1±7.9	0.096±0.007	173.0±9.3
1.8	620.8±33.9	158.0±8.6	141.7±7.9	0.105±0.007	177.4±9.3
2.4	575.6±33.9	149.4±8.6	136.7±7.9	0.092±0.007	175.9±9.3
3	653.1±33.9	152.0±8.6	155.2±7.9	0.109±0.007	184.1±9.3
Inulin (%)					
0.6	602.1±33.9	137.7±8.6	126.8±7.9	0.089±0.007	172.0±9.3
1.2	643.8±33.9	145.8±8.6	144.5±7.9	0.100±0.007	177.8±9.3
1.8	625.0±33.9	147.3±8.6	136.4±7.9	0.103±0.007	178.6±9.3
2.4	649.1±33.9	147.1±8.6	150.0±7.9	0.102±0.007	178.5±9.3
3	683.7±33.9	146.7±8.6	142.1±7.9	0.107±0.007	179.3±9.3
No feed additive	614.0±33.9	151.6±8.6	137.0±7.9	0.099±0.007	173.9±9.3
Antibiotic (BMD)[1]	678.2±33.9	146.2±8.6	155.9±7.9	0.107±0.007	181.5±9.3
P-value	0.55	0.97	0.33	0.72	0.99

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.22. Day 24 ileal intestinal histomorphology measurements of broiler chickens with dietary supplementation of *Palmaria palmata*, inulin, antibiotic and no feed additive.

Treatments	Villus height (μm)	Villus width (μm)	Crypt Depth (μm)	Villus surface area (mm^2)	Mucosal Depth (μm)
Palmaria palmata (%)					
0.6	738±33	153±9	156±9	0.125±0.008	233±15
1.2	795±33	137±9	153±9	0.136±0.008	194±15
1.8	707±33	141±9	168±9	0.129±0.008	207±15
2.4	677±33	161±9	158±9	0.122±0.008	207±15
3	743±33	172±9	173±9	0.137±0.008	215±15
Inulin (%)					
0.6	703±33	145±9	165±9	0.126±0.008	194±15
1.2	747±33	162±9	170±9	0.139±0.008	219±15
1.8	655±33	143±9	184±9	0.126±0.008	209±15
2.4	813±33	158±9	166±9	0.153±0.008	223±15
3	743±33	143±9	166±9	0.131±0.008	187±15
No feed additive	712±33	146±9	172±9	0.139±0.008	219±15
Antibiotic (BMD)[1]	729±33	147±9	172±9	0.135±0.008	186±15
P-value	0.07	0.24	0.62	0.38	0.47

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 3.23. Day 35 ileal intestinal histomorphology measurements of broiler chickens with dietary supplementation of *Palmaria palmata*, inulin, antibiotic and no feed additive.

	Villus height (μm)	Villus width (μm)	Crypt depth (μm)	Villus surface area (mm^2)	Mucosal depth (μm)
Treatments					
Palmaria palmata (%)					
0.6	889.6±25	180.8±6.3	160.2±1.7	0.153±0.008	284.2±13
1.2	851.7±25	164±6.3	158.1±1.7	0.166±0.008	264.9±13
1.8	847.5±25	166.6±6.3	156.6±1.7	0.154±0.008	292.5±13
2.4	836.8±25	160.3±6.3	154.8±1.7	0.156±0.008	270.0±13
3	832.7±25	155.6±6.3	154.1±1.7	0.147±0.008	302.8±13
Inulin (%)					
0.6	867.8±25	161.7±6.3	154.8±1.7	0.152±0.008	284.3±13
1.2	841.2±25	169.1±6.3	157.0±1.7	0.143±0.008	280.6±13
1.8	866.1±25	164±6.3	153.6±1.7	0.154±0.008	272.1±13
2.4	843.6±25	157±6.3	156.1±1.7	0.143±0.008	277.0±13
3	807.2±25	155±6.3	152.5±1.7	0.137±0.008	287.1±13
No feed additive	847.4±25	154.3±6.3	152.7±1.7	0.136±0.008	281.4±13
Antibiotic (BMD)[1]	763.6±25	158.6±6.3	156.1±1.7	0.145±0.008	293.4±13
P-value	0.10	0.20	0.09	0.30	0.70

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

There was an increasing trend in the size of villus height, width, villus surface area and mucosal depth during days 7, 14, 24 and 35 (Table 3.24). Crypt depth size had an increasing trend during days 7, 14, 24, but at day 35, the crypt depth decreased unexpectedly.

Table 3.24. The effects dietary treatments *Palmaria palmta*, inulin, antibiotic and no feed additive during days 7, 14, 24 and 35

Day	Villus Height (μm)	Villus Width (μm)	Crypt depth (μm)	Villus surface area (mm^2)	Mucosal depth (μm)
Day 7	448 ± 8^{a}	127 ± 2^{a}	120 ± 2^{d}	0.05 ± 0.002^{d}	124 ± 8^{d}
Day 14	630 ± 8^{b}	148 ± 2^{b}	141 ± 2^{c}	0.10 ± 0.002^{c}	177 ± 8^{c}
Day 24	730 ± 8^{c}	150 ± 2^{b}	166 ± 2^{a}	0.13 ± 0.002^{b}	210 ± 8^{b}
Day 35	841 ± 8^{d}	162 ± 2^{c}	155 ± 2^{b}	0.14 ± 0.002^{a}	283 ± 8^{a}

$^{a-b}$ means±SEM with different superscripts within a row are significantly different ($p\leq0.05$)

The result of microbiological analysis indicated that under normal environmental conditions for broiler grown in the cages, none of the treatments influenced ileum microbiota. Gut microbial composition can play an important role in the structure of intestinal morphology by increasing villus height in the jejunum and ileum or increasing beneficial bacteria such as *Bifidobacteria* (Awad et al., 2009). No treatments affected the production of intestinal epithelial cells either by altering bacterial populations or through fermentation into SCFA, or perhaps both.

Researchers observed different results for seaweeds used as a feed additive in their studies. These varied results could be associated with seaweeds composition difference. However, most of seaweeds contain algal fibers, which are soluble anionic polysaccharides and their sugars unique to seaweeds as well (Lahaye et al., 1993).

As case in a point, Wiseman (2012) observed that feeding Tasco® improved the histomorphological structure of the small intestine by enhancing the villi apparent area and crypt depth compared to feeding inulin during early growth (day 7). Intestinal morphological structure can be associated directly or indirectly with gut microbial composition (Baurhoo et al., 2007). Kulshreshtha et al. (2014) reported that supplementation of layer feed with the red seaweed species *Chondrus crispus* and *Sarcodiotheca gaudichaudii* increased the villus height and villus surface area, compared to the control birds. It has been reported that enhancing *Bifidobacteria* and *Lactobacilli* can affect the development of intestinal morphological structure (Pourabedin et al.,

2014). Diets, which contain large amounts of dietary fiber alter the microbial population to promote acetic acid and butyrate-producing bacteria to provide energy sources for epithelial cells, which are associated with increased villi height in the jejunum and ileum (Liu et al., 2012). From the scientific studies reporting microbiota results in chickens, many treatments have not changed the gut microbial composition. Gut microbial composition plays a vital role directly or indirectly on intestinal morphological structure, either by changing bacterial populations or through fermentation into SCFA (mainly acetate, propionate and butyrate), or perhaps both (Pourabedin et al., 2014). The production of SCFA decrease the luminal pH and also provide energy sources for epithelial cells and can also improve intestinal mucosal structure.

Furthermore, Baurhoo et al. (2007) reported that enhancing *Lactobacilli* and *Bifidobacteria* colonization improved health of broilers due to an increase in villi length. This could have given the birds an advantage in nutrient absorption, as it would increase the protrusion of the villi into the digesta, enhancing contact with nutrients present. Previous studies have shown that intestinal function depends on the intestinal mucosal architecture. A shorter crypt depth and a longer villus height provide a greater amount of surface area for nutrient absorption (Torok et al., 2009)

3.6.7 IgA level

Plasma IgA was not affected by any of the treatments (Table 3.25) at age 35. Similarly, Kim et al. (2011) observed that supplementation of broiler feed with (0.25% and 0.5%) FOS (0.586±0.45 and 0.575±0.45 mg/ml) and (0.25% and 0.5%) MOS (0.591±0.45 and 0.599±0.45 mg/ml) did not significantly alter the concentration of IgA, compared to the no feed additive group (0.586±0.45 mg/ml). In other studies however, IgA was increased in broilers by the supplementation of β1-4 mannobiose (Agunos et al., 2007). In addition, Perez-Carbajal et al. (2010) observed that challenging birds with *Eimeria* elevated the mean range of IgA from 0.051±2.0-0.140±25.2 mg/ml at day 14 to 0.325±31.2-0.369. There is inconsistency in the literature on the levels of IgA and the results of the current study are in agreement with Kim et al. (2011).

Table 3.25. Effects of *Palmaria palmata*, inulin, antibiotic and no feed additive on the IgA in the blood of broilers on day 35.

	Plasma IgA (mg/ml)
Treatments	
Palmaria palmata (%)	
0.6	0.559±0.21
1.2	0.541±0.21
1.8	0.589±0.21
2.4	0.557±021
3.0	0.620±0.21
Inulin (%)	
0.6	0.544±0.21
1.2	0.528±0.21
1.8	0.530±0.21
2.4	0.537±0.21
3.0	0.525±0.21
No feed additive	0.524±0.21
Antibiotic (BMD)[1]	0.580±0.21
P-value	0.62

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Prebiotics such as inulin have the ability to modulate the function of the immune system (Watzl et al., 2005). The level of plasma immunoglobulin indicates the response of the host to different infections, by producing antibodies. IgA plays an important role in GIT defense and secretion into the gut lumen as well as protecting the apical surface of the brush border (Lewis et al., 2010). Therefore, IgA has the potential to change the microbial population by targeting those species that the immune system considers pathogenic (Lewis et al., 2010).

Previous studies have shown that supplementation of feed with FOS leads to stimulation of mucosal immune cells, by enhancing bacterial components (e.g. peptidoglycan) and polysaccharides, all of which originate from Gram-positive bacteria within the intestine (Patterson et al., 2010). In addition, Gram-positive bacteria play an important role to stimulate the proliferation of epithelial cells by their byproduct of fermentation (SCFA)

(Hosono et al., 2003). Prebiotics are able to influence the immune system by increasing lymphocyte and/or leucocyte numbers in GALT (Field et al., 1999) and increasing IgA secretion in GALT (Kudoh et al., 1998). IgA, produced by the lymphocytes, plays an important role in mucosal immunity, responding to the intrusion of foreign substances into the body. The mucosal immune system inhibits pathogenic bacteria and neutralizes biologically active antigens by IgA secretion from the mucosal surface of the gastrointestinal tract (Ohashi et al., 2006). Therefore, it is worthwhile to evaluate the effect red seaweed on the amount of plasma immunoglobulin in broilers as plasma immunoglobulin levels indicates their potential to fight against various infections. Measuring immunoglobulin levels of birds fed red seaweed demonstrates their different stimulation aspects of the gut-associate immunity along with increased production performance and decreased pathogenic bacteria in chickens. Rezai et al. observe that feeding birds with 1% oligosaccharides from palm kernel (OoligoPKE), at days 21 (0.492±0.096 mg/ml) and 35 (0.539±0.052 mg/ml), increased the blood IgA concenteration, in comparison with no feed additives (0.204±0.097 and 0.263±0.052 mg/ml), respectively.

3.6.8 Next generation sequencing results

To determine if the *Palmaria palmata* affected the gut microbiota, the ileal microbiota of chickens on 7, 14, 24 and 35 days of age, in each treatment group, were compared. The relative abundance of OTUs was analyzed at different ranking levels from phylum to genus. At the phylum level, ileal microbiota was mainly composed of *Firmicutes* followed by *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. The majority of sequences isolated from the chicken GIT were *Firmicutes*, containing two subgroups of bacteria: bacilli, having the highest population, and clostridia (Figure 3.6).

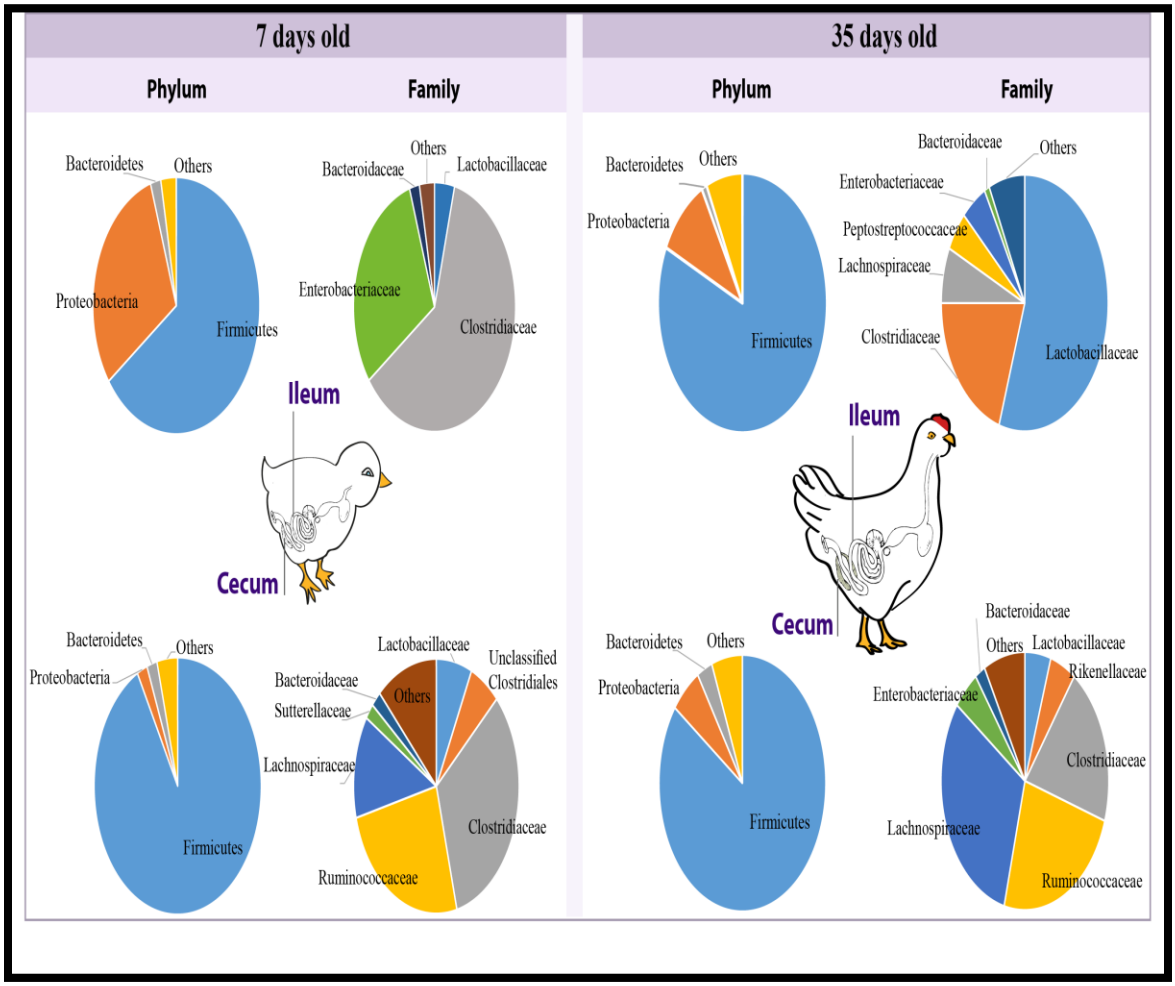
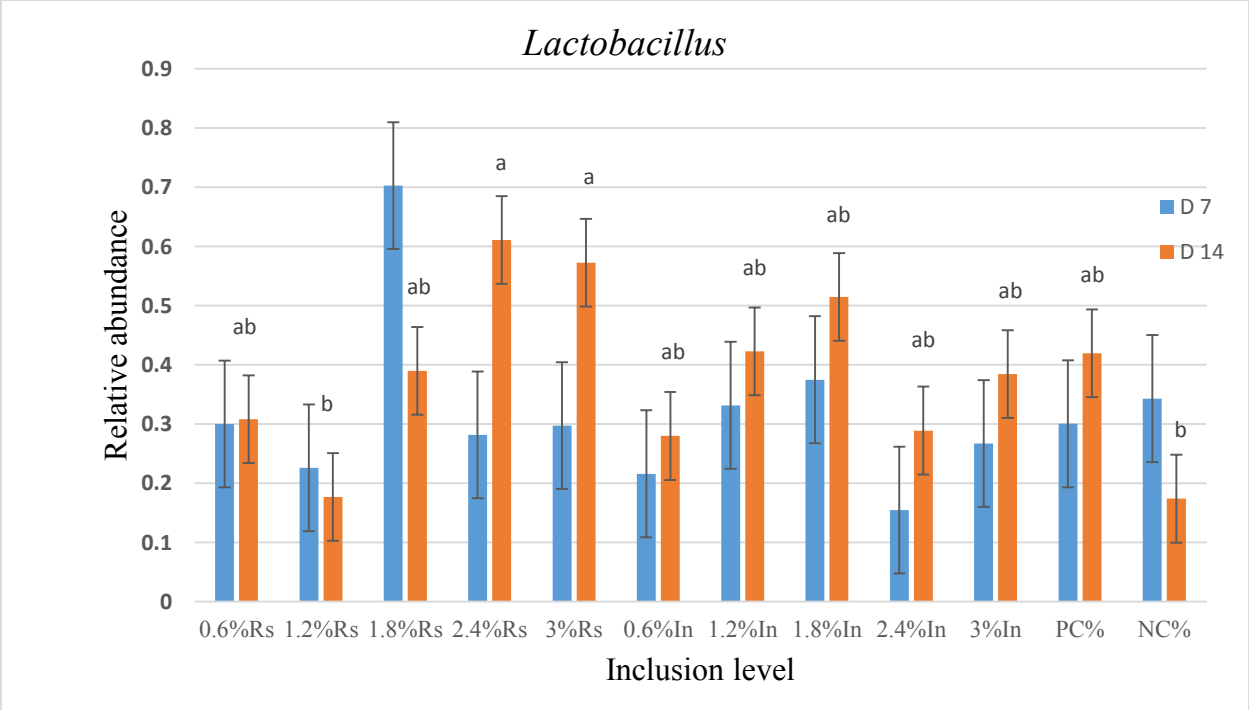


Figure 3.6. Prebiotics and gut microbiota in chickens (Pourabedin et al., 2015).

