The Microbial Modifying Properties of Re-used Chicken Litter and Iodinated Water on Poultry Health and Disease Resistance

by

Deanna Margriet Pepin

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

In animal production the overuse of antibiotics has generated numerous multidrug resistant bacteria that pose significant health risks for both humans and animals. Reducing the use of antibiotics in poultry production relies on finding ways to prevent the introduction and spread of pathogens through a flock, as well as promoting the health and development of a flock to be resilient against such infections. Water lines have been identified as a means through which pathogens can spread throughout flocks and current water sterilization methods are not effective against all bacterial pathogens and viruses. Iodinated water is one method of water sterilization that may prove to prevent pathogen spread. The use of iodine as an antimicrobial in water sterilization has long been reported, and increased iodine in poultry feed has resulted in improved production parameters. The mechanisms involved in iodine's efficacy on poultry performance are poorly understood. As well as preventing pathogens, research has been looking at ways to improve poultry resilience through the development of a healthy intestinal microbiome that excludes pathogens. The microbial community in the intestines plays an important role in disease resistance, immune development, host digestion and nutrient absorption. One way to encourage the early development of the microbiome in chicks is by placing them on already used chicken litter, which contains a diverse microbial community from adult chicken fecal droppings. Research has shown that used litter impacts the development of the chick intestinal microbial community, and has improved infection resistance to poultry pathogens such as *Salmonella*. However, the impact of used chicken litter on the intestinal development and immune education in birds is not well characterized. To better understand the mechanisms behind re-used litter and iodinated water in poultry health 1-day-old broiler chicks were supplied with regular water or two concentration of iodinated water and clean or used litter

in a 2 by 3 factorial design to determine the effect of treatment on the caecal microbiome, gene expression, and morphology over 12 days of treatment. Next, it was determined if these treatments were capable of reducing *Salmonella enterica* serovar Enteritidis colonization, comparing regular water or 1 ppm iodinated water and clean or used litter. Lastly, the long-term effect of iodinated water on poultry performance and intestinal microbial communities was analyzed over 35 days of growth. Overall, used litter had a significant effect on promoting early development of the caecal microbial community, introducing numerous bacteria not found in the clean litter treatment, and modifying short chain fatty acid profile in the caeca. As well, used litter modified caecal morphology and the caecal gene expression increasing genes related to intestinal epithelial cell homeostasis as well as activating an immune response. Used litter proved to be effective in reducing *Salmonella* colonization, and reduced potentially pathogenic members of the *Enterobacteriaceae* family, but introduced *Clostridium*, a genus also known for its pathogenic species. Iodinated water had very limited effects on the microbiome, gene expression, gut morphology and growth parameters measured in both the used litter chicks over 12 days and the adult birds over 35 days. However, the largest impact of iodinated water was seen in the clean litter treatment, with risks of increasing *Salmonella* colonization, and playing a role in intestinal epithelial cell turnover and organization as well as activating genes associated with a proinflammatory response. Overall, further research is needed to understand the effect of iodinated water to farm water in a production setting. The re-use of chicken litter has many benefits for the early development of the intestinal microbial community in poultry, however does come with risks of pathogen introduction.

Preface

This thesis is an original work by Deanna Pepin. This research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Salmonella in chickens”, No. AUP00001626, 13/08/2015.

Author contribution: conception and design of experiments: Benjamin Willing. Performance of the experiments: Deanna Pepin. Analysis of the data: Deanna Pepin, Benjamin Willing, Peris Munyaka, Andrew Forgie, Arun Kommadath. Contribution of reagents/materials/analysis tools: Deanna Pepin, Benjamin Willing and Paul Stothard.

Dedication

Dedicated

To my Husband,

Kaylan Pepin

Acknowledgments

I would like to express my deepest gratitude to my supervisor, Dr. Benjamin Willing, for this research opportunity. I have learned so much from working with him over the past few years and thank him sincerely for his support throughout my research and challenging me to grow as a scientist. I am especially grateful for his hands-on help during animal trials and his outstanding patience during the thesis writing process.

I am greatly appreciative to the members of my examining committee: Dr. Leluo Guan, Dr. Mike Steele, and Dr. Michael Gänzle. I would especially like to thank Dr. Leluo Guan for her support and invaluable advice throughout my research project, and the technical support from members of her lab. Specifically, I'd like to thank André Neves and Huizeng Sun for their support in my gene expression analysis and Yanhong Chen for her technical support in the lab. A great deal of gratitude goes to Dr. Paul Stothard for his teaching and support and Dr. Arun Kommadath for his work on the RNA sequencing data.

I would really like to thank my lab mates in the Willing lab for your hours of support, you have all become very close friends. I particularly would like to thank Tingting Ju, Dr. Janelle Fouhse, Camila Marcolla, Edward (Yi) Fan, Dr. Peris Munyaka, Andrew Forgie, Erinn Mills, Dr. Carina Yang, Benjamin Bourrie, Tausha Prisnee, Jinghong Kang, Natalie Diether, and Carla Alvarado for their contributions to my animal trials, as well as help with lab experiments and data analysis. This would not have been possible without all of your advice, support, and never-ending supply of doughnuts.

I am thankful to Sarah Collard, Simmone Kerswell, and Carol Boechler and other staff members from Science Animal Support Services for all of your help with my animal trials. Thanks goes to the staff members at the Poultry Research Center for taking great care of the animals, specifically Lyle Bouvier and Giles Hinse for all your hard work in setting up the water system. I would also like to thank Nicole Coursen for her hard work on the tissue histology. Appreciation is due to Richard Smith and Laura Patterson-Fortin from BioLargo Water Canada Inc. for making this research possible, as well as NSERC Engage and Mitacs Accelerate for their generous funding.

Most importantly, I am the most thankful to my extremely caring husband, Kaylan Pepin, for his unwavering support from start to finish. I would have never made it to this point without your care and encouragement. I owe this achievement to you. Thank-you for supporting my dreams.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

OTU: Operational taxonomic unit

SCFA: Short chain fatty acid

GF: Germ free

IL: interleukin

TNF: Tumor necrosis factor

GALT: Gut associated lymphoid tissue

CR: conventionally raised

CE: competitive exclusion

APEC: Avian pathogenic *Escherichia coli*

qRT-PCR: quantitative reverse transcribed polymerase chain reaction

PCR: polymerase chain reaction

DE: differentially expressed

DEG: differentially expressed gene

CFU: colony forming units

PCR-DGGE: polymerase chain reaction denaturing gradient gel electrophoresis

GD: gangrenous dermatitis

FPD: foot pad dermatitis

UV: ultra violet

ppm: parts per million

KI: potassium iodide

KIO_3: potassium iodate

MD: meckel's diverticulum

GC: gas chromatography

KEGG: Kyoto Encyclopedia of Genes and Genomes

DAVID: Database for Annotation, Visualization and Integrated Discovery

GO: Gene ontology

PANTHER: Protein ANnotation THrough Evolutionary Relationship

SEM: standard error mean

ANOVA: analysis of variance

MDS: multidimensional scaling plot

FC: fold change

CPM: counts per million

TMM: trimmed mean of M-values

FDR: false discovery rate

BP: biological process

MF: molecular function

CC: cellular component

ICAM: Intercellular Adhesion Molecule

LB: Luria-Bertani

ADG: average daily gain

TWG: total weight gain

Stn: *Salmonella* enterotoxin

PCoA: principle coordinate analysis

RO: reverse osmosis

FCR: feed conversion ratio

Ctrl: control

List of Appendices

- Chapter 1: Literature Review

1.0 Introduction

The overuse of antibiotics in animal production is a major generator of antibiotic resistant pathogens (Koch et al., 2017). With increasing pressure to reduce antibiotic use, there is increasing demand to identify alternatives to maintain poultry health. The gastrointestinal microbiota has proven to play significant roles in the host through aiding digestion, providing nutrients, educating the immune system, and defending against pathogens (Sommer & Bäckhed, 2013). As chicks are moved from the hatchery to clean barns shortly after hatch, their microbiomes are mostly naïve and often dominated by the *Enterobacteriaceae* family, which contains many opportunistic pathogenic bacteria (Ballou et al., 2016).

As young chicks are more susceptible to infectious pathogens such as *Salmonella,* the early establishment of a commensal microbiota is an important line of defense (Suzuki, 1994). Supplying chicks with the intestinal microbiota of adult chickens orally has been successful in reducing pathogen colonization and increasing short chain fatty acids (Corrier et al., 1992) which provide energy to the intestinal epithelial cells (Bedford & Gong, 2018; Nepelska et al., 2012). As well, the intestinal microbiota play an important role in the development of the immune system in young chicks (Honjo et al., 1993; Mwangi et al., 2010). As the initial environment a chick is introduced to plays an important role in the development of the microbiota (Ballou et al., 2016; Oakley et al., 2014), placing chicks on re-used poultry litter may prove to be an effective way to introduce an established commensal microbiota.

Infection in poultry barn can spread from the water lines throughout a flock (Pearson et al., 1996), so new techniques to keep water sterile longer could be one method to prevent infection and the need for antibiotic use. Iodine is among the oldest substance used for disinfection, and is effective against bacteria, fungi and viruses (Gottardi, 1999). As well, iodine has often been used as a feed and water additive in poultry and has shown to improve animal performance (Emeash et al., 1994; Słupczyńska et al., 2014), but the mechanisms involved are not well understood.

1.1 Poultry Intestinal Microbiota

The importance of a developed microbiome to human health has been well characterized. A review on the role the microbiome plays on host health by Sommer & Bäckhed (2013) outlines their key roles on: educating the immune system, controlling mucus thickness, developing the gastrointestinal tract, regulating host metabolism, and supporting pathogen defense. However, research on the importance of the microbiome in chickens is limited. It has been suggested that the inhibition of commensal microbiota may have a positive effect on production animals, as the use of prophylactic antibiotics has shown improved growth performance in pigs, and is known to be effective in chickens (Gaskins et al., 2002; Thomke & Elwinger, 1998). However, increasing evidence suggests that a developed gastrointestinal microbiome is essential for poultry health and production.

1.1.1 The Early Establishment and Development of the Poultry Microbiota

In mammals, the microbiome is transferred at birth, although research has identified that early development of the microbiome in the embryo prior to delivery may occur, as microbes have been identified in the umbilical cord and meconium in humans (Ardissone et al., 2014; Jiménez et al., 2005, 2008; Moles et al., 2013). Similarly in poultry, the microbiome in the egg is hypothesized to be sterile and the development of the microbiota does not begin until after hatch (Ilina et al., 2016; Maiorka et al., 2006; Mead, 1989). However, in the last decade new evidence has emerged that the chick embryo may have a microbiota developing *in ovo* as shown by live bacterial plating and microscopy techniques (Kizerwetter-Świda & Binek, 2008). More recently, one study using terminal restriction fragment length polymorphism looking at the microbiome of chick embryos discovered that up to 39 phylotypes of various microorganisms from the typical avian intestinal microbiota, from the families *Enterobacteriaceae* and *Lachnospiraceae,* and orders *Actinomycetales* and *Bifidobacteriales* (Ilina et al., 2016). As well, using metagenomic analysis, one study identified a total of 162 genera in the embryo, 65 of which were consistent in embryo, chick, and maternal hens (Ding et al., 2017). The majority of the genera fell under the *Halomonadaceae* family and *Proteobacteria* phylum (Ding et al., 2017). This research suggests that the development of the microbiome may begin *in ovo*, with the first live microbes being

inherited from the maternal hens. However, one limitation to sequencing based techniques is that they do not ensure that the bacteria are live colonizers, and DNA from proteobacteria such as *E. coli* are major contaminants found in reagents (Perez-Muñoz et al., 2017), so running a blank sample for kit contamination comparison is critical.

Post-hatching, the environment plays an important role in the development of the chick microbiome (Ballou et al., 2016; Oakley et al., 2014). Colonization of the chick begins from contact with the egg shell (Newell & Fearnley, 2003). Newly hatched chicks from hatcheries have no contact with adult birds, and the environment they are placed in will have a strong impact on their mostly naïve intestinal microbiome. The complexity of the intestinal microbial community in broilers dramatically increases with age (Van Der Wielen et al., 2002). Focussing solely on the caecal microbiota, the microbiota of the newly hatched chick from days 0 to 1 are mostly dominated by *Enterobacteriaceae* and to a lesser extent *Enterococcus* (Ballou et al., 2016). Over the first 3 days, the operational taxonomic unit (OTU) abundance increases, and by day 7 the *Firmicutes* population expands leading to greater diversity and richness with development of the *Ruminococcaceae* group which outnumber the *Enterobacteriaceae* family by day 14 (Ballou et al., 2016).

Even after 2 weeks, studies have shown that taxonomic richness and diversity continues to change, with 115 low abundance genera appearing after 6 weeks of growth that weren't present at 3 weeks in broiler chickens (Oakley & Kogut, 2016). The adult poultry caecal microbiome has been well characterized and reviewed by Oakley et al., (2014), containing the highest microbial cell densities in the tract, and playing an important role in carbohydrate fermentation. The chicken caecum microbiome is composed of 4 bacterial phyla, from most abundant to least: *Firmicutes*, *Bacteroides*, *Proteobacteria*, and *Actinobacteria*. The majority of bacterial sequences from the chicken microbiome are assigned to the *Clostridiales* order within the *Firmicutes* phylum. The *Firmicutes* phylum on average make up almost 75% of the sequences, and have a major role in short chain fatty acid (SCFA) production in the chicken caecum (Oakley et al., 2014).

1.1.2 Poultry Microbiota and its Impact on Metabolism and Intestinal Development

The commensal gut microbiota strongly affects the structure and function of the gastrointestinal tract (Dibner & Richards, 2005). Studies with germ free (GF) chickens indicate that in the absence of a commensal microbiota the total villus area and crypt depth was significantly decreased and the rate of epithelial cellular migration from the crypt villus junction to the villus tip was decreased compared to conventionally raised chicks (Cook & Bird, 1973). As well, treating conventional chickens with penicillin or chloramphenicol antibiotics significantly reduced gut weight in chickens by 14 days of age (Coates et al, 1955). The introduction of bacteria to GF mice has confirmed their effect on gut morphology resulting in increased villus height, cell turnover and overall gut weight (Abrams et al., 19 63). In poultry, research on how the introduction of a microbiota influences morphology of the gut is limited. However, there is an indication that beneficial bacterial species used as probiotics have a role in gut development. A probiotic containing multiple species of *Bacillus* fed to broiler chickens over 42 days significantly increased villus height and crypt depth in the duodenum, jejunum, and ileum (Wealleans et al., 2017). As well, *Lactobacillus salivarius* fed to broiler chicks for 42 days significantly increased villus height and villus height to crypt depth ratio in the jejunum (Shokryazdan et al., 2017).

The changes to the intestinal morphology as a result of the microbiota is likely due in part to the production of SCFAs, the by-products of microbial carbohydrate fermentation (Rinttilä & Apajalahti, 2013). SCFAs, such as acetate, butyrate, propionate, succinate, and lactate are important nutrients for the host, capable of increasing the absorptive surface area in the caecum (Dibner & Richards, 2005). SCFAs are also able to reduce the pH in the caecum, and may prove to inhibit pathogenic bacteria such as member of the *Enterobacteriaceae* family in chickens, which are more acid-sensitive than other gut microbes (Van der Wielen et al., 2000). Butyrate is a major energy source for colonic epithelial cells, and has been shown to play a role as a gene regulator in chickens by preventing histone deacetylases from condensing chromatin, keeping it in an active form for transcription (Bedford & Gong, 2018; Nepelska et al., 2012). Other studies have indicated that adding butyrate to poultry diets can reduce pathogen colonization in *Salmonella* challenge models (Fernández-Rubio et al., 2009; Sunkara et al., 2011), as well as modify intestinal development, increasing the villus height to crypt depth ratio in chicken jejunum (Hu & Guo, 2007). More research on the impact of butyrate on immune development has been seen in humans, as it can reduce proinflammatory cytokines (Segain, 2000), however in

poultry this information is limited. However, one study indicated that butyrate induces the expression of host defense peptide Avian beta-defensin 9 in the crop upon addition of butyrate to feed (Sunkara et al., 2011). As well, in chickens challenged with E. coli lipopolysaccharide, butyrate reduced serum IL-6 and TNFα, inducing anti-inflammatory effects (Zhang et al., 2011). Poultry caecal microbes are also capable of fermenting protein material when carbohydrate sources are scarce, which often results in toxic fermentation by-products such as ammonia, which has been shown to have toxic effects on enterocytes in the intestinal epithelium (Rinttilä & Apajalahti, 2013).

1.1.3 Microbiota and Intestinal Immune Development in Poultry

The presence and development of the microbiome is influential on the formation of the chicken immune system. Studies on the chicken gut-associated lymphoid tissue (GALT) are still quite young. A study by Degen et al., in 2005 determined that chickens were capable of developing the T helper Th1/Th2 cytokine balance in response to both intracellular pathogens such as viruses, and extracellular pathogens such as helminths. When comparing GF and conventionally raised (CR) chickens, the absence of a microbiome resulted in poorly developed lymphoid follicles and decreased T and B lymphocytes (Honjo et al., 1993). Another study comparing GF and CR chickens found changes in the chicken intestinal T-cell receptor beta repertoire, in the gut and spleen (Mwangi et al., 2010). Goblet cell mucin production comparing CR to birds raised in an isolator with a limited microbiota introduced resulted in changes to the small intestinal mucin profile: CR birds had lower sulfated mucin (indicative of immature goblet cells) and higher sialylated mucin (indicative of mature goblet cells), indicating that increased microbial exposure leads to mature goblet cell formation (Forder et al., 2007).

The innate immune system in chicks has been shown to be partly developed with heterophil maturation already at hatch, and develops pro-inflammatory cytokines and chemokines as it comes in contact with food and bacteria, with increases in IL-1β, 1L-8, and K203, as early as 2 days post-hatch (Bar-Shira & Friedman, 2006). Immature T and B cells is found in the GALT at hatch and a mature lymphocyte population is established over the first two weeks of life (Bar-Shira et al., 2003). As chickens begin to forage immediately post hatch, they also start to introduce a diverse community of microbes from their feed and surroundings (Hume

et al., 2003; Turk, 1982). Research has indicated correlations between the relative abundance of different bacteria and immune cell activation. *Proteobacteria*, specifically *Escherichia, Shigella*, *Parasutterella*, and *Vampirovibrio* have recently been correlated with IL-6 gene expression in 6 week old chickens (Oakley & Kogut, 2016). Interestingly, this same study showed that as the *Firmicutes* relative abundances increased, specifically *Faecalibacterium sp.*, the gene expression of proinflammatory cytokines, IL-6 and IL-18, decreased (Oakley & Kogut, 2016). *Caloramator sp.* were both negatively correlated with proinflammatory IL-6, and positively correlation with TGF-β4, an anti-inflammatory cytokine, suggesting its role in combating inflammation (Oakley & Kogut, 2016). These results suggest that each member of the microbiome in chickens likely has a different role to play when it comes to immune tolerance and education. Further research in this area using specific microbes in GF animals could further prove these correlations and indicate the specific roles of each microbe on the function of the chicken immune system.

1.1.4 Poultry Pathogens and Microbial Defense

The importance of the intestinal microbiota for pathogen defense in poultry has long been known. Poultry harbor a number of zoonotic pathogens capable of causing severe illness in humans, some of which are also capable of causing disease in poultry. One prevalent pathogen is *Salmonella*, which make up a small portion of the chicken intestinal microbial community (Oakley et al., 2014). *Salmonella* in humans causes diarrhea, fever, abdominal pain, and can be fatal in the very young, elderly, or immunocompromised (Antunes et al., 2016). *Salmonella* prevalence and incidence varies among chickens (Liljebjelke et al., 2005), and their pathogenicity in chickens depends mainly on the age of the chicken. Moreover, the strain of *Salmonella* plays a large role in determining their pathogenesis in poultry; both *Salmonella pullorum* and specific strains of *Salmonella enterica* subsp. enteritidis have shown to be infectious in poultry (Barrow, 1991; F. S. Jones, 1913). Over the first few days of growth and development, chicks are the most susceptible to *Salmonella* infection, but pathogen susceptibility is decreased in mature birds and many researchers from the early 1970's to 1990's proposed that this was due to the development of the microbiome (Rantala & Nurmi, 1973; Smith & Tucker, 1980). It was further determined that *Salmonella* infection could be significantly decreased by giving young chicks an oral gavage of either adult chicken caecal contents or the bacteria

isolated from these contents (Barnes et al., 1979; Corrier et al., 1991; Impey & Mead, 1989; Lloyd et al., 1977; Snoeyenbos et al., 1978; Stavric et al., 1985). At the time, this decreased pathogenicity was attributed to the competitive exclusion (CE) of pathogens by the commensal microbiota's ability to occupy the physical niches in the gut, compete for resources, or direct attack preventing pathogen growth and colonization (Oakley et al., 2014). This solidified that the early development of the microbiome in young chicks was highly important for infection resistance (Rantala & Nurmi, 1973).