None of the dietary treatments had a significant effect on *Bacilli*. *Lactobacillales* and *Clostridiales* were the dominant order in the ileum over time. The most dominant family in the ileum was *Peptostreptococcaceae* and *Lactobacillaceae*. *Peptostreptococcaceae* is a family of gram positive bacteria in the class Clostridia and *Lactobacillaceae* are a family of lactic acid bacteria which are beneficial bacteria. The genus *Lactobacillus* and *Candidatus Arthromitus* were the more abundant in the ileum. Pourabedin et al. (2015) observed that the addition of xylo-oligosaccharides and virginiamycin to broilers diets in different time periods changed the abundance of *Enterobacteriaceae* bacteria in the ileum

and cecum of the chickens. This study shows that sub-therapeutic levels of antibiotic did not have any specific effects on gut flora. Similarly, Lin et al. (2013) observed that dietary supplementation with a sub-therapeutic level of tylosin did not alter the population of total viable bacterial biomass in the small intestine

Supplementation with *Palmaria palmata* increased the proportion of *Lactobacillus* genus in the ileum of birds on days 7, 24 and 35. Feeding *Palmaria palmata* at 3% increased relative abundance of *Lactobacillus* genus in comparison with inulin, no feed additive and antibiotic (Table 3.26). In addition, 0.3% *Palmaraia palmata* enhanced the relative abundance of *Lactobacillus* genus compared to antibiotic and no feed additive (Figure 3.7). Furthermore, 1.8% and 2.4% *Palmaria palmata* and all inulin inclusion levels had greater relative abundance of *Lactobacillus* genus compare to no feed additive and antibiotic on day 35 (Figure 3.8). Pourabedin et al., (2015) reported that feeding xylo-oligosaccharides and virginiamycin increased *Lactobacillus* in the cecum of chicken and *Propionibacterium* and *Corynebacterium* genera were enriched in the ileum. Similarly, Kulshreshtha et al. (2014) observed that supplementation of red seaweed (*Sarcodiotheca gaudichaudii* and *Chondrus crispus*) increased the relative abundance of *bifidobacterium longum* in the small intestine. In this study, treatments with *Palmaria palmata* altered the relative abundance but not the presence or absence of specific microbial genus. Enhanced population levels of lactate-producing bacteria such as *Lactobacillus* in the GIT of chickens on day 35 supplemented *Palmaria palmata* might be an intestinal health-promoting aspect and may indeed be a prebiotic effect.

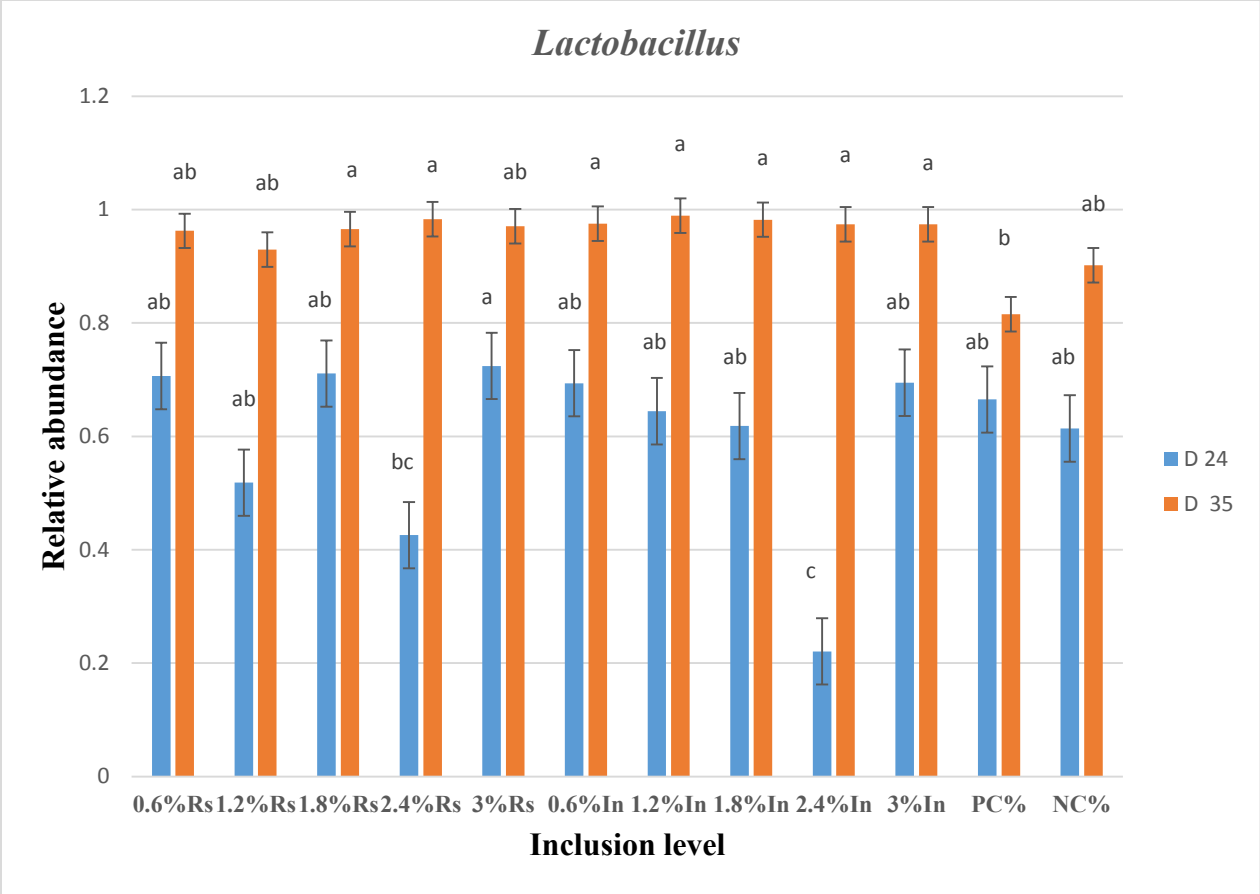


[a-b] means±SEM with different superscripts are significantly different ($p \leq 0.05$)

Figure 3.7. Effect of *Palmaria palmata*, inulin, positive control and negative control on the relative abundance of *Lactobacillus* in the ileum of chicken on Days 7 and 14. Negative control (NC = no feed additive), positive control (PC: antibiotic = Bacitracin methylene disalicylate), inulin (In: 0.6%, 1.2%, 1.8%, 2.4% and 3%) and *Palmaria palmata* (Rs: 0.6%, 1.2%, 1.8%, 2.4% and 3%).

Table 3.26. ANOVA p-values for relative abundance of *Lactobacillus* on days 7 14, 24 and 35.

ANOVA	P-value			
	Day 7	Day 14	Day 24	Day 35
Treatment	0.14	0.0017	0.0001	0.0016



[a-c] means±SEM with different superscripts are significantly different ($p \leq 0.05$)

Figure 3.8. Effect of *Palmaria palmata*, inulin, positive control and negative control on the relative abundance of *Lactobacillus* in the ileum of chicken on Days 24 and 35. Negative control (NC = no feed additive), positive control (PC: antibiotic = Bacitracin methylene disalicylate), inulin (In: 0.6%, 1.2%, 1.8%, 2.4% and 3%) and *Palmaria palmata* (Rs: 0.6%, 1.2%, 1.8%, 2.4% and 3%).

3.7 General discussion

The microbiota of the gut can affect the growth and health status of the chicken and its contents are influenced by the consumption of different feeds (Janardhana et al., 2009). Results from this trial indicated 1.8%, 2.4% and 3% *Palmaria palmata* improved beneficial bacteria in the gut. In prebiotic studies it is often found that gut environment and bacterial populations are altered while no response in growth occurs (Baurhoo et al., 2007). The change in gut microbiota is largely caused by complex polysaccharides which are fermented in the caecum and are resistant to acid hydrolysis in the upper gastrointestinal tract of higher animals (O'Sullivan et al., 2010). *Palmaria palmata* treatments altered cecal content pH on days 24 and 35, in comparison with a no feed additive diet, which resulted in an increase in bacterial fermentation. However, under the clean conditions and tight environmental control of this study, no difference in performance was observed. The change in gastrointestinal bacterial fermentation from the cecal pH could indicate that under less than ideal conditions, a greater response might be observed. Therefore a new trial, incorporating more stress, should be undertaken to further evaluate the effects of feeding *Palmaria palmata*.

3.8 Conclusion

There were indications that *Palmaria palmata* acted as a prebiotic when fed at levels of 1.8%, 2.4% and 3% as evidenced by increased beneficial bacteria including *Lactobacillus* genus in the chicken's ileum. The pH of cecal content was decreased by *Palmaria palmata*, antibiotic and inulin, which is indicative of a prebiotic activity, however, this did not translate to improve growth performance of birds. But this may have attributed to the condition of the birds grown in non-traditional environment. These results indicated that *Palmaria palmata* had improving effects by changing the fermentation patterns of microbes in the ceca (decreasing pH in the ceaca) and microbial population present in the ileum (increasing beneficial bacteria including *Lactobacillus*). Based on the hypotheses of this trial, some of them by measuring histology and microbiology were achieved. However, some others were not meet such as growth performance parameters.

Theses changes suggest that *Palmaria palmata* may indeed act as a prebiotic. Therefore, *Palmaria palmat* should be tested in the presence of a stressor.

Chapter 4 EFFECTS OF *PALMARIA PALMATA* SUPPLEMENTED DIETS FED TO BROILER CHICKENS RAISED UNDER NORMAL OR STRESSED CONDITIONS

4.1 Abstract

The effects of dietary supplementation with inulin or *Palmaria palmata* (*P.p.*) on the growth performance, small intestine microflora, immune response, ileal histomorphology and next generation sequencing of intestinal microbiota in broilers subjected to environmental stress (S) were analyzed. Environmental stress was imposed by using dirty litter, elevated humidity and temperature which was compared to a normal (US) environment. One thousand eight hundred and seventy two, 1 d-old, Ross 308, broiler chickens were randomly assigned to six dietary treatments in four rooms: no feed additive, antibiotic, inulin 2.5% or *P.p.* at 0.6%, 1.8%, and 3% of diet. The temperature were 32°C initially in the normal rooms, containing clean litter, then reduced 1°C every two day until a temperature of 23°C was reached. At d 7 S rooms were held at temperatures approximately 2-3°C above the US rooms. The average US room humidity was 6.4, 6.6, 5.1 and 5 gm $(m^3)^{-1}$on d: 7, 14, 24 and 35, respectively. Humidity in the S rooms were 7.4, 8, 8.3 and 8.5 gm $(m^3)^{-1}$on the same dates. The average US room ammonia level was 0, 1.5, 0.5 and 2 ppm on d: 7, 14, 24 and 35, respectively. Ammonia in the S rooms were 2, 2, 3 and 15 ppm on the same dates, respectively. The experiment was a randomized block design with split plots with two room treatments (U and US) as the main plots and six dietary treatments as the subplots. Each treatment had 6 replicates, of 26 birds per pen. One bird per pen at 7, 14, 24 and 33 d of age was randomly selected and euthanized by cervical dislocation. Their entire small intestines were collected. Birds fed 1.8% *P.p. P.p.* containing diets exhibited improved final body weight (2386.3± 33.4 g $bird^{-1}$ day^{-1}) ($P < 0.05$) over the antibiotic treatment (2155.2± 33.4 g $bird^{-1}$ day^{-1}). *P.p.* at 1.8% (114.4± 3.0 g $bird^{-1}$ day^{-1}) increased ($P < 0.05$) body weight gain compared with no feed additive (107.19± 3.0 g $bird^{-1}$ day^{-1}) and antibiotic (99.52± 3.0 g $bird^{-1}$ day^{-1}) at d 33. At d 14, 24 and 33, birds fed 1.8% *P.p.* (6.6± 0.01, 6.7± 0.008 and 6.84± 0.006 log_{10} cfu

g^{-1}) had greater number of lactic acid bacteria in the ileal microbiota compared to other treatments. Supplementation of broilers with 1.8% *P.p.* resulted in a significant enhancement in the number of *Bifidobacteria* on Days 14 (6.92± 000.6 $\log_{10}$ cfu g^{-1}), 24 (7.01± 0.005 $\log_{10}$ cfu g^{-1}) and 33 (7.06± 0.004 $\log_{10}$ cfu g^{-1}). *P.p.* at 0.6% (583.75± 9.04 μm) had a positive and significant ($P < 0.05$) effect on increasing villi height compared to all other treatments while at 1.8% *P.p.* (158.0 ± 3.2 μm) greater ileal villi width occurred compared to the no feed additive at d 7. Plasma immunoglobulin (IgA) was higher ($P < 0.05$) for 1.8% *P.p.* (595 ± 0.16 mg/ml) compared to no feed additive (490 ± 0.16 mg/ml) at d 33. In a stressful environment feeding *P.p.* improved growth and immune protection of the gut by increasing IgA.

Keywords: Prebiotic, red seaweed, inulin, antibiotic, environmental stress, IgA

4.2 Introduction

Results from the previous trial (chapter 3) in a clean environment demonstrated that addition of 0.6%, 1.8% and 3% *Palmaria palmata* to the diet modified the relative abundance of beneficial bacteria including *Lactobacillus* in the ileum. This current trial was designed to compare the use *Palmaria palmata* in normal environmental and environment with stress including elevated temperature and humidity in the presence of used litter (bedding material). Previous studies have shown that the effects of prebiotics on broiler growth performance were related to different factors, including environment, management, nutrition and type of feed additive (Houshmand et al., 2012). In addition, Kulshreshtha et al., (2014) observed that supplementation of red seaweeds *Chondrus crispus* and *Sarcodiotheca gaudichaudii* improved the performance and health of laying hens. It is well documented that environmental stress, due to high ambient temperature and used litter, are important challenges for the poultry industry that can affect the optimal growth performance and health of the birds (Sohail et al., 2012 and Nagaraj et al., 2007). Previous studies have shown that feed additives, including prebiotics such as MOS, can reduce the adverse effects of those stressors (Sohail et al., 2010, 2011 and 2012). Hence, it is important to test *Palmaria palmata* for its potential as a prebiotic, and as an alternative to AGP under environmental stress conditions. In the previous trial, birds were housed in cages, but in this trial, they were placed in floor pens. Santos et al. (2007) observed that birds grown on litter had improved feed conversion, lower cecal Salmonella levels, increased villi height, villi surface area, and villi height to crypt depth ratio. Better ileal histological parameters improved the health and growth of birds. (Santos et al., 2007).

Floor pens are more typical of industry production systems. Bacteria isolation from chicken was higher in birds raised in the floor pen than those in wire cages (Willis et al., 2002). The 0.6%, 1.8%, 3% *Palmaria palmata*, inulin, at the inclusion level of 2.5%, antibiotic (BMD) at the 0.04% and antibiotic were selected as the appropriate levels for further study.

4.3 Objectives

1) To determine the optimal dietary inclusion level of *Palmaria palmata* for growth performance of broilers
2) To determine the effect of *Palmaria palmata* on the beneficial gut bacterial population by microbiology and sequencing bacterial DNA.
3) To determine the effect of dietary inclusion of *Palmaria palmata* on small intestine morphology, cecal pH and plasma IgA

4.4 Hypothesis

1) Growth performance of the broiler chickens fed diets with the optimal level of *Palmaria palmata* will be better than those fed inulin or control diets.
2) *Palmaria palmata* will demonstrate the characteristic of a prebiotic specifically the gastrointestinal colonization by beneficial bacteria.
3) *Palmaria palmata* in the diet decrease cecal pH and increase plasma IgA concentrations.