Research advanced with the development of products that contained cultures of chicken caecal contents, that could be either sprayed throughout the hatchery, or added to the first drinking water at the farm (Schneitz, 2005). The research on the use of CE continued on from looking at *Salmonella*, to other well-known poultry pathogens. Certain strains of *Escherichia coli* are pathogenic in poultry, most often as a secondary infection following other pathogens such as a bronchitis virus (Smith et al., 1985). Avian pathogenic *E. coli* (APEC) is capable of invading different tissue in the chicken resulting in localized or systemic infection (De Carli et al., 2015). Only recently have the APEC virulence genes been discovered (De Carli et al., 2015). Administering commensal chicken intestinal bacteria to chickens reduced a combined infection of *S. typhimurium* and pathogenic *E. coli* O138, even if the infection was only 2 hours after the bacterial introduction (Soerjadi et al., 1981). Numerous studies support the idea that CE works to reduce many types of pathogenic poultry *E. coli*, including antibiotic resistant organisms (Hofacre et al., 2002; Weinack et al., 1981, 1982). Human pathogenic E coli 0157:H7 has been identified in poultry, and studies have shown that it can be significantly decrease by orally administering commensal chicken gut bacteria, however, non-chicken specific E. coli doesn't colonize well, and tends to decline on its own (Hakkinen & Schneitz, 1996; Stavric et al., 1992).

Campylobacter is another genus of bacteria that often inhabit the chicken intestinal tract, at amounts as high as 10^7 colony forming units per gram of contents (Stern et al., 1995). Although *Campylobacter* are not known to cause illness in poultry, they are a contributor to a human illness resulting in Campylobacteriosis, a type of enteritis caused by two species *C. jejuni* and *C. coli* (Coker et al., 2002). The colonization of young chicks with a diverse set of adult chicken commensals to poultry has been shown to significantly reduce the colonization of *Campylobacter jejuni* in chickens (Hakkinen & Schneitz, 1999; Soerjadi-Liem et al.,1984; Soerjadi et al., 1982; Stern, 1994).

Necrotic enteritis in poultry as a result of *Clostridium perfringens* infection results in severe necrosis of intestinal tissue, resulting in reduced growth and feed efficiency, and in extreme cases, mortality (Hofacre et al., 2003). The use of antibiotic growth promoters in feed kept the prevalence of *C. perfringens* infections low, but as the use of antibiotics decreased, necrotic enteritis dramatically increased (Dahiya et al., 2006; Hofacre et al., 2003). The need for antibiotic alternatives for necrotic enteritis became very important, and researchers began investigating the efficacy of CE. Many have demonstrated that the use of commercial CE products and cultures could reduce the levels of caecal *C. perfringens*, and lesions associated with necrotic enteritis (Craven et al., 1999; Hofacre et al., 2003; Kaldhusdal et al., 2001).Overall, the addition of a competitive commensal intestinal microbiota in young chicks has proven to be a very effective way of preventing and controlling pathogens in chickens.

1.2 Intestinal Microbiota and Host Gene Expression

The intestinal microbial community has a powerful impact on gut physiology, as indicated through research comparing conventionally raised mice to gnotobiotic or GF mice, playing key roles the development of the immune system (Mutch et al., 2004), lipid metabolism (Bäckhed et al., 2004; Turnbaugh et al., 2006), as well as angiogenesis (Hooper et al., 2003; Stappenbeck et al., 2002). The introduction of gene sequencing methods increased our knowledge of the impact microbial colonization on the transcription of genes in the intestine (Bäckhed et al., 2004; Fukushima et al., 2003; Hooper et al., 2001; Mutch et al., 2004), and studies conducted in GF pigs comparing the transcriptome of the small intestine via microarray to conventionally raised piglets indicated that the introduction of microbes resulted in increased expression of genes related to the maintenance of the mucosal barrier and contributing to cell proliferation, differentiation and regulation of cell growth (Chowdhury et al., 2007). Genes responsible for antigen presentation and interferon signalling cascades involved in intestinal epithelial inflammation were upregulated, as well as other genes responsible for the inhibition of inflammation, indicating that the introduced microbes generated a state of cell turnover and immune activation, while at the same time promoting intestinal homeostasis (Chowdhury et al., 2007). However, to date, research on the impact of the microbiome on caecal gene expression in poultry is limited.

1.2.1 Impact of Microbiota on Cecal Gene Expression in Poultry

Similar research to the GF piglets has been done using GF white leghorn chickens. Volf et al., (2017) compared gene and protein expression in the caecum of GF, conventionally raised, and chickens inoculated with a "tetraflora" (*E. coli Nissle*, *Enterococcus faecium*, *Lactobacillus rhamnosus*, and *Clostridium butyricum*), just *E. coli Nissle* (gram negative), or just *E. faecium* (gram-positive) mono-colonized chickens. Using protein mass spectrometry to analyze the proteins and quantitative reverse transcribed polymerase chain reaction (qRT-PCR) for analysis of gene expression to confirm the protein mass spec data, it was found that gene and protein expression in the caecum is influenced by the microbiota composition (Volf et al., 2017). Gut colonization decreased the abundance of focal adhesions and extracellular matrix proteins, and a decrease in collagen VI, the regulator of the focal adhesions in intestinal epithelial cells, however this was not seen through gene expression (Volf et al., 2017). Increases in the expression of genes related to mucin production on the intestinal epithelial cells was also found to result from microbial colonization (Volf et al., 2017). The expression of immunoglobulins was almost completely absent in the GF chickens, while the tetraflora and conventional chicks induced high antibody production, more than the *E. coli nissle* on its own, however even the *E. coli nissle* was higher than *E. faecium* (Volf et al., 2017). This suggests that individual microbes may have independent effects on the early development of the gut associated immune cells and that *E. coli* nissle may induce more inflammation than *E. faecium*.

In a different study by Volf et al., (2016), newly hatched chicks were inoculated with caecal contents from adult hens of different ages, and using the same techniques as above, the changes in both protein and gene expression were compared between the inoculated chicken, GF chicks, and uninoculated conventionally raised chicks. A total of 36 differentially expressed (DE) genes were found in the caecum at transcript or protein level, including a reduction in an enzyme responsible for metabolism of carbohydrates, which may indicate a switch from the gut epithelium deriving its energy from glucose towards butyrate, produced by the presence of microbes (Volf et al., 2016). Microbiota induced genes related to the control of the renewal of enterocytes in the crypt and may protect against apoptosis, suggesting that the host uses similar pathways to respond to pathogens as used to regulate the microbiota (Volf et al., 2016). The

presence of bacteria increased the expression of proteins involved in binding and aggregating certain bacteria in the gut as a defense mechanism, as well as the expression of genes that have been reported to be increased following infection (Volf et al., 2016). Lastly, the introduction of microbes was associated with genes responsible for the metabolism of retinol (Volf et al., 2016). The above information suggests that the early introduction of bacteria in chicks modifies host gut physiology and education of the immune system.

1.2.2 Impact of Probiotics on Cecal Gene Expression in Poultry

Changes to the existing microbiome through the use of probiotics has also been shown to change gene expression in chickens. The direct fed microbial *Bacillus subtilis* is capable of significantly altering inflammatory response genes in chickens such as inducible nitric oxide synthase 2 which produces nitric oxide, and increased pro-inflammatory cytokine tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15) (Lee et al., 2015). The addition of *B. subtilis* also upregulated genes encoding the nutrient-digesting enzymes in the digestive tract such as pancreatic lipase (PNLIP), carboxypeptidase (CPA1), chymotrypsin-like elastase family (CELA2A), chymotrypsin (CTRC), lipase (CEL), colipase (CLPS) and amylase (AMY2A), which may indicate that *B. subtilis* is able to promote digestion (Lee et al., 2015). Recently, research has explored using *in ovo* delivery of probiotics, where an injected solution is deposited inside the air cell at the 12^{th} day of embryonic development (Slawinska et al., 2016). Transcriptional analysis of caecal tonsils of adult chickens that were injected with pre and probiotics *in ovo* compared to controls showed a significant change in the expression of genes involved in the maturation of the gut associated lymphoid tissue and the inhibition of cellular and humoral immune responses. Other probiotics given in unison with *Salmonella enterica* serovar Enteritidis infection in chicks been has shown to change the expression of genes such as growth arrest-specific 2 (GAS2) and cysteine-rich angiogenic inducer 61 (CYR61), important for increasing apoptosis, and as *Salmonella* are intracellular pathogens, this could prove to help reduce *Salmonella* infection (Higgins et al., 2011).

1.2.3 Impact of Pathogens on Cecal Gene Expression in Poultry

Transcriptional regulation of GTPase-mediated signal transduction in the intestine is an important determinant of pathogen resistance. Li et al., (2011) compared the gene expression in the ceca of two broilers lines that have shown distinct responses to *Campylobacter jejuni* infection, one where 70% of birds show colonization by *C. jejuni*, and one that has reduced colonization where only 27.5% get colonized. They found that the more susceptible line generated ten times more DE genes in response to *C. jejuni* infection than the less susceptible line. Furthermore the birds that were successfully colonized by *C. jejuni* had increased expression of GTPase-mediated signal transduction, and the authors suggested that resistance to *C. jejuni* in chickens could be supported by inhibiting small GTPase-mediated signal transduction (Li et al., 2011). As well, many genes in the TNF receptor superfamily, which has a big role in the development and regulation of the immune system, were significantly up-regulated in the non-colonized birds and could play a large role in their resistance to *C. jejuni* colonization (Li et al., 2011). The effect of parasitic pathogens colonization ongene expression of the caecum in chickens has also been studied. *Eimeria tenella*, a parasitic pathogen known for causing coccidiosis in chickens, has shown to significantly impact the gene expression of the epithelial cell organization, specifically apoptosis, cell death and differentiation, signal transduction and extracellular matrix, as well as activated immune and defense responses, particularly cytokine production and interactions, natural killer cell mediated cytotoxicity, and intestinal IgA production (Guo et al., 2013).

1.3 The impact of Re-used Chicken Litter in Poultry

1.3.1 Microbial Populations in Used Chicken Litter

Used chicken litter is made up of a combination of the bedding material and chicken fecal waste, while sometimes including feed, feathers, and water (Lee et al., 2013; Lovanh et al., 2007; Lu et al., 2003; Taherparvar et al., 2016). Used chicken litter contains a diverse microbial population, that plays an important role in shaping the gut microbiota (Cressman et al., 2010; Lovanh et al., 2007). Previous studies analyzed the bacterial composition of used litter using plating techniques, showing that the bacteria contained in the used litter ranged from 10^7 up to 10^{10} colony forming units (CFU) per gram of dry material (Fries et al., 2005; Lu et al., 2003a;

Nodar et al., 1990). However, used litter sampled from different locations in a barn revealed differences in the microbial community structure, indicating that the physical conditions such as temperature and moisture could impact the presence of microbes (Lovanh et al., 2007).

Regardless of sampling location, the majority of sequences or bacteria detected in used chicken litter belong to the low G+C group of gram positive bacteria, also known as *Firmicutes*, with the remaining being mostly the high G+C group of gram positive bacteria, known as *Actinobacteria* (Lovanh et al., 2007; Lu et al., 2003a). Used chicken litter commonly contained *Bacillales*, *Lactobacillales*, *Actinomycetes*, *Staphylococcaeae*, *Firmicutes* members except *Actinomycetes* and a very low abundance of Enterococcus (Cressman et al., 2010; Fries et al., 2005; Lovanh et al., 2007; Martin et al., 1998). Interestingly, used litter contained a very small percentage of gram negative bacteria (Fries et al., 2005; Lu et al., 2003a; Martin et al., 1998). Additionally, plant and soil-related microbes were also detected in the used chicken litter, some of which are capable of degrading wood, and cycling nitrogen and sulfur (Lu et al., 2003a). The comparison of gut microbiota between clean and used chicken litter showed that the clean litter was high in members of proteobacteria, such as *Acinetobacter*, *Pseudomonas*, and Enterobacteria, while the used chicken litter contained more intestinal type bacteria such as *Lactobacillus*, *Staphylococcus*, *Jeotgalicoccus*, *Salinicoccus*, *Atopostipe* and unclassified *Bacillaceae* (Cressman et al., 2010). However, the identified bacterial groups in the used chicken litter varied in different studies due to the differences in experimental methods, environmental conditions, and sampling locations.

1.3.2 Impact of Re-used Litter on Chicken Growth Performance, Infection, and Microbiome

Re-using chicken litter between flocks is an option in some countries, such as the United States, but in other countries like Canada and the United Kingdom, used litter is removed and poultry houses are cleaned prior to the introduction of a new flock (Lu et al., 2003a; Newell & Fearnley, 2003). The effect of re-used chicken litter on animal growth performance is highly variable. Overall, used litter has little to no effect on the body weight, feed conversion, or feed consumption in chicken (Jones & Hagler, 1983; Mccartney, 1971; Vieira & Moran, 1999; Yamak et al., 2015). However, a few studies have indicated that used litter decreased the rate of mortality compared to clean litter, and one study has indicated a higher rate of growth and better

viability from chickens raised on re-used litter than that on fresh litter (Kennard & Chamberlin, 1951; Yamak et al., 2015). The variability in litter composition and microbial communities likely explain to these variations in growth performance.

Compared to the various impacts on growth performance, raising chickens on re-used litter has shown to be a highly effective method of pathogen reduction. The reduction of *Salmonella typhimurium* and *Salmonella enteritidis* have been reported in infected birds reared on used litter compared to the clean litter (Corrier et al., 1993; Corrier et al., 1992). As well, used chicken litter increased the total SCFAs compared to that in clean litter, specifically acetic, propionic, and butyric in the caecum of birds (Corrier et al., 1992). It is likely that changes in the microbial community structure from the used chicken litter resulted in increased SCFAs, and in turn may provide additional energy for the host enterocytes. The improved resistance to pathogens was likely due to the early exposure to adult chicken intestinal bacteria through the used litter, as sterilized used litter did not effectively enhance the pathogen resistance, indicating the importance of viable bacteria (Corrier et al., 1993; Corrier et al., 1992). As chickens are coprophagic and consume litter materials, it is realistic to assume that the microbes found in the litter could be introduced to the chicken (Cressman et al., 2010; Newell & Fearnley, 2003).

However, limited research is available on how used chicken litter impacts the development of the chicken intestinal microbial community. Interestingly, one study comparing the ileal mucosal microbiota of broilers reared on fresh litter and reused litter found that the birds from the clean litter group had higher bacterial diversity in the ileal mucosa and greater evenness compared to that in the used litter treatment at 7 days of age, however no difference was observed by day 42 of age (Cressman et al., 2010). Through polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rRNA gene clone libraries, it was determined that the microbial community in the fresh litter raised chicks was influenced by the type of microbes identified in the fresh litter including *Lactobacillus*, unclassified *Lachnospiraceae* and *Enterococcus*, whereas the microbiota of the chicks reared on used litter were primarily colonized by bacteria in the *Clostridiales* order which were typical intestinal bacteria (Cressman et al., 2010). Other research using next generation sequencing technologies has indicated that used litter significantly changes the microbiome of chicks over the first two weeks, making *Firmicutes* and *Actinobacteria* the predominant phyla, while the gut microbial communities in clean litter chicks were predominated by *Proteobacteria* (Cressman, 2014). In

the study, an additional five OTUs were detected in the used litter group, however, up to 56% of the identified OTUs were found in both the clean and used litter ileal mucosal microbiota by day 35, suggesting that the birds on clean litter developed slowly, but eventually shaped the microbiome similar to those on the used litter (Cressman, 2014).

It is likely that the protective effects of used litter against pathogens are not only from the competitive exclusion, but also from the stimulation and education of the avian immune system by the normal intestinal flora (Dibner et al., 1963; Lee et al., 2013; Oviedo-rondón, 2009). Two studies which compared the effect of clean litter, used litter, and used litter from a farm with a history of gangrenous dermatitis (GD) on different immune parameters determined that the used litter stimulated both the humoral and cell-mediated immune responses (Lee et al., 2013; Lee et al., 2011). Both studies found that the re-used GD litter increased the nitric oxide levels, and serum antibody titers against Eimeria or *Clostridium perfringens* compared to the clean litter and non-GD used litter group (Lee et al., 2013; Lee et al., 2011). Both the GD used litter and non-GD used litter increased the distribution of T-cell and B-cell subpopulations in the spleen and intestine as well as the mitogen-induced spleen cell proliferation compared to the clean chicken litter (Lee et al., 2013; Lee et al., 2011). Previous research has shown that the T-cell and B-cell proliferation is increased by feeding beneficial intestinal bacteria to young chicks, indicating that the microbes present in the used litter may prime the immune system to generate the beneficial outcomes (Lee et al., 2010). Used litter provided the opportunity to introduce bacteria to the chicken microbial community, and therefore influence the development of the chicken immune system. However, it has been emphasized that the litters from different farms may exert different effects, which draws attention to the quality of the litter before re-use.

1.3.3 Risks Associated with Re-use of Chicken Litter

Litter re-use poses a benefit in reducing the disposal of the staggering amounts of poultry litter produced per year, however, one of the major concerns is that it may negatively affect the health of the bird, and in turn reduce production efficiency due to the presence of certain pathogens (Cressman et al., 2010). In the United Kingdom and other European countries poultry houses are cleaned and used litter is removed between flocks (Newell & Fearnley, 2003). Introduction of *Campylobacter jejuni* is a common risk factor identified from the insufficient

cleaning and disinfection of poultry houses between flocks (Newell & Fearnley, 2003).. However, most studies using PCR and plating techniques of used chicken litter did not detect *Salmonella*, *Campylobacter*, pathogenic *E. coli* or other common pathogens in the litter (Jeffrey et al., 1998; Lu et al., 2003a; Martin et al., 1998).

An alternative health risk in re-used poultry litter is the development of foot-pad dermatitis in chickens. One major purpose of chicken litter is to absorb moisture, however at a certain point the litter reaches maximum moisture content and begins to cause caking, resulting in trapped moisture, with slippery and sticky surfaces (Yamak et al., 2015). These conditions propose a problem for the chicken as they can lead to both defects on the feet and body of the chicken (Shepherd & Fairchild, 2010; Yamak et al., 2015). Chicken litter re-use has been associated with increased incidence of chicken foot pad dermatitis (FPD) with necrotic lesions and increased inflammation, creating concern for both animal welfare and food safety (Shepherd & Fairchild, 2010; Yamak et al., 2015). However, other researches have indicated that used litter had no significant effect on FPD (Ruiz et al., 2008; Cressman, 2014), indicating that as litter is reused, it is important to prevent over-caking or moisture build-up through proper ventilation to prevent the likelihood of causing FPD (Yamak et al., 2015).

1.4 Iodine

1.4.1 Iodine Antimicrobial and Sterilization Properties

[2]Iodine has been used for many years as antimicrobial agents for wounds and external infections because of their broad-spectrum activity against bacteria, and low development of resistance (Martínez-Abad et al., 2012; Murdoch and Lagan, 2013). Iodine is an active antimicrobial ingredient used in both human and animal wound care products (Burks, 1998; Murdoch and Lagan, 2013), and is commonly been used as an udder wash (Tremblay et al., 2014). Although iodine's antimicrobial activity is not well understood, research indicates that it works on the bacterial cell walls by reacting with unsaturated fatty acids in the lipid bilayer to

[2]Paragraph modified directly from: Willing, B. P., Pepin, D. M., Marcolla, C. S., Forgie, A. J., Diether, N. E., & Bourrie, B. C. (2018). Bacterial resistance to antibiotic alternatives: a wolf in sheep's clothing?. Animal Frontiers, 8(2), 39-47.

cause leaks, as well as inactivating nuclear materials through coagulation (Murdoch & Lagan, 2013). Prior to 2013, no bacterial resistance to iodine had been identified, and studies of repeated iodine use over time did not indicate any increase in resistant bacteria (Murdoch & Lagan, 2013). Select mastitis associated bacteria have been shown to evade low concentration iodine killing *in vitro* through the generation of biofilms (Tremblay et al., 2014). Using sub-lethal concentrations of nonoxinol-9 iodine complex on *Staphylococcus aureus* strains specific to mastitis resulted in the development of resistance, although the mechanisms of tolerance are unknown (Behiry et al., 2012). Although other research has indicated cross-resistance of antibiotics with other biocides (Behiry et al., 2012), currently there is no known cross-resistance with iodine and antibiotics (Behiry et al., 2012; Murdoch & Lagan, 2013).

Iodine is among the oldest substances used for disinfection, as it has antibacterial, antifungal, and antiviral properties (Gottardi, 2001). At a concentration as low as 1 mg/L (1 ppm), iodine in water can kill bacteria in minutes (Backer & Hollowell, 2000). Iodine has been used for water sterilization since the 1940's, and like chlorine is a halogen and strong oxidant, resulting in similar biocidal activity (Backer & Hollowell, 2000). However, iodine has many advantages over chlorine for water disinfection; it is less reactive with organic contaminants in water, leaving higher concentrations of active free iodine (Shannon et al., 2008; NRC, 1980). When chlorine reacts with organic nitrogen sources in the water, it can generate toxic disinfection byproducts (DBPs), chlorine is also not effective against all water-borne pathogens (Shannon et al., 2008). While other methods of water sanitation, such as UV and ozone are very effective, they have limitations; UV is ineffective against viruses, and ozone can generate toxic DBPs in water containing bromide ions (Shannon et al., 2008).