4.5 Materials and methods

Palmaria palmata at 0.6%, 1.2% and 1.8% was compared with a commercial inulin product at 2.5% inclusion level in the diets. A positive control group including a commercially used antibiotic (Bacitracin Methylene Disalicylate (BMD)) and a negative control group with no feed additive were evaluated. This study investigated *Palmaria palmata*, in environmental stress conditions caused by elevated temperature, humidity and the presence of used litter, compared to US conditions that were typical of a well-managed facility.

4.5.1 Animal housing

One thousand eight hundred and seventy two, one-day-old, male broiler chickens (Ross 308) were obtained from Clark's Hatchery (Burtts Corner, NB). The birds obtained on the day of hatch, were immediately randomly assigned to 72 pens, (2.13 m x 1.40 m), in

four rooms. There were 26 birds per pen housed in the Atlantic Poultry Research Centre (APRC). The US room temperature program from trial 1 was applied (Appendix B). Two S rooms were kept at temperature conditions of approximately 2-3ºC above the standard temperature considered ideal for optimum broiler weight gain and feed conversion. These two rooms had from a previous flock bedding litter. The change in temperature regime for the environmentally stress rooms compared to the normal rooms started on day 7 post placement. Elevated temperature was maintained daily for stress rooms from day 7 to the end of the trial. In the normal rooms the temperature was maintained continuously at normal recommended broiler grower temperatures from day 0 to the end of the study (Appendix C). Use of these different conditions created very distinct environments in which to rear birds. The temperature and lighting of the rooms when the birds arrived were 32ºC and 20 lux, respectively. The temperature in the normal rooms was reduced reduced 1°C every two day until a temperature of 23°C was reached. The two stress environment rooms were maintained at 26ºC until day 33. The lighting in all rooms was reduced by 5 lux every 4 days until 5 lux was reached, then maintained at five lux until the end of the trial (Appendix A). Chicks were fed on cardboard trays for the first week and from tube feeders for the entire experiment. Water from 2 nipple drinkers per pen was supplied *ad libitum* to the birds throughout the trial. Birds were introduced to water by dipping beaks in the nipple drinkers. Environment temperature (Appendix B) and absolute humidity (Appendix C) were recorded by a data logger in all trial days. Ammonia levels were measured on each sampling day using ammonia tubes (Draeger, Lübeck, Germany) (Appendix D). Humidity was increased daily by spraying water on the litter in the barn passageway between the pens. Bird care was conducted in accordance with the Canadian Council on Animal Care guidelines (CCAC 2009).

4.5.2 Diets and seaweed preparations

4.5.2.1 Seaweed preparation

Samples of cultivated red seaweed *Palmaria palmata* were grown by Acadian Seaplants Limited, Nova Scotia, Canada. The seaweed was grown in an on-land

cultivation facility. Freshly harvested biomass was dried in a batch fluidized bed drier, at 50ºC (Arthur H. Thomas Co., Philadelphia, PA) until dry, and ground to a powder that passed through a screen mesh size, 0.4 mm using a micro Wiley mill, standard model 3. The crude protein content of *Palmaria palmata* was reported to be 19.0 % on dry matter basis (Lourenco et al., 2002).

4.5.2.2 Diet preparation

Diets were formulated to be isocaloric and isonitrogenous within the starter (day 0-14) (Table 4.1), grower (day 15-24) (Table 4.2) and finisher (day 25-33) (Table 4.3) phases. Feed was given in crumble form during the starter period and pelleted form during the grower and finisher periods. Diets met or exceeded the NRC (1994) nutrient requirements for birds at each phase of growth. Three dietary treatments were fed. *Palmaria palmata* at 0.6%, 1.8% and 3%. The commercial inulin, provided by Cargill Inc. (Wayzata, MN) as the product Oligo- Fiber™ DS2 inulin with average DP≤10, was fed at 2.5%. A diet with the antibiotic (bacitracin methylene disalicylate) at 0.04% level (BMD) was used as a positive control, while the negative control had no feed additive. The treatments were randomly allocated among the pens within each room.

All diets were mixed in a Hobart bowl type mixer (model L.800, The Hobart Manufacturing Co. Ltd, Don Mills, Ontario, Canada). All the starter, grower and finisher diets were formulated using MIXIT-WIN a professional feed formulation program (version 6.22, Agricultural Software Consultants, Inc.). Diets were fed in mash form through the experiment.

Table 4.1. Diet formulations for the starter period (day 0-14) with *Palmaria palmata*, inulin, antibiotic or no feed additive under normal and stressed conditions.

			Palmaria palmata			Inulin
	No feed additive	Antibiotic	0.6%	1.8%	3%	2.5%
Ingredient (% as fed)						
Corn	39.71	41.58	39.03	37.67	36.30	34.68
Soybean Meal	41.58	39.70	41.43	41.12	40.82	42.27
Wheat	10.00	10.00	10.00	10.00	10.00	10.00
Tallow-Grease (An/Veg Fat)	3.95	3.96	4.21	4.70	5.20	5.78
Limestone, Ground	1.45	1.45	1.45	1.44	1.44	1.43
Mono-Dicalcium Phosphorus	1.76	1.76	1.76	1.76	1.77	1.77
Methionine Premix[1]	0.60	0.60	0.61	0.62	0.64	0.62
Vitamin/mineral Premix[2]	0.50	0.50	0.50	0.50	0.54	0.50
Iodized salt	0.45	0.45	0.43	0.38	0.34	0.45
BMD[3]	-	0.04	-	-	-	-
Palmaria palmata[4]	-	-	0.60	1.80	3.00	-
Inulin[5]	-	-	-	-	-	2.5
Total	100	100	100	100	100	100
Calculated analysis						
Metabolizable energy (kcal/kg)	3025	3025	3025	3025	3025	3025
Protein (%)	23.0	23.0	23.0	23.0	23.0	23.0
Calcium (%)	1.05	1.05	1.05	1.05	1.05	1.05
Lysine (%)	1.30	1.30	1.30	1.28	1.27	1.31
Tryptophan (%)	0.26	0.26	0.26	0.25	0.25	0.26
Methionine+Cysteine (%)	0.94	0.94	0.94	0.94	0.94	0.94
Sodium (%)	0.19	0.19	0.19	0.19	0.19	0.19
Available phosphorous (%)	0.50	0.50	0.50	0.50	0.50	0.50
Determined analysis						
Protein (%)	24.0	24.5	25.0	25.1	24.3	24.4
Total calcium (%)	1.0	1.1	1.2	1.1	1.0	0.81
Total phosphorus (%)	0.74	0.83	0.81	0.79	0.77	0.71
Sodium (%)	0.17	0.22	0.23	0.21	0.23	0.18
Crude fat (%)	5.86	6.12	6.24	6.81	6.88	9.37

[1] The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vitamin/mineral premix containing the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3]BMD is medicated bacitracin methylene disalicylate premix at 0.04%

[4] Red seaweed provided by Acadian Seaplants Ltd. (Dartmouth, NS)

[5] Oliggo-Fiber DS2 inulin (Cargill Inc., Wayzata, MN)

Table 4.2. Diet formulations for the grower period (day 15-24) with *Palmaria palmata*, inulin, antibiotic or no feed additive under normal and stressed conditions.

			Palmaria Palmata			Inulin
	No feed additive	Antibiotic	0.6%	1.8%	3%	2.5%
Ingredient (% as fed)						
Corn	42.74	42.73	42.06	40.70	39.34	37.71
Soybean Meal	36.85	36.85	36.70	36.40	36.10	37.55
Wheat	10.00	10.00	10.00	10.00	10.00	10.00
Tallow-Grease (An/Veg Fat)	5.73	5.73	5.97	6.74	6.96	7.55
Limestone, Ground	1.20	1.20	1.20	1.20	1.19	1.19
Mono-Dicalcium Phosphorus	1.55	1.55	1.55	1.56	1.49	1.57
Methionine Premix[1]	0.50	0.50	0.51	0.52	0.54	0.51
Vitamin/mineral Premix[2]	0.50	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.42	0.42	0.40	0.36	0.31	0.43
BMD[3]	-	0.04	-	-	-	-
Red seaweed[4]	-	-	0.60	1.80	3.00	-
Inulin[5]	-	-	-	-	-	2.5
Total	100	100	100	100	100	100
Calculated analsis						
Metabolizable energy (kcal/kg)	3150	3150	3150	3150	3150	3150
Protein (%)	21.0	21.0	21.0	21.0	21.0	21.0
Calcium (%)	0.90	0.90	0.90	0.90	0.90	0.90
Lysine (%)	1.17	1.17	1.17	1.17	1.18	1.18
Tryptophan (%)	0.23	0.23	0.23	0.23	0.23	0.23
Methionine+Cysteine (%)	0.84	0.84	0.84	0.84	0.84	0.84
Sodium (%)	0.18	0.18	0.18	0.18	0.18	0.18
Available phosphorous (%)	0.45	0.45	0.45	0.45	0.45	0.45
Determined analysis						
Protein (%)	22.1	22.1	21.6	21.8	21.6	21.4
Total calcium (%)	0.81	0.87	0.87	0.85	0.90	0.85
Total phosphorus (%)	0.71	0.70	0.71	0.71	0.72	0.74
Sodium (%)	0.18	0.19	0.20	0.20	0.21	0.24
Crude fat (%)	7.71	7.67	7.65	8.13	8.76	7.70

[1]The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vitamin/mineral premix containing the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3] BMD is medicated bacitracin methylene disalicylate premix at 0.04%

[4] Red seaweed provided by Acadian Seaplants Ltd. (Dartmouth, NS)

[5] Oliggo-Fiber DS2 inulin (Cargill Inc., Wayzata, MN)

Table 4.3. Diet formulations for the finisher period (day 25-33) with red seaweed, inulin, antibiotic and no feed additive under normal and stressed conditions.

			Palmaria palmata			Inulin
	No feed additive	Antibiotic	0.6%	1.8%	3%	2.5%
Ingredient (% as fed)						
Corn	51.10	51.09	50.44	49.13	36.85	46.25
Soybean Meal	29.24	29.24	29.09	28.78	29.99	29.91
Wheat	10.00	10.00	10.00	10.00	10.00	10.00
Tallow-Grease (An/Veg Fat)	5.07	5.07	5.30	5.75	10.00	6.75
Limestone, Ground	1.22	1.22	1.21	1.21	1.18	1.20
Mono-Dicalcium Phosphorus	1.45	1.45	1.45	1.46	1.49	1.46
Methionine Premix[1]	0.49	0.49	0.50	0.51	0.54	0.50
Vitamin/mineral Premix[2]	0.50	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.42	0.42	0.40	0.36	0.32	0.43
BMD[3]	-	0.04	-	-	-	-
Red seaweed[4]	-	-	0.60	1.80	3.00	-
Inulin[5]	-	-	-	-	-	2.5
Total	100	100	100	100	100	100
Calculated analysis						
Metabolizable energy (kcal/kg)	3150	3150	3150	3150	3150	3150
Protein (%)	18.0	18.0	18.0	18.0	18.0	18.0
Calcium (%)	0.85	0.85	0.85	0.85	0.85	0.85
Lysine (%)	1.30	1.30	1.30	1.28	1.27	1.31
Tryptophan (%)	0.26	0.26	0.26	0.25	0.25	0.26
Methionine+Cysteine (%)	0.94	0.94	0.94	0.94	0.94	0.94
Sodium (%)	0.19	0.19	0.19	0.19	0.19	0.19
Available phosphorous (%)	0.50	0.50	0.50	0.50	0.50	0.50
Determined analysis						
Protein (%)	21.2	18.7	18.8	19.2	19.0	18.2
Total calcium (%)	0.80	0.77	0.73	0.79	0.76	0.83
Total phosphorus (%)	0.64	0.66	0.65	0.66	0.64	0.66
Sodium (%)	0.18	0.19	0.20	0.21	0.21	0.20
Crude fiber (%)	5.69	6.98	7.41	7.47	7.80	8.66

[1]The methionine premix contained 0.5kg/kg DL- Methionine and 0.5kg/kg wheat middlings

[2]Vitamin/mineral premix containing the following per kg of diet: 9750 IU vitamin A; 2000 IU vitamin D3; 25 IU vitamin E; 2.97 mg vitamin K; 7.6 mg riboflavin; 13.5 mg Dl Ca-pantothenate; 0.012 mg vitamin B12; 29.7 mg niacin; 1.0 mg folic acid, 801 mg choline;0. 3 mg biotin; 4.9 mg pyridoxine; 2.9 mg thiamine; 70.2 mg manganese; 80.0 mg zinc; 25 mg copper; 0.15 mg selenium; 50 mg ethoxyquin; 1543mg wheat middlings; 500 mg ground limestone

[3]BMD is medicated bacitracin methylene disalicylate Premix at 0.04%

[4] Red seaweed provided by Acadian Seaplants Ltd. (Dartmouth, NS)

[5] Oliggo-Fiber DS2 inulin (Cargill Inc., Wayzata, MN)

4.5.3 Analysis of growth performance

At 0, 7, 14, 24 and 33 days of age, the birds were group weighed per pen and feed was weighed back and recorded. Feed provided was weighed and recorded each day at feeding. When mortalities occurred, it was recorded, the dead birds were weighed, and the feed of that pen weighed back (Animal Pathology Lab, Nova Scotia). The cause of mortality was determined from necropsy by a veterinary pathologist. Feed intake, body weight, body weight gain and feed conversion ratio on a per bird basis and % mortality were measured or calculated during each stage of growth.

4.5.4 Sample collection

One bird per pen was randomly selected and euthanized by cervical dislocation at 7, 14, 24 days of age. At day 33, birds were euthanized with an electric knife. Each bird was weighed and the entire ileum was removed between Meckel's diverticulum (identified as the start of ileum) and the ileocaecal junction. Two pieces, each 0.5-1.0 cm, in length were cut from the midpoint of ileum for NGS and histology analysis respectively. The rest of the ileum contents were harvested, placed in sterile plastic bags for conventional microbiology analysis. For NGS analysis, on day 7 the entire ileum was used, while on Day 14, 24 and 33, a 0.5-1.0 cm section was removed from midpoint of the ileum and immediately placed on ice and then frozen at -80°C within two hours and held at that temperature until analysis.

For histological analysis, the 0.5-1.0 cm section was removed from midpoint of the ileum and rinsed in deionized water. Each sample was then placed in 10% buffered formalin for storage and subsequent histological analysis of paraffin embedded sections. Both caeca were removed. The left one was immediately placed on ice and subsequently stored at -80°C until NGS was conducted. The pH of the right caecum was measured with a VWR 8000 Series bench-model pH meter (Fisher Scientific, Ottawa, ON) based on the procedure applied by Catala-Gregori et al., (2008). At day 33 of the experiment, one animal was randomly selected from each pen, euthanized by cervical dislocation and 5 ml of blood were collected individually from the neck into heparinized tubes and stored on ice for IgA analysis.

4.5.5 Intestinal histomorphology

Intestinal histomorphology analysis was performed using the same procedure as in the first trial (Section 3.4.8). Parameters measured were villus height, width, crypt depth, mucosal depth and villus surface area.

4.5.6 Analysis of serum immunoglobulin

Analysis of serum immunoglobulin was performed using the same procedure as the first trial section (3.5.6). In this trial, the same three dilutions sample prepared (1:100, 1:1000 and 1:100000) and dilution 1:1000 was within the dynamic range of standard curve. As described previously the ELISA kit (Bethyl Labratories Inc., Montgonery, TX) was used for analyzing of IgA. The absorbance was measured on a plate reader (Model Synergy HT, Bio-Tek Instrument, Inc., Winooski, USA) at 450 nm. A calibration curve was made using standard solutions and the IgA concentration was quantified by fitting the absorbance into the standard curve in accordance with ELISA kit data sheet. IgA was reported as mg/ml.

4.5.7 DNA extraction

Extraction of DNA was performed using the same procedure as in section 3.4.10. No modification of the procedure was used during the DNA extracting in this trial. Based on results from the last trial, where greater variation occurred in data collected in day 35, ileum samples on day 33 were prepared and sent for NGS analysis to Genome Quebec Innovation Center.

4.5.8 Microbiota analysis

Microbial analysis was performed at the same time points, following the same procedures as described in section 3.4.7, to determine whether different diet treatment groups affected ileal microbiota. A conventional culturing technique was used for measuring

Lactobacillus, *Bifidobacterium*, aerobic, anaerobic, *E. coli*, Coliform and *Clostridium perfringens*.