1.4.2 Iodine use in Animal Production

Bromide ions have been found in ground water and public water systems often resulting as a by-product of coal-fired power plants waste run-off, and have been shown to cause negative health outcomes in poultry production (du Toit & Casey, 2010; Good & Vanbriesen, 2017; Hitchon et al., 1977). Bromine in water can reduce both feed and water intake in broilers, and increase thyroid gland hormones and goiters in rats (du Toit & Casey, 2010; Velický et al., 2004). However, adding iodine to their water at 0.7 mg I/L in the drinking water, resulting in the

intake of 1.1 mg I/L (1.1 ppm) per bird per day, ameliorated these negative effects (du Toit & Casey, 2010, 2012). Iodine treated water's effect on poultry performance was evaluated in the late 80's, showing that 2 ppm iodine in combination with high stocking densities could improve the growth rate of broilers at 6 and 8 weeks of age, proposing at this time that the mechanism of action could be alteration of the gut microbiota resulting in increased nutrient availability for the chicken (Stanley et al., 1989). Other research indicated that 10 ppm iodine in water fed to broilers resulted in increased feeding, drinking, body weight and feed efficiency, as well as positive behavioral changes such as increased resting, sleeping and body preening (Emeash et al., 1994).

Iodine is an essential trace element for both animals and humans, as it is required for the production of thyroid hormones and required for normal thyroid function (Röttger et al., 2011). Iodine is a component of thyroxin synthesis, which plays a role in regulating metabolism and influences growth and performance of birds (Opalinski et al., 2012). Inadequate iodine consumption causes hypothyroidism and other iodine deficiency disorders (De Benoist et al., 2004). To counteract hypothyroidism, salt is iodized to increase iodine consumption. This has decreased goiter and hypothyroidism worldwide, but has not eliminated the problem (Backer & Hollowell, 2000; Röttger et al., 2011). Iodine has also been supplemented to hens in order to produce eggs as a source of dietary iodine, as the research has shown that the iodine doesn't accumulate in the meat (Röttger et al., 2012). In poultry diets, iodine is often provided in the form of potassium iodide (KI) and Calcium iodate ($Ca(IO_3)_2$), and both of these are rapidly reduced to iodide in the stomach, having no antimicrobial effects (Backer & Hollowell, 2000; Medicine, 2006; Block, 1991). Studies of excess iodine in laying hen diets have found varied effects on production parameters. One study found that iodide and iodate at 0.25, 0.5, 2.5 and 5 mg I/kg of feed had no effect on feed intake or hen performance (Röttger et al., 2012, 2011). Another trial that fed layers yeast enriched with $Ca(IO_3)2{\cdot}H_2O$ at 1 mg/kg and 2 mg/kg also found no impact of iodine on body weight gain (Opalinski et al., 2012). However, a different study providing layers KI or KIO_3 as an iodine source at 1, 3, or 5 mg of supplemented I/kg of feed, did find that increased iodine resulted in increased egg weight, improved feed efficiency in egg production, reduced damaged eggs, and overall improved performance (Słupczyńska et al., 2014).

1.4.3 Iodine Absorption and Toxicity

Most dietary iodine is rapidly converted to iodide in the stomach and absorbed into the blood stream, however some iodine-containing compounds such as thyroid hormones are directly absorbed without conversion (Backer & Hollowell, 2000; Medicine, 2006). Once in circulation, iodine is transported into the thyroid gland via a sodium/iodide transporter, using a concentration gradient to ensure the proper amounts of iodine are available for hormone synthesis (Medicine, 2006). Excess iodine is excreted primarily through urine, but also in feces (Medicine, 2006). The upper limit for dietary intake of iodine for adults is 1.14 mg/day, and iodine intake concentration from foods in a diet is unlikely to go above the upper limit (Medicine, 2006). As iodine concentrations in foods increases, understanding the risk of over consumption is important, and research has shown that excessive iodine may disrupt normal thyroid function (Backer & Hollowell, 2000). Excessive iodine intake can cause hypothyroidism, as well as hyperthyroidism, and autoimmune thyroid disease in humans (Andersen et al., 2009; Burek & Talor, 2009; Delange et al., 1999). One study giving KI at 0, 2, or 20 mg per deciliter to chickens that are genetically susceptible to thyroiditis determined that excessive consumption of iodine causes increased incidence of autoimmune thyroiditis, indicating that elevated levels of dietary iodine may increase or induce disease in genetically susceptible individuals (Bagchi et al., 1985). Limited information is available regarding thyroiditis development in healthy chickens, as well as iodine requirements and absorption.

1.5 Research Objectives

Re-using chicken litter has proven to be beneficial in poultry production, improving performance and reducing pathogen colonization, likely through modifying the intestinal microbial community. However, understanding what these bacterial modifications do to the intestinal development and how they impact the expression of genes in the chicken has yet to be determined. As well, the addition of iodine has proven to promote health benefits and provide better production parameters in chickens, but the underlying mechanisms involved are yet to be determined. In this thesis, we aimed to understand the underlying mechanisms of iodinated water and re-using poultry litter on poultry performance through their effect on the caecal microbiota

and gene regulation in young chickens. We hypothesized that iodinated water and re-used litter improve animal performance by promoting a healthy gut microbiome, increase infection resistance, are modify the host response through immune development.

The main objectives of this study were as follows:

1. To understand how introducing used litter and feeding iodinated water changes the caecal microbiota, morphology, and gene expression in chicks.
2. To determine if used litter and iodinated water could reduce pathogen colonization in a *Salmonella enterica* Sarovar Enteritidis challenge model in chicks.
3. To examine the effects of iodinated water on growth and feed intake in adult broilers.

- Chapter 2: Effect of Iodinated Water and Used Chicken Litter on Poultry Intestinal Microbiota, Gene Expression, and Growth Performance

2.0 Introduction

Reducing antibiotic use in animal production is critical with the increased prevalence of multi-drug resistant pathogens that have evolved as a result of antibiotic use in both human medicine and animal agriculture (Koch et al., 2017). To reduce the need for antibiotics, we must find ways to improve animal health through husbandry practices. This can be achieved through the prevention of infection, as well as the development of the host microbiome and immune system so as to prevent or better tolerate infection.

It is well recognized that the establishment and maintenance of host-specific gastrointestinal microbiome plays important roles in host digestion, nutrient absorption, immune modulation, and pathogen defense in humans (Sommer & Bäckhed, 2013). However, in poultry our understanding of this is limited. Administering an oral gavage of adult chicken intestinal contents to young chicks has been shown to substantially reduce the impacts of common chicken enteropathogens such as Salmonella, as well as increase short-chain fatty acid production, a preferred energy source for enterocytes (Corrier et al. 1991; Impey & Mead 1989; Lloyd et al.,1977; Snoeyenbos et al., 1978; Stavric et al., 1985). Promoting the development of the microbiota in young chicks through direct fed microbials, or probiotics, can improve gut morphology and increase absorptive surface area of the intestine (Markovic et al., 2009; Shakouri et al., 2009).

The re-use of poultry chicken litter (bedding material and fecal waste) between flocks has been common practice in the United States of America, but in other countries such as the United Kingdom and other European countries, barns must be cleaned prior to the placement of a new flock (Newell & Fearnley, 2003). Young chicks moving from hatchery to a clean barn have a very naïve, mostly vacant microbiota, leaving an open space for others to flourish, and the early establishment of potentially pathogenic bacteria such as the *Enterobacteriaceae* family (Ballou

et al., 2016; Nurmi & Rantala, 1973). Re-using poultry litter is one strategy that has been shown to reduce *Salmonella* colonization of species such as *S. typhimurium* and *S. enteritidis* in young chicks (Corrier et al. 1992; Corrier et al. 1993). Currently there is limited research on the underlying mechanism of the benefits of the re-use of chicken litter. One study using PCR-DGGE and 16S rRNA gene clone libraries found that the clean litter birds had higher bacterial diversity in the ileal mucosa and greater evenness than birds raised on used litter. However, there is greater evidence indicating that reducing infection (Corrier et al., 1993; Corrier et al., 1992), increased short chain fatty acids (SCFA's)(Corrier et al., 1992), improved growth performance (Kennard & Chamberlin, 1951), and reduced mortality (Yamak et al., 2015) suggest otherwise.

Preventing infection from spreading throughout a flock is imperative and an important means to reduce antibiotic use. One method of pathogen spread in chicken houses is through contaminated water lines (Pearson et al., 1996). As current methods of water sterilization are not effective against all pathogens and creates disinfection by-products that can have negative effects on production (du Toit & Casey, 2012; Shannon et al., 2008), there is a need for alternative methods. Iodine is among the oldest substances used for disinfection, and it is effective against bacteria, fungi, and viruses (Gottardi, 1999). Iodine has also been used as a feed additive in poultry showing improved feed efficiency in laying hens (Słupczyńska et al., 2014), growth promotion and positive behavioural changes such as increased rest and preening (Emeash et al., 1994). However, research on the subject is limited, and the underlying mechanism has yet to be described. Given the antimicrobial properties of iodine, it is plausible the production benefits are through impacts on the microbiota.

To better understand the mechanisms behind the positive impact of re-used litter as well as iodinated water we designed an experiment to test both of these treatments in young chicks up to 12 days of age. In this study, we raised chicks on clean or re-used chicken litter, as well as water containing 1 ppm or 10 ppm iodine and analyzed the changes in chicken growth, caecal morphology, microbiota, and caecal transcriptome. We hypothesized that both used chicken litter and iodinated water influence the microbiota, reducing pathogenic bacteria and modifying the caecal gene regulation in chickens.

2.1 Materials and Methods

2.1.1 Ethics Statement

The animal use in this study was approved by the University of Alberta Animal Care and Use Committee (AUP00001626) and performed according to the guidelines of the Canadian Council on Animal Care. Chickens used in this study were managed by approved protocols at the Science Animal Support Services.

2.1.2 Animals and Experimental Design

Forty-eight 1-day old Ross 708 broiler chicks were randomly separated into 24 sterile microisolators with 2 chicks per isolator. 4 isolators (n = 8) were randomly assigned to 6 treatments for 12 days in a 2X3 factorial design with 2 litter treatments (clean litter, or clean litter supplemented with used adult broiler litter), and 3 levels of water (double distilled water (H_2O), 1 ppm iodinated water, or 10 ppm iodinated water). Food and water were administered *ad libitum*. Each chick was weighed on days 1, 5, 8 and 12. Water was supplied in 500 mL bell drinkers and chicks were terminated and samples collected after 12 days on treatment.