4.5.9 Statistical design

The experimental design was a randomized split plot block design with, two environments (US and S) as the main plots and six dietary treatments as the subplots. The response was measured using the repeated measure statement of SAS at Day 7, 14, 24 and 33. Dietary treatments were 0.6%, 1.8% or 3% *Palmaria palmata*, 2.5% inulin, antibiotic (BMD), and no feed additive. Data were analyzed using ANOVA in SAS 9.3 (SAS Institute Inc., Cary, NC) (Littell et al. 1996). Growth data (FI, BW, BWG and FCR) were the within-subject factors repeated over time. Data on physiological variables were analyzed as a single time measurement. Any significant main or interaction effects were analyzed using the Tukey Kramer method at to differentiate the means (Gbur et al., 2012). The level for significance was (α=0.05). Before analyzing the data for microbiological analysis, it was transformed to $\log_{10}$.

4.5.9.1 Statistical analysis of growth performance data

The FI, BW, BWG and FCR were analyzed as repeated measures when time was a factor. The model statement for Trial 2 repeated measure analysis was:

$$\mathbf{Y_{ijkl} = \mu + Treatment_i + Environment_j + Day_k + Treatment*Environment_{ij} + Treatment*Day_{ik} + Environment*Day_{jk} + Treatment*Environment*Day_{ijk} + \varepsilon_{ijkl}}$$

Where:

Y_{ijkl} was the response of the variable (BW, BWG, FI and FCR).

μ was the overall mean response of that parameter.

$Treatment_i$ was the effect of treatments (i=1-6).

$Environment_j$ was the effect of two environment conditions (S or US) (j=1-2).

Day_k was the effect of the k^{th} level of day (k=1-4).

ε_{ijkl} was the effect of the uncontrollable factors for the i^{th} level of treatment.

The statistical model for a single measurement analysis is likewise displayed above where all terms are the same, with the exception that day is no longer a factor.

$$\mathbf{Y_{ijkl} = \mu + Treatment_i + Environment_j + Treatment*Environment_{ij} + \varepsilon_{ijkl}}$$

4.5.9.2 Statistical analysis of microbiology analysis data

Before analyzing the data for microbiology analysis, they were transformed to $\log_{10}$. Y was the response variable for total aerobes, anaerobes, coliforms, *E. coli*, Clostridium perfringens and Bifidobacterium. The statistical model for single measurement analysis was the same as previously described.

4.5.9.3 Statistical analysis of histomorphology and IgA analysis data

The intestinal measurements evaluated were villus height, width, surface area and mucosal depth and crypt depth. IgA were analyzed. The statistical model for single measurement analysis was the same as that previously described.

4.5.9.10 Statistical analysis of NGS

For NGS analysis, the most abundant sequences were merged by selecting those classifications where the taxa had ≥0.5% representation in at least one sample. The data files were merged into the final file (Quince et al., 2011). The data of relative abundance of bacteria was run as a randomized block design using SAS. The statistical model analysis was the same as previously described.

4.6 Results and discussion

4.6.1 Analyzed nutrient composition of diets

The analyzed CP content of the starter (23%), grower (21%) and finisher (19%) diet (Table 4.1, Table 4.2 and Table 4.3) was higher than the calculated CP content for all treatments. In the starter period, the analyzed total calcium was lower than the calculated calcium (1.05%) for all treatments, except for antibiotic was higher than calculated analysis (Table 4.1). In the grower period, the analyzed total calcium was lower than the calculated calcium (0.90%) for all treatments (4.2). In the finisher period, the analyzed total calcium was lower than the calculated calcium (0.85%) for all treatments (Table 4.3). Nutrient composition of diets was analyzed twice, as the analyzed CP and calcium were lower than NRC (1994) requirements. At the second analysis, some of the values corrected. Although analyzed nutrition composition of diets indicated that analyzed CP and calcium were lower than NRC (1994) requirements, no symptoms of disease or mortality were observed during the trial. Therefore, this shortage did not endanger the health of the birds.

4.6.2. Growth performance

Treatment had no influence on FI ($p \leq 0.05$); however, there was an interaction effect between environment and bird age ($p \leq 0.05$). During all experimental periods, birds in the US had significantly higher FI than those in the S (Table 4.4).

There was an interaction effect for treatment and environment, treatment and bird age and environment *day for FCR (Table 4.4). Feeding birds with 0.5% brown seaweed showed improved BW and BWG (Wiseman 2012) in unstressed condition. Treatment*environment*day interaction for BW and BWG was significant ($p \leq 0.05$) (Table 4.4). During the finisher period, birds in US environment fed 1.8% *Palmaria palmat*had significantly greater BW, in comparison with other treatments (Table 4.5). On days 14 and 24, bird BW was not influenced by treatment (Table 4.5). Effect of S environment was observed for BW (Table 4.5). Birds raised in the US environment had significantly greater BW, in comparison with birds raised in S. Environmental stress,

caused by heat stress and dirty litter conditions, can reduce bird growth performance (Sohail et al., 2012).

Table 4.4. ANOVA P-values for (normal and stressed conditions) growth variable analysis and mortality

Growth Variable	Body weight	Body Weight Gain	Feed Intake	Feed to Gain	Mortality
ANOVA					
Treatment	<.0001	<.0001	0.66	0.45	0.82
Environment	<.0001	0.04	<.0001	0.60	0.76
Treatment* environment	<.0001	0.01	0.07	<.0001	0.45
Day	<.0001	<.0001	<.0001	<.0001	0.02
Treatment*day	0.03	0.31	0.47	0.03	0.99
Environment *day	<.0001	0.41	<.0001	0.02	0.88
Treatment* environment *day	<.0001	0.04	0.35	0.36	0.54

This condition can influence endocrine disorders by decreasing metabolic rate, increasing feed conversion ratio, intestinal microbial imbalance and reducing feed consumption (Sohail et al., 2012). However, prebiotics such as MOS increased the BWG of birds (754±6 gram) when the birds were subjected to chronic heat stress condition in comparison with control heat stress birds (698±6 gram). The poor performance of birds when they were subjected to environmental stress may be attributed to a smaller appetite result in lower feed intake. This may be a defense mechanism to help reduce heat production (Sohail et al., 2012). Prebiotics can increase beneficial bacteria and promote intestinal function and disease resistance (Rehman et al., 2007; Awad et al., 2008). They may increase nutrient absorption from the intestine and balance the negative effect of environmental stress (Sohail et al., 2012). Feeding birds with 0.5% brown seaweed showed improved BW and BWG (Wiseman 2012) in unstressed condition.

Table 4.5. Body weight (g bird^{-1}day^{-1}) of broiler chickens during the starter (day 0-14), grower (day 15-24), and finisher (day 25-33) experimental periods with dietary supplement of *Palmaria palmata*, inulin, antibiotic and no feed additive in normal and stressed conditions.

Environment/ Treatment	Starter 0-14d	Grower 15-24d	Finisher 25-33d
Normal environment			
Palmaria palmata (%)			
0.6	387.72±7.6	1265.22±17.2	2219.45±33.4^{bc}
1.8	420.92±7.6	1331.42±17.2	2386.31±33.4^{a}
3	413.81±7.6	1289.65±17.2	2197.15±33.4^{bc}
Inulin 2.5%	425.23±7.6	1329.90±17.2	2278.55±33.4^{b}
Antibiotic (BMD)[1]	408.27±7.6	1304.61±17.2	2240.39±33.4^{bc}
No feed additive	392.71±7.6	1285.54±17.2	2226.91±33.4^{bc}
Stressed environment			
Palmaria palmata (%)			
0.6	396.27±7.6	1252.94±17.2	2175.28±33.4^{bc}
1.8	397.64±7.6	1272.3±17.2	2194.17±33.4^{bc}
3	403.52±7.6	1257.44±17.2	2184.80±33.4^{bc}
Inulin 2.5%	401.44±7.6	1267.36±17.2	2215.12±33.4^{bc}
Antibiotic (BMD)[1]	386.18±7.6	1240.51±17.2	2155.42±33.4^{c}
No feed additive	407.47±7.6	1284.97±17.2	2269.99±33.4^{b}
Day mean	403±7.3^{c}	1281±7.3^{b}	2216±7.3^{a}

$^{a-c}$ means±SEM with different superscripts within a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Dietary 1.8% *Palmaria palmata* addition significantly increased BWG of broilers during the finisher period, but bird BWG were not influenced by treatment during the starter and grower periods (Table 4.6). Three percent *Palmaria palmata*, in comparison with lower levels of *Palmaria palmata* (0.6% and 1.8%), had the lowest BWG. The birds given

supplemented treatments in the US environment had higher BWG, in comparison with bird growth in the S environment.

Table 4.6. Body weight gain (g bird $^{-1}$day^{-1}) of broiler chickens over the starter (day 0-14), grower (day 15-24) and finisher (day 25-33) experimental periods with dietary treatment of *Palmaria palmata*, inulin, antibiotic and no feed additive in normal and stressed environment.

	Starter 0-14d	Grower 15-24d	Finisher 25-33d
Environment/ Treatment			
Normal environment			
Palmaria palmata (%)			
0.6	23.77±0.5	85.31±1.0^{f}	102.96±3.0^{b}
1.8	26.28±0.5	88.90±1.0^{cdef}	114.42±3.0^{a}
3	25.73±0.5	85.29±1.0^{f}	97.91±3.^{bcde}
Inulin 2.5%	26.57±0.5	88.38±1.0^{def}	102.66±3.0^{b}
Antibiotic (BMD)[1]	25.49±0.5	87.82±1.0^{ef}	101.49±3.0^{b}
No feed additive	24.45±0.5	87.65±20^{ef}	102.21±3.0^{b}
Stressed environment			
Palmaria palmata (%)			
0.6	25.58±0.5	85.51±1.0^{f}	100.47±3.0^{b}
1.8	25.74±0.5	87.30±1.0^{ef}	100.28±3.0^{bc}
3	25.97±0.5	85.46±1.0^{f}	101.09±3.0^{b}
Inulin 2.5%	26.07±0.5	86.21±1.0^{f}	103.38±3.0^{ab}
Antibiotic (BMD)[1]	24.88±0.5	85.26±1.0^{f}	99.52±3.0^{bcd}
No feed additive	26.39±0.5	87.58±1.0^{ef}	107.19±3.0^{ab}
Day mean	25.82±0.7^{c}	86.72±0.7^{b}	102.80±0.7^{a}

$^{a-g}$ means±SEM with different superscripts within a column or a row are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

On the contrary, Rebole et al. (2010) observed that feeding birds with 10 and 20 g of inulin/kg improved final BWG. Xu et al. (2003) reported that dietary supplementation with 4g/kg fructooligosaccharide improved BWG. Studies using dietary inulin did not show consistent results. Rehman et al. (2008) observed that feeding broilers with 10 g/kg of diet did not improve growth performance. BMD inclusion did not improve broilers growth performance in this trial two and one. It has been suggested that there are more

beneficial effects resulting from feeding prebiotics when they are examined under suboptimal experimental conditions (Orban et al., 1997, Allen et al., 2001). Baurhoo et al. (2009) observed that feeding birds with mannanoligosaccharides or antibiotics had no effects on the growth variables, under sanitary conditions. Furthermore, Coates et al. (1963) found that a germ-free environment for housing birds, compared with a conventional environment, produced optimal conditions for growth. However, Wiseman (2012) observed that feeding Tasco® at 0.5% improved BW in sanitary conditons. This finding is inconsistent with current study, since birds rose in the conventional envirоonments had greater BW and BWG due to implying high bacterial challenge (Baurhoo et al., 2009). Environmental conditions such as poultry litter can affect significantly their intestinal microbiota. Cressman et al. (2010) observed that bird's grawn in the freshed-litter in comparison with those raised in reused-litter their intestinal microbiota had more bacteria of the fresh-litter origin, while the gut of reused-litter birds was mostly colonized with bacteria of intestinal origin, possibly excreted from the previous flocks.

Dietary treatment had no effect on FI (Table 4.7). The stress environment did not affect feed intake (Table 4.7). Sohail et al. (2012) observed that heat stress decreased appetite and resulted in lower feed intake. A possible explanation is that the heat stress in this study was not enough to decrease the feed consumption. The normal body temperature of birds is in a range of 40-42°C (Lan et al., 2004). There was inconsistency and lack of broiler response in FI and feed conversion ratio was generally observed (Rebole et al., 2010; Janardhana et al., 2009; Rehman et al., 2008).

Table 4.7. Feed intake (g bird^{-1}day^{-1}) of broiler chickens over the starter (day 0-14), grower (day 14-24) and finisher (day 25-33) experimental periods with dietary supplement of *Palmaria palmata*, inulin, antibiotic and no feed additive in normal and stressed conditions.

Environment/treatment	Starter 0-14d	Grower 15-24d	Finisher 25-33d
Normal environment			
Palmaria palmata (%)			
0.6	33±2	115±2	162±2
1.8	35±2	124±2	167±2
3	34±2	123±2	158±2
Inulin 2.5%	32±2	123±2	162±2
Antibiotic (BMD)[1]	33±2	123±2	163±2
No feed additive	36±2	119±2	160±2
Stressed environment			
Palmaria palmata (%)			
0.6	35±2	116±2	162±2
1.8	36±2	117±2	157±2
3	35±2	116±2	161±2
Inulin 2.5%	36±2	116±2	163±2
Antibiotic (BMD)[1]	35±2	114±2	159±2
No feed additive	36±2	116±2	159±2
Day mean	35±0.8^{c}	119±0.8^{b}	161±0.8^{a}
Environment*day			
Normal	34±1.2^{c}	121±1.2^{b}	162±1.2^{a}
Stressed	35±1.2^{c}	116±1.2^{b}	160±1.2^{a}

$^{a-c}$ means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Interactions for FCR were: treatment by environment (Table 4.8), environment by day (Table 4.9) and treatment by day (Table 4.10). Birds fed no feed additive in the clean room had poorer FCR, in comparison with birds fed at 1.8% red seaweed in S rooms.

Table 4.8. Feed conversion ratio of broiler chickens averaged over the starter (day 0-14), grower (day 15-24), and finisher (day 25-33) experimental periods with dietary supplementation of *Palmaria palmata,* inulin, antibiotic and no feed additive under normal and stressed environment.

Environment/treatment	Feed conversion ratio
Normal environment	1.43±0.03[ab]
Palmaria palmata (%)	1.41±0.03[ab]
0.6	
1.8	1.47±0.03[ab]
3	1.41±0.03[ab]
Inulin 2.5%	1.46±0.03[ab]
Antibiotic (BMD) [1]	1.52±0.03[a]
No feed additive	
Stressed environment	
Palmaria palmata (%)	
0.6	1.45±0.03[ab]
1.8	1.39±0.03[b]
3	1.45±0.03[ab]
Inulin 2.5%	1.44±0.03[ab]
Antibiotic (BMD) [1]	1.45±0.03[ab]
No feed additive	1.43±0.03[ab]

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.9 Feed conversion ratio of broiler chickens during the starter (day 0-14), grower (day 15-24), and finisher (day 25-33) experimental periods with placement in normal or stressed rooms.

Day/ Environment	day	Feed conversion ratio
Normal environment	33	1.57±0.02[a]
	24	1.41±0.02[b]
	14	1.37±0.02[b]
Stressed environment	33	1.57±0.02[a]
	24	1.40±0.02[b]
	14	1.35±0.02[b]

[a-b]means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)

Table 4.10. Feed conversion ratio of broiler chickens during the starter (day 0-14), grower (day 15-24), and finisher (day 25-33) experimental periods with placement in normal or stressed environments.

Day	0-14	15-24	25-33
Treatment			
Palmaria palmata (%)			
0.6	1.41 ±0.03^{cde}	1.33±0.03^{e}	1.59±0.03^{a}
1.8	1.38 ±0.03^{de}	1.37±0.03^{e}	1.51±0.03^{abcd}
3	1.36 ±0.03^{e}	1.41±0.03^{cde}	1.61±0.03^{a}
Inulin 2.5%	1.33 ±0.03^{e}	1.37±0.03^{e}	1.58±0.03^{ab}
Antibiotic (BMD) [1]	1.39 ±0.03^{bcde}	1.37±0.03e	1.61±0.03^{a}
No feed additive	1.43 ±0.03^{cde}	1.41±0.03^{cde}	1.52±0.03^{abc}

$^{a-e}$ means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Kulshreshtha et al. (2014) observed that feeding birds feeding (2% CC and SG) significantly improved FCR in layer hens. Awad et al. (2009) observed that feeding birds *Lactobacillus spp.* in comparison with control groups improved BW, average daily gain and feed conversion ratio in broilers In addition, Catalá-Gregori et al. (2008) reported that ProFeed (a candidate prebiotic made of sugar beet short chain FOS) increased BWG and improved feed conversion ratio in broilers. Conditions, such as heat stress and reused litter, can affect the intestinal microbiota and therefore, decrease growth and increase the occurrence of various infection diseases (Lan et al., 2004 and Cressman et al., 2010). Results from this trial indicate that 1.8% *Palmaria palmata* improved FCR by changing the bacterial composition in the chicken ileum, which was subsequently associated with improving of BWG and FCR. Taken together, the FCR data shows that *Palmaria palmata* improved the microbial balance in the ileum and therefore, promoted the growth and improve FCR.

4.6.3 Bird health

No treatment differences occurred in mortalities throughout the experiment (Table 4.11). Mortality rate was significantly higher during starter periods. The most common causes of mortality were septicemia, leg deformities, peritonitis, and ascites, as well as omphalitis in the early growth period, and were not treatment related. The total mortality in the growth study was 2.5% (US environment 1.6% and S environment 0.85%). The mortality incidence during the starter, grower and finisher periods in the US environment were 9, 7 and 10 birds, for S environment were 9, 7, 0). Since the data did not satisfy the normality assumption, the Chi-squared test was used to analyze the mortality.

Table 4.11. Effect of *Palmaria palmata,* inulin, antibiotic and no feed additive on mortality (%) during starter, grower and finisher periods.

Environment/treatment	Starter	Grower	Finisher	Total treatment
Normal environment				
Palmaria palmata (%)				
0.6	3	3	0	6
1.8	1	2	1	4
3.0	3	1	2	6
Inulin 2.5%	4	2	2	8
No feed additive	2	0	0	2
Antibiotic (BMD) [1]	1	2	1	4
Total	14	10	6	30
Stressed environment				
Palmaria palmata (%)				
0.6	1	0	0	1
1.8	4	1	0	5
3.0	1	2	0	3
Inulin 2.5%	1	1	0	2
No feed additive	1	2	0	3
Antibiotic (BMD) [1]	1	1	0	2
Total	9	7	0	16
	P-value			
Mortality	0.12			

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Results indicated that there was no significant difference between treatment in normal and stressed environments. Previous studies have shown that the environmental stresses can affect of mortality. Sohail et al. (2012) observed that mortality increased when the birds were subjected to chronic heat stress (10ºC over normal ambient temperature), which decreased bird immunity. In addition, heat stress decreased feed consumption, which limited body weight gain and increased mortality in broilers (Suzuki et al., 1983). Used litter may have endangered bird health and decreased bird growth performance by concentrating the ammonia (NH_3) generated by microbial breakdown of excreta materials in the used litter material (Shepherd and Fairchild 2010). Performance and immunity of birds can be compromised by NH_3 levels higher than 10 ppm (Nagaraj et al., 2007). However, in the current trial, at day 33, the ammonia level increased by 15 ppm but it did not affect the health and mortality.

4.6.4 Intestinal pH

The pH of cecal contents (Table 4.12 and Table 4.13) were affected by *Palmaria palmata*, indicating a change in fermentation. Birds housed on new litter in US environment had cecal contents with significantly greater pH than those housed on previously used litter in S environment, on days 7, 14, 24 and 33 (Table 4.14). Red seaweed at 1.8% decreased cecal content pH on days 24 and 33.

Table 4.12. pH of the cecal contents of broiler chickens on days 7, 14, 24 and 33 posthatch with dietary treatment of *Palmaria palmata* inulin, antibiotic or no feed additive in normal and stressed condition.

Age (Day)	7	14	24	33
Normal environment				
Palmaria palmata (%)				
0.6	6.09±0.1	6.30±0.1	6.03±0.1	6.38±0.1
1.8	6.03±0.1	6.41±0.1	5.94±0.1	6.24±0.1
3	6.11±0.1	6.22±0.1	5.96±0.1	6.39±0.1
Inulin 2.5%	6.13±0.1	6.39±0.1	6.12±0.1	6.40±0.1
Antibiotic (BMD) [1]	6.21±0.1	6.34±0.1	6.50±0.1	5.57±0.1
No feed additive	6.10±0.1	6.16±0.1	6.10±0.1	6.40±0.1
Stressed environment				
Palmaria palmata (%)				
0.6	5.81±0.1	5.93±0.1	5.83±0.1	6.10±0.1
1.8	5.51±0.1	5.89±0.1	5.56±0.1	5.60±0.1
3	5.75±0.1	5.89±0.1	5.77±0.1	5.87±0.1
Inulin 2.5%	6.02±0.1	5.74±0.1	5.69±0.1	6.06±0.1
Antibiotic (BMD)[1]	5.99±0.1	5.86±0.1	6.11±0.1	6.21±0.1
No feed additive	5.75±0.1	6.06±0.1	5.90±0.1	6.26±0.1
ANOVA	P-value			
Treatment	<0.001			
Environment	0.41			
Day	<0.001			
Day*environment	0.82			
Day*treatment	0.49			
Treatment* environment	0.74			
Treatment*environment*day	0.96			

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.13. Effects of *Palmaria palmata*, inulin, antibiotic or no feed additive on the pH of cecal contents on days 24 and 33.

Treatment	day 24	day 33
Palmaria palmata (%)		
0.6	5.93±0.1[ab]	6.24±0.1[ab]
1.8	5.75±0.1[b]	5.92±0.1[b]
3.0	5.87±0.1[ab]	6.13±0.1[ab]
2.5% Inulin	5.91±0.1[ab]	6.23±0.1[ab]
Antibiotic (BMD) [1]	6.00±0.1[a]	6.39±0.1[a]
No feed additive	6.31±0.1[ab]	6.33±0.1[ab]
ANOVA (P-value)		
Treatment	0.01	0.03

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.14. Effects of normal and stressed environment on the pH of cecal contents on days 7, 14, 24 and 33

Environment	day 7	day 14	day 24	day 33
Normal	6.12±0.08[bc]	6.30±0.08[ab]	6.11±0.08[abc]	6.40±0.08[a]
Stressed	5.89±0.08[cd]	5.98±0.08[bcd]	5.76±0.08[d]	6.10±0.08[abcd]

[a-d] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Feeding red seaweed resulted in lower cecal content pH, in comparison with other treatments. High fermentation activity in the chicken gut has been found to be related with a lower pH in the ceca and this can inhibit the growth of acid-sensitive bacteria such as *E. coli* (Russell and Diez-Gonzalez 1997). Gut pH was affected by the presence of fermentation products, such as SCFA, and the composition of non-digested material, such as seaweed, which could be fermented by intestinal microbiota to produce beneficial by-products, such as SCFA. The major reduction in pH and elevated VFA concentrations are thought to occur in response to prebiotic supplementation and is thought to be related to growth of beneficial species of *Lactobacillus* and *Bifidobacteria* (Stoidis et al., 2010). These bacteria are considered to be inhibitory towards pathogens (Dunkley et al., 2009).

By increasing beneficial bacterial populations, prebiotics alter microbial fermentation and nutrient metabolism (Gibson and Roberfroid 1995). Birds in the US environment had significantly higher pH of cecal contents than those in S environmental on days 7, 14, 24 and 33. The deposition of excreta onto the used litter could change the biotic and abiotic environments, and therefore the bacteria population during the study. The lower pH of ileal contents would be consistent with a high degree of fermentation occurring within the beneficial microbe populations.

4.6.5 Ileal microbial analysis

On days 14, 24 and 33, the bacterial populations examined: cultureable aerobic, anaerobic, coliforms, *E. coli*, *Lactobacillus* bacteria, *Bifidobacteria* and *Clostridium perfringens*. Feeding *Palmaria palmata* at different inclusion levels had no significant effect on total culturable aerobes (Table 4.15). Birds raised in a normal environment had a greater total number of culturable aerobes, in comparison with those in a stressed environment (Table 4.16). Environmental stressed conditions affect the intestinal microbial ecology, causing an imbalance in chicken gut flora (Sohail et al., 2011). These effects reduce the total number of aerobic bacteria in the ileum from chickens in the stressed environment in comparison with normal environment.

Table 4.15. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total aerobic (log_{10} cfu g^{-1}) in the ileum of broiler chickens days 14, 24 and 33 in normal and stressed conditions.

Age (days)	14	24	33
Normal environment			
Palmaria palmata			
0.6%	6.59±0.01	6.69±0.01	6.80±0.01
1.8%	6.60±0.01	6.69±0.01	6.78±0.01
3%	6.61±0.01	6.70±0.01	6.79±0.01
Inulin 2.5%	6.62±0.01	6.70±0.01	6.78±0.01
Antibiotic (BMD) [1]	6.59±0.01	6.69±0.01	6.76±0.01
No feed additive	6.57±0.01	6.67±0.01	6.75±0.01
Stressed environment			
Palmaria palmata			
0.6%	6.57±0.01	6.63±0.01	6.75±0.01
1.8%	6.43±0.01	6.57±0.01	6.71±0.01
3%	6.51±0.01	6.63±0.01	6.73±0.01
Inulin 2.5%	6.53±0.01	6.64±0.01	6.74±0.01
Antibiotic (BMD) [1]	6.47±0.01	6.60±0.01	6.72±0.01
No feed additive	6.53±0.01	6.64±0.01	6.76±0.01
ANOVA (P-value)			
Treatment	0.18		
Environment	0.04		
Day	<.0001		
Day*environment	<.0001		
Day*treatment	0.68		
Treatment* environment	0.53		
Treatment*environment*day	0.91		

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.16. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total aerobic (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14, 24 and 33.

	Age (day)		
	14	24	33
Environment			
Normal	6.60±0.01[c]	6.68±0.01[b]	6.79±0.01[a]
Stressed	6.52±0.01[d]	6.64±0.01[bc]	6.76±0.007[a]

[a-d] means±SEM with different superscripts for the interaction are significantly different ($p \leq 0.05$).

The total aerobic bacteria measured in this experiment included obligate aerobes and facultative anaerobes. There was increasing trend for aerobic bacteria during days 14, 24 and 35. Birds raised in the stressed environment had lower aerobic bacteria compared to normal rooms (Table 4.15). Silmilarly, Budgell (2008) observed that feeding *Saccharomyces cerevisiae* cell wall extract, as an alternative to antibiotics in broilers did not affect the levels of aerobic microbes in the ileum. The levels of intestinal aerobes increased from 5.67 log_{10} cfu g^{-1} to 6.00 log_{10} cfu g^{-1}, however, Jin et al., (1998) observed no age effect for the total aerobe levels in the small intestine under normal conditions. Gut microbiota play an important role in bird health by increasing nutrient digestion and absorption by affecting intestinal morphological structure (Yang et al., 2009). The function of the small intestine is nutrient absorption and digestion (Józefiak et al., 2004). Disturbances of the intestinal microbiota may delay growth, weaken resistance, and result in infectious diseases (Sohail et al., 2012). Increasing beneficial bacteria, including *Lactobacillus* species, improved the humoral immunity of broilers subjected to chronic heat stress (Sohail et al., 2013). Previous studies have shown that there are reciprocal effects between the microbiota present in the litter and those in the intestines of broilers (Cressman et al., 2010). Therefore, the environment in which birds are raised should be examined as an important factor for their health and growth.

The counts of total anaerobic bacteria were not affected by any treatments (Table 4.17), but were affected by stressed and normal environments. The number of anaerobic bacteria decreased from day 14 to 24 in the stressed and normal environments, while between days 24 and 33, their numbers were decreased in both environments (Table 4.18).

In the ileum, the majority of total anaerobes are *Lactobacillus*. Clostridia are also present (Barnes, 1972). The first bacteria to colonize the gut of broilers were reported to have originated from aerobic and facultative anaerobes, such as *Escherichia, Klebsiella, Enterobacter* (Yoshioka et al., 1983), *Lactobacillus*, and *Streptococcus* (Mackie et al., 1999; Dibner and Richards 2005). The majority of the adult microbiota have been found to be obligate anaerobes (Dibner and Richards 2005). Levels of intestinal aerobes increased from 5.67 $\log_{10}$ cfu g^{-1} to 6.00 $\log_{10}$ cfu g^{-1} as the birds age ($p<0.1$).

Table 4.17. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total anaerobic (log_{10} cfu g^{-1}) in the ileum of broiler chickens days 14, 24 and 33 in normal and stressed environments.

Age (days)	14	24	33
Normal environment			
Palmaria palmata (%)			
0.6	6.55±0.01	6.72±0.01	6.81±0.01
1.8	6.56±0.01	6.71 ±0.01	6.78±0.01
3	6.56±0.01	6.68±0.01	6.80±0.01
Inulin 2.5%	6.57±0.01	6.69±0.01	6.79±0.01
Antibiotic (BMD) [1]	6.56±0.01	6.68±0.01	6.76±0.01
No feed additive	6.57±0.01	6.70±0.01	6.77±0.01
Stressed environment			
Palmaria palmata (%)			
0.6	6.60±0.01	6.66±0.01	6.76±0.01
1.8	6.59±0.01	6.69±0.01	6.71±0.01
3	6.60±0.01	6.67±0.01	6.75±0.01
Inulin 2.5%	6.59±0.01	6.68±0.01	6.74±0.01
Antibiotic (BMD) [1]	6.57±0.01	6.65±0.01	6.72±0.01
No feed additive	6.59±0.01	6.64±0.01	6.77±0.01
ANOVA (P-value)			
Treatment	0.18		
Environment	0.04		
Day	<.0001		
Day*environment	<.0001		
Day*treatment	0.68		
Treatment* environment	0.53		
Treatment*environment*day	0.91		

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.18. Effects of stressed or normal environment on total anaerobic bacteria in the ileum of broiler chickens days 14, 24 and 33 in normal or stressed environments.

	Age (day)		
	14	24	33
Environment			
Normal	6.57±0.007[d]	6. 79±0.007[a]	6.68 ±0.007[c]
Stressed	6.60±0.007[d]	6.74±0.007[b]	6.70±0.007[c]

[a-d] means±SEM with different superscripts for the interaction are significantly different ($p \leq 0.05$)

Palmaria palmata supplementation at 1.8% increased the number of *Lactobacillus* in the chicken gut microbiota compared to other treatments on days 14, 24 and 33 (Table 4.19). There was a positive linear relationship between treatment lactic acid bacteria during all periods.

There was an increasing trend in *Lactobacillus* number during days 14, 24 and 33 (Table 4.20). Birds raised in the normal rooms had higher Lactobacillus, in comparison with stressed rooms (Table 4.20). These results indicate that feeding *Palmaria palmata* caused changes in gut microbiota as a result of complex polysaccharides in the Palmaria palmata. These results are in agreement with Cressman et al. (2010), who observed that the ileal mucosal of birds raised in fresh litter, in comparison with reused litter, had higher *Lactobacillus* spp., while in the reused litter, *Clostridiales* was dominant. These changes improve host health by increasing beneficial bacteria such as *Lactobacillus* and decreasing pH, which act to suppress pathogen growth. *Lactobacillus* in the GIT of young chicks is the dominant species bacteria and the predominant bacterial species in the small intestine of mature chicks, (Amit –Romach et al., 2004).

Table 4.19. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total *Lactobacillus* (log10 cfu g-1) in the ileum of broiler chickens days 14, 24 and 33 in normal and stressed conditions.

Treatments	*Lactobacillus*
Palmaria palmata (%)	
0.6	5.66±0.04[ab]
1.8	5.67±0.04[a]
3	5.65±0.04[abc]
Inulin 2.5%	5.65±0.04[abc]
Antibiotic (BMD)[1]	6.65±0.04[abc]
No feed additive	5.64±0.04[c]
ANOVA (P-value)	
Treatment	<.0001
Environment	0.15
Day	<.0001
Day*environment	<.0001
Day*treatment	<.0001
Treatment*environment	0.07
Treatment*environment*day	0.63

[a-d] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$).
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.20. Total *Lactobacillus* (log10 cfu g-1) in the ileum of broiler chickens on days 14, 24 and 33 in normal and stressed environments.

Age (Day)	14	24	33
Palmaria palmata (%)			
0.6	6.59±0.007^{ab}	6.69±0.006^{a}	6.82^{ab}
1.8	6.60±0.007^{a}	6.70±0.006^{a}	6.83^{a}
3	6.58±0.007^{abc}	6.67±0.006^{ab}	6.81^{ab}
Inulin 2.5%	6.58±0.007^{abc}	6.69±0.006^{a}	6.82^{ab}
Antibiotic (BMD)[1]	6.57±0.007^{bc}	6.67±0.006^{ab}	6.80^{bc}
No feed additive	6.54±0.007^{c}	6.65±0.006^{b}	6.79^{c}
ANOVA (P-value)			
Treatment	<.0001	<.0001	<.0001
Environment	<.0001	<.0001	<.0001
Treatment*environment	0.14	0.09	0.12
		Age (day)	
Environment	6.59±0.005^{b}	6.55±0.005^{c}	6.82±0.005^{a}
Normal	6.59±0.005^{b}	6.55±0.005^{c}	6.82±0.005^{a}
Stressed	6.57±0.005^{b}	6.59±0.005^{d}	6.80±0.005^{a}

$^{a-d}$ means±SEM with different superscripts for the interaction are significantly different ($p \leq 0.05$).
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Feeding broilers with 0.6%, 1.8%, 3% *Palmaria palmata* and 2.5% inulin resulted in a significant enhancement in the number of *Bifidobacteria* on days 14, 24 and 33 (Table 4.21). There was an increasing trend in the proportion of *Bifidobacteria* during day 14, 24 and 33 (Table 4.22). The birds grown in the normal environment, compared to stressed environment, had greater *Bifidobacteria* (Table 4.22). There is interactive effects among environmental stressed and supplement response. Beneficial bacteria, including

Table 4.21. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total *Bifidobacterium* (log10 cfu g^{-1}) in the ileum of broiler chickens Days 14, 24 and 33 in normal and stressed conditions.