2.1.3 Water Treatment and Measurements

Iodinated water was made and supplied by BioLargo Water Inc. using their iSAN precision iodine dosing system, using reverse osmosis water. Iodinated water was made to 25 ppm and diluted to 10 ppm and 1 ppm concentration using Milli-Q sterile double distilled water (H_2O). Each cage received 500 mL of water, either 1 ppm, 10 ppm, or H_2O control. The remaining water in the bell drinkers was measured into a 500 mL graduated cylinder on days 3, 5, and 6 through 12, and replaced with freshly made water.

2.1.4 Litter Treatment

On the first day, all 24 cages received sterile pine shavings to cover the bottom of the isolators, and half of the cages received an additional 5 grams of used litter, which is a mixture of pine shavings and poultry fecal waste from the floor of adult chicken pens at the Poultry

Research Centre at the University of Alberta. Cages were changed when the litter was determined to wet and caked, and at each cage change, 5 grams of bedding material was transferred from the used isolator to the clean isolators in both litter treatments.

2.1.5 Animal Euthanasia and Sample Collection

After 12 days on treatment, all 48 chickens were euthanized by cervical dislocation, which severs the spinal cord and separated the vertebrae so that the bird does not recover consciousness. The body cavity of the chicken was sprayed with 70% ethanol and opened with sterile surgical scissors and forceps. The gastrointestinal tract was removed and both caeca were carefully dissected. Five mm of the tip of one caecum was collected and fixed in formalin for ileal histological analysis (10% formalin, Fisher Scientific). Approximately 100 mg of caecal digesta was collected aseptically into sterile 1.5 mL Eppendorf tubes and snap frozen in liquid nitrogen and stored at -80^{o}C for microbial and SCFA analysis. Contents were removed and one entire caecum was collected into tin foil and snap frozen in liquid nitrogen until storage at -80^{o}C for gene expression analysis.

2.1.6 Microbial Analysis

2.1.6.1 16S rRNA gene amplification and sequencing

Total DNA was extracted from caecal contents using the QIAamp® FAST DNA stool mini kit (Qiagen Inc., Valencia, CA), according to manufacturer's instructions with the addition of 2.0 mm diameter garnet beads (BioSpec Products, Bartlesville, OK) and a 60 second bead-beating step at a speed of 6.0 meters per second (FastPrep instrument, MP Biomedicals, Solon, OH, USA). The V3-V4 region of the 16S rRNA gene was amplified according to the Illumina protocol (16S Metagenome sequencing library preparation) with the following forward and reverse primers: Forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and Reverse (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-

3') (Klindworth et al., 2013). Extracted samples were sequenced on a paired-end sequencing run with 2x300 cycles on the Illumina Miseq Platform (Illumina Inc. San Diego, CA).

2.1.6.2 Microbial composition analysis

The sequencing reads were assessed for quality using FastQC developed by Babraham Institute bioinformatics (Andrews, 2010), after which primers were removed from the high-quality paired end reads and merged using PANDAseq (Masella et al., 2012). The paired reads were dereplicated and singletons were removed using VSEARCH 2.7.1 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Chimeras were removed using USEARCH 7.1 with the database "gold.fa" (Navas-Molina et al., 2013). Merged reads were mapped to this reference database using VSEARCH 2.7.1 usearch_global command with 97% identity. A tab delimited OTU table was generated using the USEARCH 7.1 python uc2otutab.py. Subsequent analysis was conducted using the QIIME pipeline (MacQIIME 1.9.1), assigning taxonomy with the QIIME default RDP classifier (Wang, Garrity, Tiedje, & Cole, 2007). Beta diversity analysis was conducted using the QIIME core_diversity_analysis.py, and normalized to a sampling depth set by the sample with the lowest number of reads (>950). Shannon, Chao1, and inverse Simpson diversity indices were calculated with 'alpha_diverstiy.py' in QIIME. Both alpha and betadiversity were plotted with R using ggplot2. Abundance heatmap was visualized in R with the 'pheatmap' function.

2.1.7 Histology

Cecal tissues were placed in 10% buffered formalin (Fisher) for 24 hours, and then placed into 70% ethanol. Tissues were embedded in paraffin wax, and sectioned into 5 µm slices and fixed to glass slides. The sections were stained with Haematoxylin and Eosin and images and measurements were taken at 200X using an EVOS FL Auto Imaging System (Thermo Scientific, Nepean, ON). Tissues from 4 chickens per treatment from the following 4 treatments were analyzed; 1 ppm used, 1 ppm clean, H_2O used, and H_2O clean. Three sections of each caeca were analyzed with 3 images per section, equaling 9 images per chicken. Measurements included

villus height, width, crypt depth, as well as counting the number of villi in the length of 200 micrometers on each image.

2.1.8 Short chain fatty acid measurement

Short chain fatty acids were extracted from approximately 40 mg of caecal contents. Samples were mixed with 0.8 mL of 25% phosphoric acid and homogenized by vortexing. Samples were centrifuged at 15,000 × g for 10 min at 4°C and the supernatant was removed, and then centrifuged at 15,000 × g for 10 min at 4°C and the supernatant was removed a second time. The resulting 0.8 mL of supernatant was filtered through a 0.45 μm filter (Fisher), mixed with 0.2 mL of internal standard solution containing 24.5 mmol/L isocaproic acid in a GC vial (12 × 32mm, Thermo Scientific). Short chain fatty acids were quantified using gas chromatography as previously described (J. Li et al., 2017).

2.1.9 RNA Sequencing

2.1.9.1 RNA Isolation, Gene amplification, and Next generation sequencing

RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific, Nepean, ON) using manufacturer's instructions from the caecum of 16 samples, 4 from each of the following treatment groups; 1 ppm used, 1 ppm clean, H_2O used, and H_2O clean. RNA quality was verified using the Agilent 2200 TapeStation System (Agilent, Santa Clara, CA, US) using manufacturer's instructions to determine the RNA Integrity Number (RIN) to be above 7 for all samples. RNA was stored at -80ºC, prior to being shipped on dry ice to Génome Québec (Génome Québec Innovation Centre, Montréal, QB, CA). Total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies). Libraries were generated from 250 ng of total RNA as follows: mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). cDNA synthesis was achieved with the NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs). The remaining steps of library preparation were done using and the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Adapters

and PCR primers were purchased from New England BioLabs. Libraries were quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument. Samples were used for paired end next generation sequencing (NGS) on a HiSeq4000 PE100. The 16 samples were run on 2 lanes, 8 samples per lane, generating an average of 38,824,357 reads per sample.

2.1.9.3 Gene expression analysis

The raw sequence data was checked for quality using FastQC v0.11.5 (Andrews, 2010). The reads were trimmed using Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2014). The trimmed quality reads were next mapped to the chicken genome file (Gallus_gallus.Gallus_gallus-5.0.dna.toplevel.fa) using Spliced Transcripts Alignment to a Reference software (STAR v2.5.3a, Dobin et al. 2013). Read summarization was conducted using the featureCounts program (v1.5.3) to count the number of reads assigned to each gene (Liao, Smyth, & Shi, 2014). Bioconductor (version 3.5) package refGenome (v1.7.3) was used to parse the chicken Gene Transfer File (GTF, Gallus_gallus.Gallus_gallus-5.0.90.gtf) to create a gene annotation file. The generated Ensembl gene ID's were mapped to Entrez gene ID's using the package org.Gg.eg.db (v3.4.1).

Analysis was performed using R statistical programming language version 3.4.2 to determine genes with differential expression (DE) by edgeR (version 3.18.1). Power estimation and sample size was estimated using RNASeqPower (version 1.16.0). The gene expression data was normalized using the trimmed mean of M-values (TMM) in EdgeR, and a design matrix was constructed to compare the following 4 contrasts: Used.1ppm vs Used.H_2O, Clean.1ppm vs Clean.H_2O, Used.1ppm vs Clean.1ppm, and Used.H_2O vs Clean.H_2O. Applying a logFC 1 (log2(2)) and a false discovery rate (FDR) of 0.01 cut-off criteria, DE genes were identified between each comparison. Differentially expressed genes in the 4 comparisons were entered into Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Recourses to be matched to the Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to investigate their related functional categories (Huang, Sherman, & Lempicki, 2009b, 2009a). Terms and pathways with *P*-values greater than

0.05 were kept and used to analyze the potential functions of the DE genes. These genes were input into the Protein ANnotation THrough Evolutionary Relationship (PANTHER) classification system for GO term analysis.

2.1.10 Statistical Analysis

The data is expressed as mean +/- SEM. The data generated from the water, weight, histology, SCFAs, and alpha diversity were analyzed for outliers using the Robust regression and Outlier removal (ROUT) method in Prism (Motulsky & Brown, 2006), and tested for normality using the Shapiro-Wilk W statistical measures (SAS University Edition). The water, weight, and alpha diversity were analyzed as a 2 by 3 factorial and the histology and SCFA data were analyzed as a 2 by 2 factorial to determine if there was any combined effect of treatment using a mixed model ANOVA (SAS university edition, Bonferroni adjusted P value for multiple comparisons). Non-normal data was transformed using a log transformation prior to analysis in SAS. If no combined effects were observed between water and litter the main effects of treatment on weight gain and water consumption was further analyzed as a repeated measures ANOVA (SAS university edition, Bonferroni adjusted P value). Total weight gain and average daily gain between litter treatments was analyzed by a student's t-test in prism (Motulsky & Brown, 2006). Differences in beta diversity was tested using Adonis. The phylum, family, and genus level percent relative abundances were transformed by $Log_{10}(X+1)$, where X is the percent relative abundance. Next, the data was analyzed with a mixed model ANOVA to determine combined effect of treatment (SAS university edition, Satterthwaite, Bonferroni adjusted P value for multiple comparisons).

2.2 Results

2.2.1 Water consumption *1 ppm iodinated water was consumed more that 10 ppm iodinated water*

There was little effect of litter treatment or combined effect of water and litter on water consumption (Table 2.1), but type of water had a significant effect on days 3, 5-8, and 11 (Figure

2.1A). When analyzed through a repeated measures ANOVA, there was significantly lower water disappearance for 10 ppm than 1 ppm water on day 5 (Figure 2.1A, $P = 0.007$). The 1 ppm iodinated water had significantly more water disappearance than the H_2O group on days 7 and 8 (Figure 2.1A $P = 0.03$). Both 1 ppm and 10 ppm had significantly more water remaining in the bell drinkers on day 11 (Figure 2.1A, $P < 0.05$). Over the 12 days, 1 ppm had more water disappearance that 10 ppm (ANOVA $P = 0.03$), but neither were significantly different than the H_2O control (Figure 2.1B)

2.2.2 Weight gain *Used litter significantly decreased weight gain up to day 8*

There was no effect of water treatment or combined effect of water and litter treatment on weight gain, so the main effects of litter treatment on weight gain are shown. Used litter significantly reduced the weight gain of the chicks over the first 8 days but was no longer significant from days 8 to day 12 (Figure 2.2). Overall, used litter significantly decreased total weight gain and average daily gain of the chicks over the 12 days of the experiment (Figure 2.3 A & B).