Age (days)	
Normal	*Bifidobacterium*
Palmaria palmata (%)	
0.6	6.91±0.006[a]
1.8	6.92±0.006[a]
3	6.91±0.006[a]
Inulin 2.5%	6.92±0.006[a]
Antibiotic (BMD) [1]	6.89±0.006[ab]
No feed additive	6.87±0.006[bc]
Stressed environment	
Palmaria palmata (%)	
0.6	6.86±0.006[cd]
1.8	6.85±0.006[cd]
3	6.86±0.006[cd]
Inulin 2.5%	6.85±0.006[cd]
Antibiotic (BMD) [1]	6.85±0.006[d]
No feed additive	6.85±0.006[cd]
ANOVA (P-value)	
Treatment	<.0001
Environment	<.0001
Day	<.0001
Day*environment	<.0001
Day*treatment	0.99
Treatment* environment	<.0001
Treatment*environment*day	0.99

[a-d] means±SEM with different superscripts for treatment by interaction significantly different ($p \leq 0.05$).
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.22. Effects of normal or stressed environments on *Bifdobacterium* (log10 cfu g^{-1}) in the ileum of broiler chickens days 14, 24 and 33.

	Age		
Environment	day 14	day 24	day 33
Normal	6.90±0.002[e]	6.99±0.002c[c]	7.04±0.002[a]
Stressed	6.83±0.002[f]	6.97±0.002[d]	7.02±0.002[b]

[a-f] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$).

Bifidobacteria, positively influenced the health of birds by increasing the proliferation of intestinal epithelial cells along the length of the villus and improving intestinal function (Yang et al., 2009). *Bifidobacteria* can protect intestinal epithelial cells against enteropathogenic infection by producing acetate (Fukuda et al., 2011).

Diets with no feed additives had a greater number of *Clostridium perfringens,* in comparison with other treatments (Table 4.23). Dietary treatments decreased adverse bacteria including *Clostridium perfringens*. There was an increasing trend in the number of *Clostridium perfringens* on days 14, 24 and 35 (Table 4.24). Feeding 0.6% *Palmaria palmata* decreased *Clostridium perfringens* numbers, compared to all other dietary treatments on day 24. There was an increasing trend in the number of *Clostridium perfringens* on days 14, 24 and 35 (Table 4.24). Birds raised in the stressed environment had more *Clostridium perfringens,* compared to normal environment during days 14, 24 and 33 (Table 4.23 and 4.24). Kulshreshtha et al. (2014) observed that feeding birds with red seaweed (*S.g.* and *C.C.*) reduced the prevalence of *Clostridium perfringens*. A potential mechanism for decreasing *Clostridium perfringens,* is the prebiotic role of red seaweed. Red seaweed can alter the gut microbiota by increasing beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, decreasing the growth of pathogens by mechanism such as competitive exclusion, producing SCFA (Kulshreshtha et al., 2014). SCFA products could decrease the gut pH to levels below at which pathogens activity (Gibson et al., 2005).

Table 4.23. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens days 14, 24 and 33.

Treatments	*Clostridium perfringens*
Palmaria palmata (%)	
0.6	1.46±0.04[b]
1.8	1.54±0.04[ab]
3	1.56±0.04[ab]
Inulin 2.5%	1.55±0.04[ab]
Antibiotic (BMD)[1]	1.51±0.04[ab]
No feed additive	1.10±0.04[a]
ANOVA (P-value)	
Treatment	0.03
Environment	<.0001
Day	<.0001
Day*environment	0.69
Day*treatment	0.97
Treatment* environment	0.25
Treatment*environment*day	0.99

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.24. Effects of stressed and normal environment on *Clostridium perfringens* in the ileum of broiler chickens on days 14, 24 and 33.

	Age (days)		
	14	24	33
Environment			
Normal	1.12±0.05[d]	1.47±0.05[c]	1.67±0.05[b]
Stressed	1.32±0.05[cd]	1.72±0.05[b]	1.94±0.05[a]

[a-d] means±SEM with different superscripts in the environment by age interaction are significantly different ($p \leq 0.05$)

The birds raised in the S environment had more coliforms compared to US environment (Table 4.25). Dietary treatment affect the number of coliforms in the chicken ilea. Antibiotic, inulin 2.5% and *Palmaria palmata* supplementation at 1.8% decreased the number of coliforms during days 14, 24 and 35. There was an increasing trend in the number of coliforms during days 14, 24 and 35 (Table 4.26). Baurhoo et al. (2007) observed that when birds challenged with *E. coli* were fed 2.5% of the potential prebiotic Alcell lignin, *E. coli* levels decreased.

Table 4.25. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total coliform (log_{10} cfu g^{-1}) in the ileum of broiler chickens days 14, 24 and 33.

Age	14
Treatments/rooms	
Palmaria palmata (%)	
0.6	3.65±0.004[ab]
1.8	3.64±0.004[b]
3.0	3.65±0.004[ab]
Inulin 2.5%	3.64±0.004[b]
Antibiotic (BMD) [1]	3.64±0.004[b]
No feed additive	3.66±0.004[a]
ANOVA (P-value)	
Treatment	<.0001
Environment	<.0001
Day	<.0001
Day*environment	<.0001
Day*treatment	0.99
Treatment* environment	0.46
Treatment*environment*day	0.99

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.26. Effects of normal and stressed environment on total coliform (log_{10} cfu g^{-1}) in the ileum of broiler chickens days 14, 24 and 33.

	Age		
	14	24	33
Environment			
Normal	3.50±0.005[d]	3.62±0.005[c]	3.74±0.005[b]
Stressed	3.51±0.005[d]	3.72±0.005[b]	3.81±0.005[a]

[a-d] means±SEM with different superscripts for the interaction are significantly different ($p \leq 0.05$)

On Days 14, 24 and 33, number of *E. coli* in the ilea were affected by antibiotic, inulin 2.5% and *Palmaria palmata* at 1.8% inclusion level during days 14, 24 and 33 (Table 4.27). There was an increasing trend in the number of *E. coli* during days 14, 24 and 33 (Table 4.28). The birds raised in the stressed environment had greater *E. coli*, compared to normal environment.

Table 4.27. Effect of inclusion level of *Palmaria palmata*, inulin, antibiotic and no feed additive on total *E. coli* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14, 24 and 33.

Age (days)	14
Palmaria palmata (%)	
0.6	3.58 ± 0.05^{ab}
1.8	3.57 ± 0.05^{b}
3	3.58 ± 0.05^{ab}
Inulin 2.5%	3.57 ± 0.05^{b}
Antibiotic (BMD)[1]	3.57 ± 0.05^{b}
No feed additive	3.59 ± 0.05^{a}
ANOVA (P-value)	
Treatment	<. 0001
Environment	<. 0001
Day	<. 0001
Day*environment	0.08
Day*treatment	0.99
Treatment* environment	0.50
Treatment*environment*day	0.99

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.28. Effects of normal or stressed environment on total *E. coli* (log_{10} cfu g^{-1}) in the ileum of broiler chickens days 14, 24 and 33.

	Age		
	14	24	33
Environment			
Normal	3.44 ± 0.006^{c}	3.58 ± 0.006^{b}	3.70 ± 0.006^{a}
Stressed	3.46 ± 0.006^{c}	3.59 ± 0.006^{b}	3.70 ± 0.006^{a}

[a-c] means±SEM with different superscripts for the interaction are significantly different ($p\leq0.05$

Li et al. (2009) observed that feeding birds with the candidate prebiotic Astragalus polysaccharide reduced *E. coli* in the ileum and cecum. In the current study, none of treatments influenced *E.coli* number. Perhaps, there was not enough patoghenic *E.coli* to make a disease. Except for extraintestinal pathogenic *E. coli*, which are disease-causing, most strains are harmless (Ron et al., 2006). However, the number of *E. coli* was higher

in the stressed rooms, compared to the normal rooms. This difference can be associated with using dirty litter, elevated humidity and higher temperature in stressed room.

The number of total *Lactobacillus* and *Bifidobacteria* in the ileum, which was influenced by S environment and treatment, were greater in the birds fed with 1.8% *Palmaria palmata*. Feeding 0.6% *Palmaria palmata*, reduced the number of *Clostridium perfringens* in the chicken ileum. In another study, red seaweeds *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, in layers, increased the relative abundance of *Bifidobacterium longum* and *Streptococcus salivarius* (Kulshrehtha et al., 2014). These result indicate that feeding red seaweed can increase the population of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* in order to decrease binding sites for pathogens and increase beneficial fermentation products like SCFA. The production of SCFA reduce the pH in the gastrointestinal tract, which is consider that have an important role in pathogen inhibition (Dunkley et al., 2009). Xu et al., (2003) observed that feeding birds with 4.0 g/kg of FOS increased the growth of *Bifidobacterium* (8.11±0.36) and *Lactobacillus* (8.47±0.31), in comparison with a no feed additive diet (7.22±0.36 and 7.46±0.31). Establishment of beneficial bacteria including, *Lactobacilli* and *Bifidobacteria* in the intestine has been related to competitive exclusion of pathogen (Baurhoo et al., 2009). Seaweeds have low molecular weight polysaccharides and oligosaccharides that can have prebiotic activity as a source of soluble fiber when fermented in the gut (Ramnani et al., 2012). Hence, this prebiotic activity can improve health of the host by limiting the growth of undesirable bacteria via different methods such as competitive exclusion and on secretion of SCFA (Torok et al., 2009). Current results support the finding of Kulshrehtha et al. (2014) who concluded that the use of red seaweed as a prebiotic improved the population of beneficial bacteria and a reduced pathogenic bacteria in the gut.

4.6.6 Histomorphological analysis

To determine if treatments have effects on Day 7, 14, 24 and 33 histomorphological parameters, ileal sections were analyzed. On Day 7, dietary treatments had an effect on villus height, width and mucosal depth (Table 4.29 and Table 4.30).

Table 4.29. Ileal intestinal histomorphology measurements of broiler chickens day 7 posthatch with dietary supplementation of *Palmaria palmata*, inulin, antibiotic and no feed additive in normal and stressed environments.

	Villus height (μm)	Villus width (μm)	Crypt depth (μm)	Mucosal depth (μm)	Villus surface area (mm^2)
Environment/treatment					
Normal environment					
Palmaria palmata (%)					
0.6	608.5±12.7	146.1±3.2^{abc}	120±3.9	145±4.1^{a}	0.089±0.001
1.8	537.8±12.7	147.0±3.2^{abc}	117±3.9	128±4.1^{ab}	0.088±0.001
3	537.6±12.7	150.1±3.2^{abc}	113±3.9	136±4.1^{ab}	0.085±0.001
Inulin 2.5%	545.0±12.7	139.3±3.2^{bc}	114±3.9	123±4.1^{b}	0.087±0.001
Antibiotic (BMD) [1]	541.1±12.7	140.6±3.2^{bc}	120±3.9	135±4.1^{ab}	0.087±0.001
No feed additive	522.3±12.7	154.5±3.2^{ab}	124±3.9	143±4.1^{a}	0.089±0.001
Stressed environment					
Palmaria palmata (%)					
0.6	559.0±12.7	154.1±3.2^{ab}	114±3.9	143±4.1^{a}	0.091±0.001
1.8	533.5±12.7	158.0±3.2^{a}	107±3.9	129±4.1^{ab}	0.086±0.001
3	529.0±12.7	149.1±3.2^{abc}	109±3.9	143±4.1^{a}	0.090±0.001
Inulin 2.5%	545.1±12.7	145.1±3.2^{abc}	124±3.9	138±4.1^{ab}	0.086±0.001
Antibiotic (BMD) [1]	524.3±12.7	143.3±3.2^{abc}	109±3.9	138±4.1^{ab}	0.087±0.001
No feed additive	505.1±12.7	136.5±3.2^{c}	115±3.9	146±4.1^{a}	0.085±0.001
ANOVA (P-value)					
Treatment	<. 0001	<. 0001	0.17	<. 0001	0.46
Environment	0.03	0.45	0.05	0.05	0.82
Treatment* Environment	0.45	<. 0001	0.09	0.37	0.28

[a-c] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$).
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Table 4.30. Ileal intestinal histomorphology measurements of broiler chickens day 7 posthatch with dietary treatment of *Palmaria palmata*, inulin, antibiotic and no feed additive.

Days Posthatch	day 7
	Villus height (μm)
Treatment	
Palmaria palmata (%)	
0.6	583.75±9.04[a]
1.8	535.67±9.04[b]
3	533.33±9.04[b]
Inulin 2.5%	545.08±9.04[b]
Antibiotic (BMD) [1]	532.75±9.04[b]
No feed additive	513.75±9.04[b]

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$).
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

No significant changes were seen among dietary treatments on villus surface area and crypt (Table 4.29). On Day 7, the interaction of feeding 1.8% *Pamlaria palmata* and stressed environment increased villus width, compared to inulin, no feed additive and antibiotic (Table 4.29). The mucosal depth was lower for birds raised in the stressed environment compared to the normal environment. On Day 7, inulin had the lowest mucosal depth in comparison with other treatments. An increased villus height and width, as occurred in this study, is related to less turnover and more absorptive surface area in the intestinal cells (Rehman et al., 2007). That may be associated with good nutrient absorption; contrarily, a deeper crypt depth has been associated with poor nutrient absorption (Awad et al., 2009).

In trial two, birds grown in the normal environment had greater crypt depth in comparison with those in the stressed environment, which indicates increased cell turnover via greater production of epithelial cells (Rehman et al., 2007).

On day 7, feeding 0.6% *Palamria palmata* resulted in larger villus height over other treatments (Table 4.30). Results of the current study agreed with Wiseman (2012) who reported that villi apparent area, crypt depth, and villi length increased on broilers at day 7 in the unstressed environment. Feeding 2.5% inulin in the US environment resulted in shorter mucosal depth in comparison with other treatments (Table 4.30). This may indicate an improvement in nutrient absorption at a time soon after the chicks switched from being reliant on the yolk sac for nutrients to being completely reliant on the feed, as 85% of the yolk sac is absorbed by day 5 posthatch (Noble and Ogunyemi 1989) A larger villi height can increase nutrient absorption by providing a greater surface area, enhanced mucosal enzymes and nutrient transport system (Amat et al., 1996). Intestinal morphological can be affected by altering microbial composition or through fermentation of prebiotic compounds into SCFA, or perhaps both (Catala- Gregori et al., 2008). In the small intestine the largest bacterial population is in the ileum. Any change in gut microbiota can have an effect on the intestinal histomorphology of this area (Catala-Gregori et al., 2008).

Dietary treatment had no effects on villus height and width, crypt depth, mucosal depth and villus surface area on day 14 (Table 4.31). There were no significant differences among treatments or between environments. As the birds' bacterial populations underwent the transitional period observed on days 12-17 posthatch (Torok et al., 2009), it may be that fewer species of beneficial bacteria were present which could ferment prebiotics, resulting in less effect on intestinal histomorphology.

Table 4.31. Ileal intestinal histomorphology measurements of broiler chickens on day 14 posthatch with *Palmaria palmata*, inulin, antibiotic and no feed additive in normal and stressed environments.

Dietary treatment	Villus height (μm)	Villus width (μm)	Crypt Depth (μm)	Mucosal depth (μm)	Villus surface area (mm^2)
Normal environment					
Palmaria palmata (%)					
0.6	718±33	160±12	141±7	189±16	0.143±0.01
1.8	688±33	147±12	141±7	184±16	0.120±0.01
3	686±33	164±12	142±7	196±16	0.134±0.01
Inulin 2.5%	675±33	174±12	138±7	205±16	0.131±0.01
Antibiotic (BMD) [1]	668±33	155±12	142±7	180±16	0.113±0.01
No feed additive	667±33	190±12	137±7	214±16	0.135±0.01
Stress rooms					
Palmaria palmata (%)					
0.6	764±33	176±12	147±7	192±16	0.141±0.01
1.8	740±33	164±12	156±7	204±16	0.134±0.01
3	678±33	141±12	141±7	180±16	0.105±0.01
Inulin 2.5%	722±33	147±12	150±7	196±16	0.118±0.01
Antibiotic (BMD) [1]	741±33	161±12	147±7	185±16	0.132±0.01
No feed additive	632±33	168±12	134±7	183±16	0.111±0.01
ANOVA (P-value)					
Treatment	0.13	0.30	0.69	0.90	0.49
Treatment	0.13	0.45	0.20	0.64	0.41
Treatment* Environment	0.55	0.21	0.74	0.72	0.27

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Dietary treatment had no significant effect on any of the Day 14 intestinal histomorphological measurements, except villus height (Table 4.31). At Day 24, birds given the diet containing 1.8% red seaweed had longer villus height, compared with other treatments. Furthermore, the stressed environment affected villus height (Table 4.32). Similarly, Baurhoo et al. (2007) observed that supplementation of broiler feed with MOS resulted increased villi height and goblet cell numbers on Day 24. An increase in villi

length due to MOS has been associated with increased *Lactobacilli* and *Bifidobacteria* colonization of broiler intestine in normal envoienment (Baurhoo et al., 2007).

Table 4.32. Ileal intestinal histomorphology measurements of broiler chickens day 24 posthatch with dietary supplementation of *Palmaria palmata.*, inulin, antibiotic and no feed additive in normal and stressed conditions.

Environment/treatment	Villus height (μm)	Villus width (μm)	Crypt depth (μm)	Mucosal depth (μm)	Villus surface area (mm2)
Normal environment					
Palmaria palmata (%)					
0.6	803±64	175±14	153±8	278±28	0.168±0.02
1.8	884±64	207±14	181±8	229±28	0.194±0.02
3	900±64	199±14	147±8	275±28	0.183±0.02
Inulin 2.5%	740±64	186±14	156±8	291±28	0.167±0.02
Antibiotic (BMD) [1]	887±64	193±14	168±8	262±28	0.182±0.02
No feed additive	712±64	234±14	169±8	289±28	0.188±0.02
Stressed rooms					
Palmaria palmata (%)					
0.6	865±64	196±14	158±8	233±28	0.178±0.02
1.8	833±64	177±14	148±8	234±28	0.153±0.02
3	897±64	184±14	167±8	211±28	0.186±0.02
Inulin 2.5%	674±64	181±14	152±8	266±28	0.141±0.02
Antibiotic (BMD) [1]	872±64	171±14	163±8	265±28	0.159±0.02
No feed additive	734±64	192±14	159±8	288±28	0.181±0.02
ANOVA (P-value)					
Treatment	0.01	0.29	0.58	0.38	0.68
Environment	0.82	0.06	0.31	0.21	0.23
Treatment*Environment	0.93	0.33	0.06	0.78	0.81

[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

No statistical differences were observed among dietary treatments in villus width, crypt depth, mucosal depth and villus apparent area on Day 24 (Table 4.33). Birds raised in the stressed environment rooms had significantly, lower villus width compared to the normal environments. Villus height was significantly different among treatments. *Palmaria palmata* at 3% increased villus height in comparison with 2.5% Inulin (Table 4.32). Sohail et al. (2012) observed that heat stress decreased villus height, width, crypt depth and villus surface area in birds subject to chronic heat stress.

Table 4.33. Intestinal histomorphology measurements of broiler chickens day 24 posthatch with dietary supplementation *Palmaria palmata*, inulin, antibiotic and no feed additive .

	Villus Height (µm)	Villus Width (µm)	Crypt Depth (µm)	Mucosal Depth (µm)	Villus Apparent Area (mm2)
Treatment					
Palmaria palmata (%)					
0.6	834±45[ab]	185±10	155±6	256±20	0.173±0.01
1.8	858±45[ab]	192±10	164±6	231±20	0.173±0.01
3	898±45[a]	191±10	157±6	243±20	0.185±0.01
Inulin 2.5%	707±45[b]	183±10	153±6	278±20	0.154±0.01
Antibiotic (BMD)[1]	879±45[ab]	182±10	165±6	263±20	0.171±0.01
No feed additive	723±45[ab]	213±10	163±6	288±20	0.184±0.01

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$).
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

At day 33, interaction effects of treatment and environment were observed for villus height and surface area (Table 4.34). S environment affected villus surface area on day 33. Birds placed in the stressed rooms had lower villus surface area, in comparison with birds grown in the normal environment. The interaction of treatment and stressed environment, increased villus height and surface area in birds fed 1.8% *Palmaria palmata* (Table 4.34).

Table 4.34. Ileal intestinal histomorphology measurements of broiler chickens on day 33 posthatch with *Palmaria palmata*, inulin, antibiotic and no feed additive under normal and stressed conditions.

Environment/treatment	Villus Height (μm)	Villus width (μm)	Crypt Depth (μm)	Mucosal Depth (μm)	Villus Surface Area (mm2)
Unstressed environment					
Palmaria palmata (%)					
0.6	796±63^{ab}	152±10	135±7	284±27	0.155±0.01^{abc}
1.8	994±63^{a}	191±10	156±7	226±27	0.203±0.01^{a}
3	930±63^{ab}	180±10	142±7	247±27	0.168±0.01^{abc}
2.5% Inulin	890±63^{ab}	189±10	163±7	251±27	0.198±0.01^{ab}
Antibiotic (BMD)	674±63^{b}	161±10	139±7	332±27	0.120±0.01^{c}
No feed additive	830±63^{ab}	180±10	141±7	321±27	0.162±0.01^{abc}
Stressed envoirnment					
Palmaria palmata					
0.6	925±63^{ab}	175±10	153±7	278±27	0.173±0.01^{abc}
1.8	882±63^{ab}	161±10	148.±7	297±27	0.150±0.01^{abc}
3	880±63^{ab}	167±10	146±7	260±27	0.143±0.01^{abc}
2.5% Inulin	692±63^{ab}	160±10	150±7	325±27	0.127±0.01^{c}
Antibiotic (BMD)	813±63^{ab}	164±10	134±7	293±27	0.135±0.01^{bc}
No feed additive	691±63^{ab}	156±10	153±7	359±27	0.119±0.01^{c}
ANOVA(P-value)					
Treatment	0.01	0.72	0.14	0.26	0.01
Environment	0.29	0.06	0.78	0.12	0.001
Treatment* Environment	0.039	0.09	0.32	0.27	0.007

[a-c] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$).
[1]Medicated Bacitracin Methylene Disalicylate Premix (BMD) at 0.04% level

This indicates that *Palmaria palmata* was able to improve the development of morphological structures of intestine. As were observed in this trial, 1.8% *Palmaria*

palmata improved the number of *Bifidobacteria* in the ileum is indicative of an increased villi width and therefore symbol of less turnover and more absorptive surface area (Rehman et al., 2007). The increased nutrient absorptive area may be responsible for the increased growth performance of birds fed *Palmaria palmata.*

Increased dietary fiber shifts the microbial population towards acetic acid production and butyrate-producing bacteria (Liu et al., 2012), which are associated with increased villus height in the jejunum and ileum (Kuzmuk et al. 2005). Therefore, it can be conclude that the reduction of pH, increasing beneficial bacteria including *Lactobacillus* and *Bifidobacterium* at this day and also increased villus height is related to prebiotic like effects of *Palmaria palmata*.

There were increasing trends for villus height, width, mucosal depth, villus surface area and crypt depth during days 7, 14, 24 and 33 (Table 4.35). Theses parameters increased as the birds got older, which indicates the growth and development of the gastrointestinal tract of birds during starter, grower and finisher periods.

Table 4.35. Effects of dietary treatments *Palmaria palmata*, inulin, antibiotic and no feed additive during days 7, 14, 24 and 33.

	Villus height (μm)	Villus width (μm)	Crypt depth (μm)	Mucosal depth (μm)	Villus surface area (μm)
Day					
7	556±18^{d}	145±^{c}	115±3^{c}	79±8^{d}	0.08±0.005^{d}
14	714±18^{c}	161±4^{b}	141+3^{b}	184±8^{c}	0.12±0.005^{c}
24	823±18^{b}	168±4^{b}	160±3^{a}	252±8^{b}	0.17±0.005^{a}
33	827±18^{a}	190±4^{a}	146±3^{b}	282±8^{a}	0.15±0.005^{b}

$^{a-d}$ means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)

In the present study, stressed environment did not affect the ileum morphology of chickens on most sampling days. Sohail et al. (2012), observed that villus height, width, crypt depth, and villus apparent surface area were decreased when birds were exposed to heat stress. In this trial, birds were not affected by environmental stress which included a heat component. The present experiment results are in agreement with Wiseman (2012),

who observed that supplementing brown seaweed (Tasco®) in broiler diets increased the villus apparent area, compared to inulin and had deeper crypt than inulin fed birds. Tasco® supplementation resulted in taller villi than inulin supplementation on days 7 and 21. Similarly, the results of current trial indicated that feeding seaweeds increased villus with and apparent area. This increase could be associated with an enhance digestive and absorptive function of the intestine due to increased absorptive surface area and nutrient transport system (Awad et al., 2009). Furthermore, Kulshreshtha et al. (2014) reported that feeding 2% red seaweed (C*C and SC*) in layer hens increased the average villus height (909.4±35.5µm) and crypt depths (328.8±26.5 µm) were deeper for layers fed with 2% CC, compared with control birds (658.8±35.5µm, 159.8±26.5). Similarly, Baurhoo et al. (2009) observed that feeding broilers with MOS improved morphological structure of the intestine by increasing villi length and goblet cells. Antibiotics did not affect any of the intestinal morphological parameters measured.

4.6.7 IgA level

IgA level at day 33 was higher than the no feed additive diet ($P < 0.05$) for broilers fed the 1.8% red seaweed treatment (Table 4.36). Environmental stress did not affect the plasma immunoglobulin of chickens.

Table 4.36. Effects of dietary supplement of *Palmaria palmata*, inulin, antibiotic and no feed additives in the blood of broilers (mg/ml).

Treatment	Day 33
Palmaria palmata (%)	
0.6	0.523 ± 0.16^{bc}
1.8	0.594 ± 0.16^{a}
3	0.534 ± 0.16^{abc}
Inulin 2.5%	0.576 ± 0.16^{ab}
Antibiotic (BMD)[1]	0.516 ± 0.16^{bc}
No feed additive	0.489 ± 0.16^{c}
ANOVA	P-value
Treatment	<. 0001
Environment	0.66
Treatment*environment	0.20

[a-c] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

Birds protect against various infections by secreting plasma immunoglobulins. Avian immunoglobulin is classified into three classes in which IgG, IgM, and IgA mainly represent the immune response of an individual. Salim et al. (2013) observed that supplementation of broilers with direct-fed microbials (0.381 ±11.8 mg/ml) (a mixture of *L.reuteri*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*) increased the plasma immunoglobulin levels in comparison with control groups (0.277 ±11.8 mg/ml). In another study, Kim et al., (2011) observed that supplementation of broiler feed with (0.25% and 0.5%) FOS (0.586±0.45 and 0.575±0.45 mg/ml) and (0.25% and 0.5%) MOS (0.591±0.45 and 0.599±0.45 mg/ml) did not significantly alter the concentration of IgA, compared to the no feed additive group (0.586±0.45 mg/ml). In the current experiment, the IgA levels for 1.8% *P.p.* (0.594±0.16 mg/ml) was higher than what Salim et al. (2013) reported. This difference may attribute to the condition of trial. In the current trial,

a combination of environmental stressors increased IgA, compared to the previous trial, which was conducted without any environmental stresses.

The improved plasma immunoglobulin levels of broilers fed 1.8% *Palmaria palmata* in the present experiment might be attributed to the shifting bacteria including *Lactobacillus* which occurred in the gut. It has been shown that *Lactobacillus* species stimulated the different aspects of the gut-associated immunity along with increased production performance and decreased the pathogenic bacteria in chickens (Mountzouris et al., 2007).

4.6.8 Next generation sequencing results

On the basis of the NGS results from Trial 1, most prebiotic like activity of *Palmaria palmata* was observed on 33 day old chickens. Therefore, the ileal microbiotas of chickens of 33 days of age were analyzed for NGS. The relative abundance of OTUs was examined at different ranking levels from phylum to genus. At the kingdom level, K-bacteria were affected by the S environment and birds in S environments had higher K-bacteria, in comparison to birds grown in the normal environment (Table 4.37). *Firmicutes* species bacteria, which are a part of K-bacteria, are dominant in young chickens (Pourabedin et al., 2015).

Table 4.37. Effect of *Palmaria palmata*, inulin, antibiotic and no feed additive on the relative abundance of K-bacteria in the ileum of chicken on day 33.

Treatment	day 33
Palmaria palmata (%)	
0.6	0.9888±0.001
1.8	0.9978±0.001
3	0.9881±0.001
Inulin 2.5%	0.9867±0.001
Antibiotic (BMD)[1]	0.9890±0.001
No feed additive	0.9878±0.001
ANOVA	P-value
Treatment	0.87±0.001
Environment	0.01±0.001
Treatment*environment	0.83±0.001
Environment	P-value
Unstressed environment	0.9895±0.001[a]
Stressed environment	0.9866±0.001[b]

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]BMD is medicated bacitracin methylene disalicylate premix at 0.04% level

At the phylum level, *Firmicutes*, *Protobacteria*, *Actinobacteria* and *Bacteroidetes* (Figure 3.6). *Firmicutes,* which was the most abundant phylum in the chicken GIT, contained two subgroups of bacteria ***b****acilli*, having the highest population, and *clostridia*. There were no significant differences among treatments for the number of *Bacili* and *Clostridiales*. Depending on the location in the chicken gut the number and species of bacteria were different (Pourabedin et al., 2015). Genus *Lactobacillus* has the highest bacterial proportion in the chicken crop, gizzard and duodenum (Pourabedin et al., 2015). The crop has the highest diversity of *Lactobacillus* which is dominant in the ileum (Pourabedin et al., 2015). The dominant orders in the ileum included *Lactobacillales* and *Closttridiales*. *Peptostreptococcaceae* and *Lactobacillaceae* were the most dominant families in the

ileum. The genus *Lactobacillus* and *Candidatus Arthromitus* were more abundant in the ileum. *Lactobacillus*, *Ruminococcus* and *Clostridium* are the three most abundant bacterial phylotypes, which are related with performance enhancement (Torok et al., 2009). Several bacterial phylotypes, more specifically within genera *Lactobacillus*, *Ruminococcus* and Clostridium, were associated with performance enhancement (Torok et al., 2009). Feeding 1.8% *Palmaria palmata* enhanced the relative abundance of *Lactobacillus* genus in comparison with other treatments (Table 4.38). Kim et al. (2011) observed that feeding birds with 0.025 and 0.05% of MOS beneficially affect the chicken by enhancing the relative proportion of beneficial bacteria including *Lactobacillus* and decreased the adverse bacteria such as *Clostridium perfringens*. In addition, Pourabedin et al. (2014) observed that feeding birds under suboptimal conditions with MOS (1 g/kg) enhanced the diversity of cecal bacteria and increased the growth of *Lactobacillus* and *Bifidobacterium* species in the cecum (Pourabedin et al., 2014).

Table 4.38. Effect of *Palmaria palmata*, inulin, antibiotic and no feed additive on the relative abundance of *Lactobacillus* in the ileum of chicken on day 33.