2.2.3 Microbial composition *Used litter had a significant impact on the microbial composition in the caeca*

There was a significant effect of treatment on the caecal microbiota of the chicks (Adonis $P = 0.001$), indicated in both the relative abundance (weighted UniFrac distances) and presence or absence of OTUs (unweighted UniFrac distances) (Figure 2.4). When comparing each treatment, used litter had the main effect on the microbiome in both the weighted and unweighted UniFrac, although 1 ppm iodinated water affected the microbiota only in the presence of clean litter (Table 2.2). Next the groups were separated to determine the individual effects of water and litter treatments on the microbiome. First, we compared the effect of iodinated water on the caecal microbiome in the presence or absence of used chicken litter and found that iodinated water had no effect on the microbiome in the presence of used litter but did have an impact in birds on the clean litter treatment, indicated in both the weighted (Adonis, $P = 0.003$) and unweighted (Adonis, $P = 0.03$) Unifrac (Figure 2.5). Next, we compared the effect of used and clean litter on

the caecal microbiome in the 3 water treatment groups, and found that used litter had a significant effect on the caecal microbiome regardless of water treatment, as indicated by both the weighted and unweighted unifrac analyses (Adonis, $P < 0.01$, Figure 2.6). Alpha diversity analysis indicated that used chicken litter had a more significant impact on the microbiome than iodinated water. In almost all treatments, regardless of water type, used litter significantly increased the within sample diversity indicated by the species richness from the Chao1 index, as well as the richness and evenness of the species present as shown by the Shannon index (Table 2.3, Figure 2.7). There was an effect of water, and combined effect of treatment on the inverse Simpson index, however with multiple comparisons analysis this effect was not significant between treatments (Figure 2.7C).

There was almost no combined effect of water and litter treatment on the relative abundance of the bacterial families, so the main effect of litter is described (Table 2.4). Figure 2.8 shows that used litter had a significant effect on 4 bacterial phyla, increasing *Actinobacteria* ($P = 0.04$), *Firmicutes* ($P = 0.003$), and *Tenericutes* ($P = 0.004$), while decreasing the relative abundance of *Proteobacteria* ($P < 0.0001$). When comparing the relative percent abundance at the family level, the heatmap in figure 2.9 shows that used litter significantly increased *Coriobacteriaceae* ($P = 0.007$), *Christensenellaceae* ($P = 0.004$), *Ruminococcaceae* ($P = 0.0002$), *Mogibacteriaceae* ($P = 0.008$), *Erysipelotrichaceae* ($P = 0.02$), and unclassified *Clostridiales* ($P < 0.0001$) and *RF39* orders ($P = 0.01$), and significantly decreased *Enterococcaceae* ($P = 0.007$), *Enterobacteriaceae* ($P = <0.0001$), and *Leuconostocaceae* ($P = 0.03$) (Table 2.4, Figure 2.9). As well, both *Campylobacteraceae* ($P = 0.06$) and unclassified *Streptophyta* ($P = 0.09$) showed a trend to be more abundant in the clean litter treatment (Figure 2.9). There was a significant interaction between the water and litter treatment on *Bifidobacteriaceae* ($P = 0.003$), found in the samples at a very low average percent abundance of 0.006% in clean litter and 0.00006% in used litter (Figure 2.10a). When looking at the percent relative abundance at the genus level, Figure 2.11 shows that used litter significantly increased *Ruminococcus* ($P < 0.0001$), *Faecalibacterium* ($P < 0.0001$), *Erysipelotrichaceae* Cc 115 ($P = 0.006$), *Clostridium* ($P = 0.01$), *Lachnospira* ($P = 0.004$), *Eggerthella* ($P = 0.008$), *Coprococcus* ($P = 0.0001$), *Oscillospira* ($P = 0.004$), and *Anaerotruncus* ($P = 0.006$). Used litter decreased *Klebsiella* ($P = 0.04$), *Proteus* ($P < 0.0001$), and *Enterococcus* ($P = 0.007$). There was a significant effect of litter, water and interaction between treatments on *Blautia* (litter: $P = 0.007$,

water: $P = 0.003$, interaction: $P = 0.02$), *Bifidobacterium* (litter: $P = 0.003$, water: $P = 0.007$, interaction: $P = 0.006$), and *Holdemania* (litter: $P = 0.02$, water: $P = 0.02$, interaction: $P = 0.005$), (Figure 2.12). Out of the 304 OTUs identified, a total of 149 unique OTUs were introduced by the used litter treatment, while only 7 unique OTUs were identified in the clean litter treatment (Figure 2.13). Most of these OTUs belonged to the *Firmicutes* phylum, as well as *Actinobacteria*, *Bacteroidetes*, and *Tenericutes* (Table 2.5).

Iodinated water treatment on clean litter significantly changed the relative percent abundance of 4 bacterial families, *Enterobacteriaceae*, *Streptococcaceae*, *Bifidobacteriaceae*, and *Bacteroidaceae* (Figure 2.14). Both water and 10 ppm iodinated water contained higher relative abundance of *Enterobacteriaceae* and *Bifidobacteriaceae*, while both iodinated water groups reduced *Streptococcaceae* and 1 ppm increased *Bacteroidaceae* (Figure 2.15). Iodinated water did not have any impact on the alpha diversity on clean litter (Figure 2.7), however introduced 5 unique OTUs on clean litter, 3 were unassigned to taxonomy, and 2 OTUs were assigned to *Bacteroides fragilis* (Figure 2.13).

2.2.4 Histology

Used chicken litter had a significant impact on the caecal morphology in the chicken gut. The addition of used litter resulted in increased villus height, increased crypt depth, increased number of villi in 200 micrometer section, and decreased the villus width, regardless of water treatment (Figure 2.16). Although iodinated water on its own had no effect on the caecal morphology, there was a combined effect of litter*water on the villus width, indicating that iodinated water on used litter increased villus height compared to H_2O on used litter (Figure 2.16B). The effects of litter on gut morphology were visible and representative sections are shown in Figure 2.17.

2.2.5 Short chain fatty acids

Used chicken litter significantly increased the relative concentration of butyric and valeric acid compared to clean litter ($P < 0.0001$), while 1 ppm iodinated water on clean litter significantly increased the amount of propionic acid compared to 1 ppm used litter, used litter on

1 ppm iodinated water and used litter on H_2O ($P < 0.05$, Figure 2.18). Neither treatment had an effect on acetic acid, or the total short chain fatty acids.

2.2.6 RNA Sequencing

2.2.6.1 Differential gene expression profiling

Hierarchical clustering of the samples, using Euclidean distance (Method = ward.D), revealed that the samples clustered more so by litter type than water treatment (Figure 2.19). A multi-dimensional scaling (MDS) plot based on the top 500 genes with the greatest log fold change (Log_2FC) differences between groups also indicated that litter had a stronger effect on overall separation in the treatments based on gene expression (Figure 2.20). Read counts were found for a total of 24,881 genes, of which 14,748 genes were expressed at a level that passed the following criteria: keeping genes with at least 9 reads, corresponding to counts per million (CMP) of 0.4, in at least 8 of the 16 samples. The data was analyzed by CPM, which is a standardized measure that accounts for library size and a gene is considered expressed if it has a read count between 5-10 reads. Using the sample with the smallest library size the read counts and corresponding CPM values were used to determine the CPM cut-off for further filtering, choosing a read count of 9 reads and above corresponding to CPM above 0.43, thus a CPM 0.4 as a cut-off threshold to filter out the extremely low expressed genes. The qualifying genes are visualized with volcano plots in Figure 2.21.

2.2.6.2 Differential gene functional annotation

2.2.6.2.1 Used.H_2O vs Clean.H_2O

A total of 250 differentially expressed genes were identified between the used litter and clean litter treatment on H_2O; 162 upregulated and 88 downregulated (Appendix 1 & 2). From those genes, 74 GO terms were identified from the upregulated genes, 60 biological processes (BP), 8 molecular functions (MF), and 6 cellular components (CC), and 17 GO terms were identified from the downregulated genes (10 BP, 3 MF, and 4 CC) (Appendix 3 & 4). Further analysis using PANTHER indicated that for the upregulated genes, the biological processes were

associated with 13 higher categories, with the most relevant being cellular process, metabolic process, and biological regulation accounting for 61% of the up-regulated DE genes (Figure 2.22). The down-regulated genes were associated with 10 higher categories of biological processes, the top being cellular process, metabolic process and cellular component organization or biogenesis accounting for 67% of the down regulated genes (Figure 2.22). The most relevant molecular functions in both up and down regulated genes were in 6 higher categories, with binding and catalytic activity making up 84% of the up regulated genes and 73% of the down regulated genes (Figure 2.22). Lastly, the down regulated genes made up 6 higher cellular component ontologies for up-regulated genes, and 7 for down-regulated genes, with cell part, organelle, and membrane making up 77% of up-regulated and 81% of the down regulated genes (Figure 2.22). Further analysis of the DEGs with DAVID generated 40 annotation clusters for the up-regulated genes and 35 for the down regulated genes, with significance indicated by the enrichment scores, in which the top 5 clusters are shown (Appendix 5 & 6). Lastly, 4 significant KEGG pathways were identified in the upregulated genes; steroid biosynthesis (gga00100), influenza A (gga05164), biosynthesis of antibiotics (gga01130), and ErbB signaling pathway (gga04012). No KEGG pathways were found for the down-regulated genes.

2.2.6.2.2 Clean.1ppm vs Clean.H_2O

The iodinated water treatment on clean litter resulted in 472 DE genes, 361 up regulated and 111 down regulated (Appendix 7 & 8). The up regulated genes annotated to 115 GO terms (62 BP, 30 MF, and 23 CC) and the down regulated genes were annotated to 98 GO terms (73 BP, 4 MF, and 21 CC) (Appendix 9 & 10). Using PANTHER analysis 13 higher level BP GO term categories were identified, the most relevant for upregulated genes were cellular process, metabolic process, and cellular component organization or biogenesis, making up 66% of the up-regulated gene hits (Figure 2.23). For down-regulated genes, 12 higher level GO categories were identified, with cellular process, metabolic process, and multicellular organismal process making up 53% (Figure 2.23). For MF, 8 categories were found for up regulated and 7 for down, both with binding and catalytic activity being the most relevant, making up 75% and 72% of gene hits, respectively (Figure 2.23). Lastly, upregulated genes corresponded to 6 CC categories, with cell part and organelle making up 69%, down regulated to 8 CC categories with cell junction and

organelle making up 54% of the gene hits (Figure 2.23). Further analysis of the DEGs was conducted using DAVID, identifying 108 and 14 annotation clusters for the up and down regulated genes respectively, where the top 5 based on enrichment score are shown (Appendix 11 & 12). Lastly, DAVID was used to determine if the DEGs correlated to any KEGG pathways. For the up-regulated genes, 6 significant ($P < 0.05$) pathways were identified; metabolic pathways (gga01100), phosphatidylinositol signaling system (gga04070), mucin type O-Glycan biosynthesis (gga00512), arachidonic acid metabolism (gga00590), drug metabolism - cytochrome P450 (gga00982), and pantothenate and CoA biosynthesis (gga00770). No KEGG pathways were found for the down-regulated genes.