Treatment	day 33
Palmaria palmata (%)	
0.6	0.46±0.09[ab]
1.8	0.82±0.09[a]
3	0.49±0.09[ab]
Inulin 2.5%	0.54±0.09[ab]
Antibiotic (BMD)[1]	0.52±0.09[ab]
No feed additive	0.39±0.09[b]
ANOVA	
Treatment	0.05
Environment	0.96
Treatment*environment	0.36

[a-b] means±SEM with different superscripts within a column are significantly different ($p \leq 0.05$)
[1]Medicated Bacitracin Methylene Disalicylate Premix (BMD) at 0.04% level

4.7 General discussion

Results of this trial indicated that by increasing IgA, the population of *Bifidobacterium* and *Lactobacillus* rose, resulting in improved gut health by changing the morphology of the intestine (villus width, height and surface area). *Bifidobacterium* and *Lactobacillus* produce lactic and acetic acids which cause a pH reduction in the gut, resulting in unfavorable conditions for pathogen growth. Feeding *Palmaria palmata* at 1.8% increased birds' growth performance by enhancing BW and BWG at Day 33. In addition, NGS results proved that feeding birds with 1.8% *Palmaria palmata* elevated the proportion of *Lactobacillus*, in comparison with other treatments. In this trial, birds were exposed to a combination of environmental stresses caused by (used litter, elevated humidity, ammonia and heat). It is well documented that the environmental conditions play a major role in chicken gut microbiota (Cressman et al., 2010) observed that *Lactobacillus* species were dominant in the ileal mucosal microbiota of birds rose with fresh litter, while birds that were raised with reused litter, had *Clostridiales* bacteria as the dominant species. In addition, heat stress conditions and elevated ammonia and humidity had negative effects on growth performance and health of birds by decreasing feed consumption and increasing mortality (Sohail et al., 2012; Suzuki et al., 1983). Conventional microbiology results from the current trial indicated that stresed environment decreased beneficial bacteria, including *Lactobacillus* and *Bifidobacterium*, in comparison with normal environment, which had higher *Lactobacillus* and *Bifidobacterium* proportions. Furthermore, adverse bacteria, such as coliform, *E. coli*, *Clostridium perfringens*, were higher in stressed environment, compared to US environment. At day 33, NGS results of Trial 2 showed that stressed environment decreased k-bacteria, which include the *Firmicutes* bacteria species, including *Lactobacillus*. However, S environment did not influence relative abundance of this specific microbial species (*Lactobacillus*). From both conventional microbiology and NGS results, it can be concluded that *Palmaria palmata* at 1.8% improved the balance of the birds' gut commensal microbiota. Gut microbiota can produce SCFA by fermenting prebiotics, like *Palmaria palmata* to make acetate, propionate and butyrate (Gibson and Roberfroid 1995). These products can be a source of energy for epithelial cells and also improve intestinal mucosal structure. In addition, beneficial bacteria, including

Bifidobacteria, can increase the proliferation of intestinal epithelial cells along the length of the villus and improve intestinal function. Other important defensive factors in the gastrointestinal tract, which can be influenced by prebiotics, are the secreted immunoglobulins (Ig), particularly IgA. The secretion of IgA, which is affected by mucosal immunity and mucosal immunity, is an important part of humoral immunity. In this trial, IgA levels were not significantly different for US and S environments. However, the IgA level affected the birds fed with 1.8% *Palnaria palmata,* in comparison with other treatments. Prebiotics can provide energy resources (by producing SCFA) for epithelial cells which are made in the intestinal mucosal crypts and which migrate to the top of the villi (Schat and Myers, 1991). Therefore, prebiotics can directly influence the health status of the gastrointestinal tract by improving intestinal morphology. The crypt, also known as the villus factory, inflates due to pathogens and their toxins, which increase to provide more energy for faster turnover (Awad et al., 2009). Furthermore, butyrate has been shown to increase the height of villi, which results in improved digestibility by enhancing the absorptive function due to an increased absorptive area on the villus (Awad et al., 2009). Therefore, dietary supplementation of *Palmaria palmata* at 1.8%, as an alternative for AGP, may reduce the detrimental effects on broiler productivity and health, caused by suboptimal growing condition.

4.8 Conclusion

Palmaria palmata at 1.8% improved BW and BWG of broilers in a stressed environment. Furthermore, 1.8% *Palmaria palmata* increased villi height, villi apparent area, and villi width, as well as improved immune protection of the gut by increasing plasma IgA. The 1.8% *Palmaria palmata* positively influenced the number of beneficial bacteria, including *Lactobacillus* and *Bifidobacterium* and decreased the number of undesirable bacteria, including *clostridium perfringens*. Environmental stress reduced BW, BWG, as well as the population of total anaerobic bacteria and *Clostridium perfringens*. These results suggest that *Palmaria palmata* shows promise as a prebiotic and antibiotic alternative, when fed at a level of 1.8 % in floor raised birds.

Chapter 5 COMPARISON BETWEEN TRIAL 1 (CAGE) AND TRIAL 2 (PEN)

5.1 Cross trial comparison

The first trial with normal environmental conditions was conducted in 48 cages in two climate-controlled rooms. The second trial was performed, under two different environments. In the S environment rooms, temperature and humidity were elevated and excrement was present from a previous flock. Raising birds in the floor pens is more applicable to the industry In Trial 2, male birds were obtained from the hatchery; however, only female birds were available for the first trial. The growth of males, in comparison to females, is faster as their intestinal tracts, and hence, their absorptive capacity, develops more rapidly (Miles et al., 2006). Birds raised in the cage trial had higher average BW and BWG, in comparison with birds in the pen trial for the starter period. However, birds in the pen trial had higher average BW and BWG, in comparison with the cage trial for the grower and finisher periods. There were inconsistent results regarding poultry housing systems in the literature. Santos et al. (2008) observed that birds, from days 0 to 42, fed in pens with litter had improved feed conversion, increased villi height, villi surface area, villi height to crypt depth ratio. However, Garcia et al. (2006) reported that in a study of the growth of birds from days 21 to 38, birds raised in cages versus pens had better growth performance.

When the birds are raised in floor pens with litter, they are exposed to feces of other birds and this can enhance their pathogen exposures. There was a dietary difference between Trials 1 and 2. In the first trial, the diet form was mash, while in the second, during the starter period, diets were crumbled and then switched to pelleted diets for the rest of the trial (grower and finisher periods). The form of diet can affect the growth performance of birds. As a case in point, feeding birds with pellet form is thought to save energy and therefore, they have more energy for growth (Jensen et al. 1962). The second trial results indicated that BW, BWG and FI were higher than in the first trial and feed efficiency seemed to be improved.

Feeding *Palmaria palmata* at 1.8% improved the relative abundance of beneficial bacteria in the ileum in both trials. *Palmaria palmata* at 1.8% increased growth

performance of the broilers, beneficial bacteria in ileum and serum IgA and villus width, height and surface area in broilers.

In the first trial, none of the treatment improved growth performance traits, serum IgA and ileal intestinal morphology of the broilers. Raising birds in the floor pens and using environmental S resulted in prebiotic like effects of *Palmaria palmata*. Environmental S decreased the proportion of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium,* compared to the US environment. Based on the results from these two experiments, raising birds in the pens with stressed environmental conditions, in comparison with pens, helps to identify the prebiotic like effects of *Palmaria palmata* and demonstrated the optimal inclusion level of *Palmaria palmata* for growth and health. In the second trial, a combination of stresses was used, which were elevated temperature, humidity and the presence of used litter. This combination of environmental stressors classified the prebiotic effects of *Palmaria palmata*.

Chapter 6 CONCLUSION

6.1 Conclusion

The supplementation of *Palmaria palmata* improved broiler growth and health, compared to inulin and AGP, in stressed environment conditions (temperature, humidity and presence of excreta). Environmental stress decreased the number of aerobic, anaerobic, *Lactobacillus*, K-bacteria and *Bifidobacterium* bacteria. The inclusion of 1.8% *Palmaria palmata* had a positive effect on BW, BWG, beneficial bacteria such as *Lactobacuillus* and *Bifidobacterium*, villus height, width, surface area and IgA in stressed and normal environment. These effects are indicative of the presence of *Palmaria palmata* in lower gastrointestinal tract and its fermentation by microflora. Shifts in physiological variables were generally beneficial when *Palmaria palmata* were added as a feed additive. The results indicated that cultivated strain of *Palmaria palmata* could be used as a prebiotic for broilers at a 1.8% inclusion level.

6.2 Recommendations

In the future research, investigating the effects of *Palmaria palmata* on the bird cecal bacterial populations by NGS is recommended, as there were changes in the cecal content pH that likely cause to change in bacteria profile. Furthermore, examining the combination of fermentable *Palmaria palmata* as a feed additive combine with a feeding probiotic species could help to find the optimal dietary inclusion level of *Palmaria palmata.*

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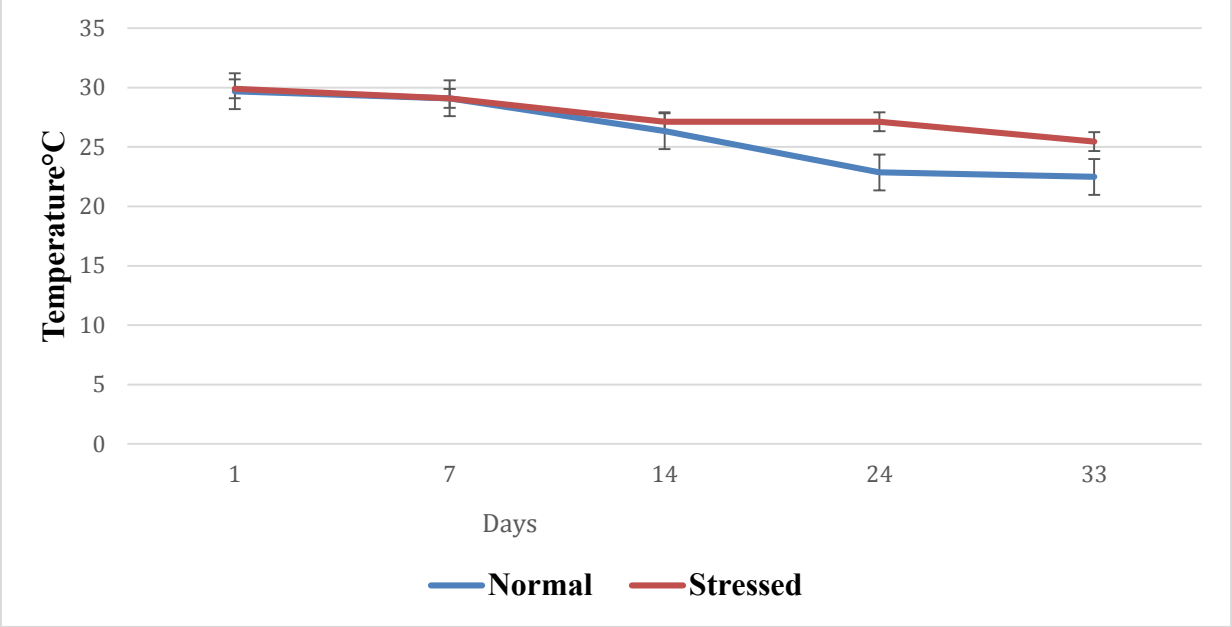
APPENDICES

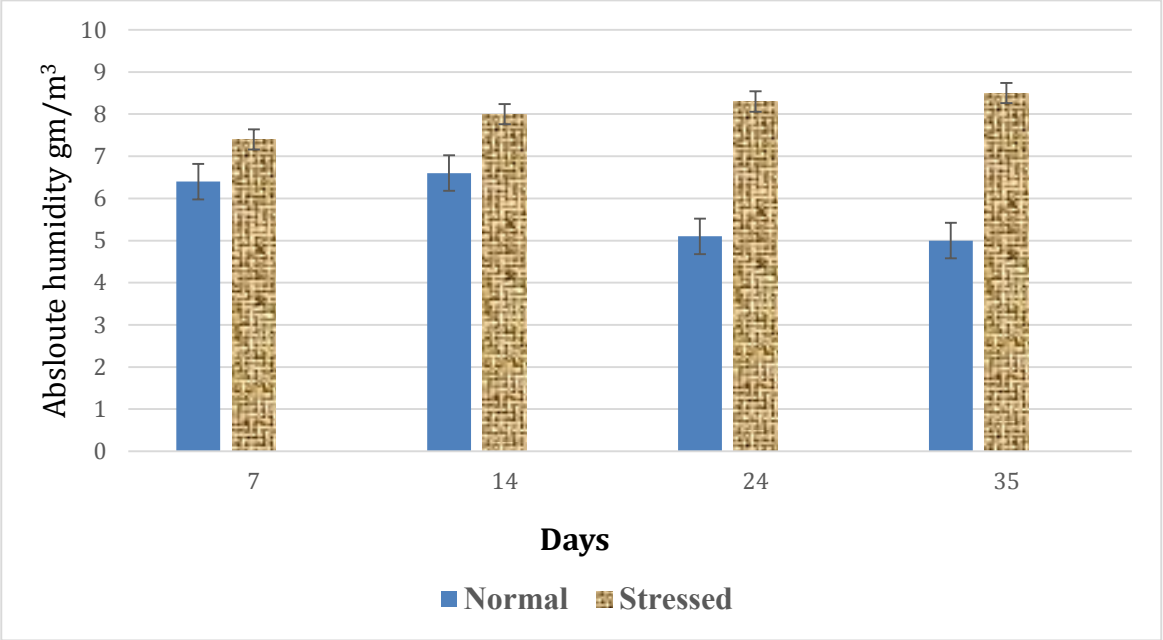
Appendix A. Lighting schedules for cage and floor

Days post hatch	Light hours	Light intensity (lux)
0-2	24	20
3-4	23	20
5-6	16	15
7-9	16	10
10-11	16	5
12-13	16	5
14-16	16	5
17-18	16	5
19-20	16	5
21	16	5
22-23	16	5
24-27	16	5
28	17	5
29-32	18	5
34-35	19	5

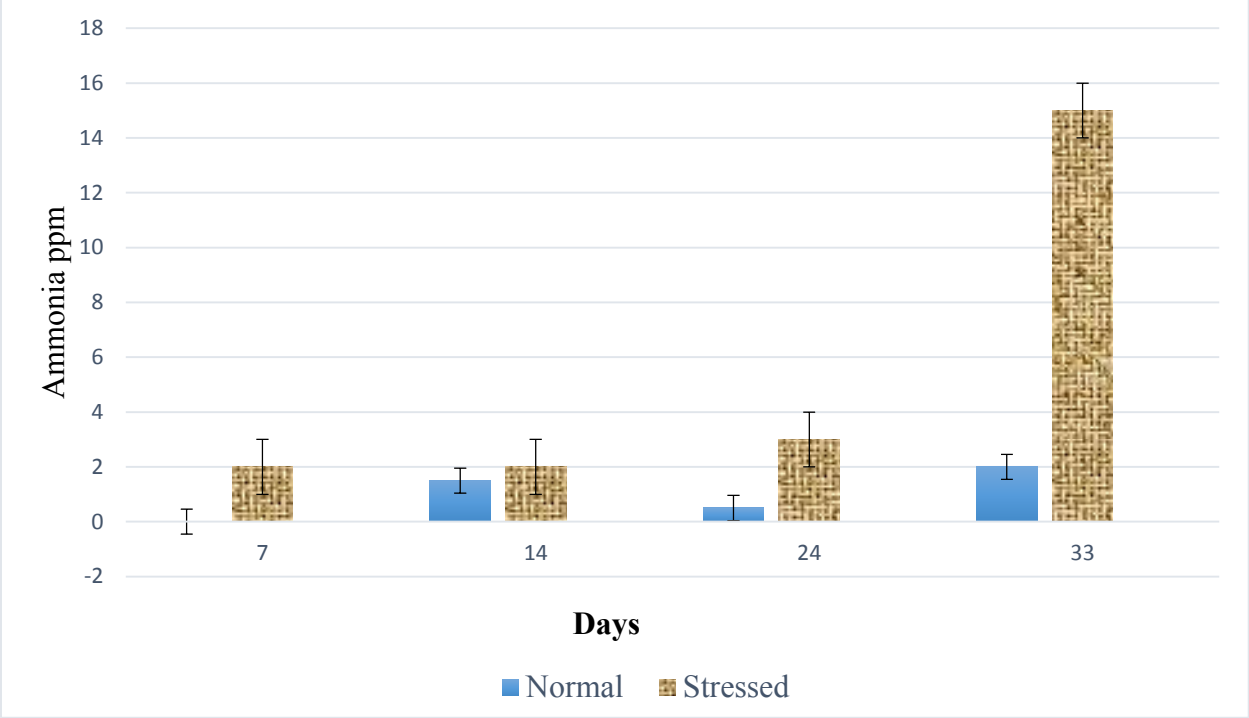
Appendix B. temperature recorded by data logger for floor trial (stressed and normal environment program)



Appendix C. humidity recorded by data logger for floor trial (Environmentally stressed condition.



Appendix D. ammonia measured by using ammonia tubes for floor trial (Environmentally stressed conditions).



Appendix E. Chemical composition of brown seaweed and red seaweed samples.

Component	Brown Seaweed		Red Seaweed	
	% as is	% DM	% as is	% DM
Dry matter	91.3		87.6	
Acid detergent fiber (ADF)	24.2	26.6	4.03	4.60
Neutral detergent fiber (NDF)	45.0	49.3	20.5	23.4
Total dietary fiber	57.7	63.2	39.0	44.5
Non-starch polysaccharides (NSP)	24.9	27.3	33.2	37.9
NSP component sugars				
Rhamnose	9.50	10.4	0.00	0.00
Arabinose	0.00	0.00	0.00	0.00
Xylose	1.87	2.05	28.1	32.1
Mannose	2.42	2.65	0.00	0.00
Galactose	0.63	0.69	2.01	2.29
Glucose	2.66	2.91	1.90	2.16
Uronic acids	7.81	8.55	1.21	1.38
Lignin	27.1	29.7	1.10	1.26
Glycoproteins	5.70	6.25	4.70	5.37
Carbohydrates				
Simple sugars*	7.09	7.79	0.16	0.18
Sucrose	0.30	0.33	0.00	0.00
Oligosaccharides **	0.83	0.91	0.49	0.56
Starch	0.27	0.30	1.47	1.68

* Includes glucose and fructose
**Includes raffinose and stachyose

Appendix F. Chemical composition of inulin

Component	Inulin	
	% as is	% DM
Dry Matter	94.7	
Fructans (minimum)	87.0	91.9
Simple sugars*	0.75	0.79
Sucrose	1.09	1.15
Oligosaccharides	3.68	3.89
Starch	0.30	0.32
Acid detergent fiber (ADF)	0.00	0.00
Neutral detergent fiber (NDF)	0.00	0.00

*Includes glucose and fructose