2.2.6.2.3 Used.1ppm vs Clean.1ppm

The used chicken litter with 1 ppm iodinated water resulted in 129 DE genes, 44 up regulated and 85 down regulated (Appendix 13 & 14). The up regulated genes corresponded to 18 GO terms (5 BP and 13 MF), while the down regulated genes corresponded to 22 GO terms (15 BP, 5 MF, and 2 CC) (Appendix 15 & 16). PANTHER analysis indicated that the up-regulated genes were assigned to 11 higher categories of GO terms, the most relevant BP GO terms were cellular process, metabolic process, multicellular organismal process, and response to stimulus, making up 64% of gene hits (Figure 2.24). For down regulated genes, cellular process and metabolic process made up 63% of the down regulated gene hits (Figure 2.24). The most relevant MFs of the 5 identified for up regulated and 6 for down regulated, were binding and catalytic activity, making up 81% of genes for both up regulated and down regulated genes. Lastly, for CC's, of the 6 higher categories identified, 71% of the up-regulated genes were most associated with extracellular region, cell part, and membrane, while 88% of the down regulated were most associated with cell part, organelle, and membrane (Figure 2.24). The DEGs were analyzed using DAVID, resulting in 7 annotation clusters for the up-regulated genes, and 21 for the down regulated genes. The top 5 clusters based on enrichment score are shown in appendix 17 & 18. Lastly, 5 KEGG pathways were identified using the down regulated genes including retinol metabolism (gga00830), drug metabolism - cytochrome P450 (gga00982), metabolism of xenobiotics by cytochrome P450 (gga00980), metabolic pathways (gga01100), and arachidonic acid metabolism (gga00590). No KEGG pathways were found for the up-regulated genes.

2.2.6.2.4 Used.1ppm vs Used.H_2O

Iodinated water in chickens reared on used litter resulted in 7 DE genes, 5 up regulated and 2 down regulated (Appendix 19 & 20). The down regulated genes did not correspond to any GO terms, and the 5 upregulated corresponded to 2 GO terms, but they did not pass set cut-off of $P < 0.05$. Therefore, no further gene expression analysis was conducted on the effect of iodinated water on used chicken litter.

2.3 Discussion

The benefits of re-used chicken litter and increased iodine in poultry health and performance has been shown, however the mechanisms of action behind these two treatments are not well understood. Our research is the first to study the underlying mechanisms of used litter and iodinated water on animal performance through their effect on the caecal microbiota and impact on gene expression in young chickens. Overall, used litter had a significant effect on the microbial composition and morphology in the caeca, and genes related to intestinal epithelial cell turnover and regulation, as well as immune activation. Iodinated water had a limited effect on both the microbiome and gene expression in the presence of used litter. However, on clean litter iodine modified the microbial community in the caeca and played a role in activating genes related to cellular homeostasis and immune activation.

Previous research has indicated that adding iodine to poultry feed and poultry water can improve animal performance, through growth and animal behaviour in broilers (Emeash et al., 1994), however in our study we did not see the positive impact of iodine on growth of the birds over the 12 days, which may be due to the limited sample number of our trial, or may be because our control group was drinking sterile double distilled water that is much cleaner that what might be exhibited in a barn setting. The weight gain of the chicks over the first 8 days was reduced by used chicken litter. Enriched networks related to used litter treatment on H_2O and used litter on 1 ppm involved response to virus, biotic stimulus and symbiosis/mutualism. Used litter also changed immune-associated genes, indicated by enriched networks of response to interferon-gamma (IFN-γ), complement activation, and B cell mediated immunity. IFN-γ is produced by

Th1 cells, CD8+ T cells and natural killer cells when activated by specific antigens, and induces B cell class switching to IgG, which supports phagocytosis and fixation of complement (Owen, 2013). Annotation clusters corresponded to increased steroid biosynthesis and cholesterol metabolic processes. Bacterial interaction with the intestinal epithelial cells (IECs) causes them to produce immunoregulatory glucocorticoids (cortisol in humans, corticosterone in chickens), and although it was originally thought these steroids were only made in the adrenal cortex, many studies indicate they are also made in multiple places, including the gut (Bouguen et al., 2015). These steroids are converted from cholesterol and therefore increased cholesterol synthesis may promote inflammation in the gut as a result of increased bacterial interactions. Therefore, the transcriptome data suggests that the reduced early weight gain is likely due to an initial bacterial and viral challenge introduced through the litter, and energy demand associated with immune activation. Interestingly, there was no difference in weight gain from days 8-12 which is consistent with previous research that indicates that used litter had no significant impact on the long term growth of broilers (Jones & Hagler, 1983; Mccartney, 1971). However, another study has shown that used litter increases growth and reduces mortality of leghorn chickens (Kennard & Chamberlin, 1951), but as this research was conducted over 12 weeks and ours 12 days, we may see changes in weight gain in broilers over a longer time on used litter. It is likely that the introduction of disease challenge through used litter factored into this study, reducing the initial weight gain of the chicks.

Overall, used litter had the most significant effect on the microbiome in all water treatments, increasing diversity, richness and evenness of the microbial community. Used litter increased the genus *Eggerthela* from the *Coriobacteriaeae* family, a family that is considered a core member of the mammalian microbiome (Rosenberg et al., 2014) which has previously been identified in commercially raised chickens and Guinea fowl (Bhogoju et al., 2018). Of the 7 major bacterial families that were upregulated by used litter, 5 families were from the phylum *Firmicutes,* and 4 of those were in the class *Clostridiales*. An increase in *Firmicutes* in humans was associated with an increase in nutrient absorption (Jumpertz et al., 2011), while *Clostridiales* order is specifically known to be major contributors to SCFA production (Oakley et al., 2014). Accordingly, used litter significantly increased the relative concentration of SCFAs butyrate and valerate on both H_2O and 1 ppm iodinated water. However, one genus within the *Clostridiales* order that increased in used chicken litter was *Clostridium*, a genus that contains some

pathogenic species such as *Clostridium perfringens,* which is known to cause necrotic enteritis in chickens (Hofacre et al., 2003) and could indicate one potential risk to re-using litter. However, with 16s rRNA sequencing we were not able to target at the species level and therefore cannot conclude the type of *Clostridium* introduced, as well there are many species within the *Clostridium* genus, specifically *Clostridium* cluster *XIVa* and *IV* that make up a substantial percent of the human commensal microbiome that play important roles in infection resistance and immune education (Lopetuso et al., 2013).

Not only did used litter increase the relative abundance of select bacteria, it also introduced 149 unique OTUs representing the four major bacterial phyla in chickens: *Actinobacteria, Bacteroidetes, Firmicutes*, and *Tenericutes*, with the majority being *Firmicutes,* likely to also be contributing to the changes in SCFAs, suggesting that without this exposure, birds are missing a substantial number of bacteria that they are not being exposed to from the hatchery. The used litter group contained significantly lower relative abundance of *Enterococcaceae*, *Leuconostocaceae*, and *Enterobacteriaceae*. Research has shown that both *Enterococcaceae* and *Enterobacteriaceae* were the dominant species in birds at day 0 and day 1 of life, but that over the first week the microbiota shift with increased gram positive bacteria mostly from the *Clostridiales* group, and by day 28, the percentage of gram negative bacteria is at 6% and is mostly *Enterobacteriaceae* (Ballou et al., 2016), interestingly detecting extremely low levels of *Bacteroidetes* in the caeca. The *Enterobacteriaceae* family includes many pathogenic bacteria such as *Salmonella*, *Klebsiella*, *Proteus* and *E. coli* (Ballou et al. 2016; Oakley et al. 2014), indicating a beneficial effect of pathogen reduction through re-used chicken litter, and in our study a reduction in *Proteus* and *Klebsiella* genera was noted by used litter. Some bacteria that have been associated with beneficial effects were also depleted such as *Enterococcaceae* and *Leuconostocaceae*, both members of the *Firmicutes* phylum, and certain genus members of these two families have been used as probiotics in the poultry industry (Pourakbari et al., 2016; Seo et al., 2012).

Iodinated water had a significant effect on the beta diversity of the caecal microbiota of chicks reared on clean litter, but this effect was mitigated through the addition of used litter. Iodinated water at 1 ppm concentration compared to 10 ppm and water control decreased the relative abundance of *Enterobacteriaceae* and *Bifidobacteriaceae*, while *Bacteroidaceae* increased in 1 ppm iodinated water, which may indicate that the effect of iodinated water is

dosage dependent, and that higher concentrations impact the microbiome differently. Lastly, both 1 ppm and 10 ppm decreased the relative abundance of *Streptococcaceae*, a bacterial family that contains antibiotic resistance genes to multiple antibiotics (Molitoris et al., 1986). Five OTUs were uniquely detected with the addition of iodinated water, 3 of which were unassigned, while the remaining 2 were assigned to *Bacteroides fragilis*, which have been isolated in poultry feces, and contain strains that are multi-drug resistant and enterotoxigenic that have been associated with inflammatory gastrointestinal tract diseases in humans (Garcia et al., 2012).

Consistent with the microbiome data, iodinated water only had a significant effect on gene expression on clean chicken litter, as on used litter no significant GO terms could be identified by the 7 DEGs. The most enriched networks related to the up-regulated DEGs of 1ppm iodinated water on clean litter were related to cell regulation; mostly GO terms involved in cell communication, cell-cell signaling, and signal transduction, as well as the KEGG pathway phosphatidylinositol (PI) signaling system. PI are lipids in cell plasma that regulate cellular processes, and in our samples, inositol polyphosphate-4-phosphatase A (INPP4A) and inositol-trisphosphate 3-kinase A (ITPKA) were significantly up-regulated, which are thought to influence membrane trafficking and actin cytoskeleton organization respectively (Sasaki et al., 2009). The top 4 enriched GO clusters from the down-regulated DEGs related to muscle cell differentiation, actin cytoskeleton, cell-cell adhesion, extracellular matrix, tissue development, cell motility, differentiation and proliferation, while the 5th was downregulation of cell death, apoptosis and programmed cell death. Both these up and down regulated processes indicate that iodinated water is impacting cell organization in the caecum, either directly or indirectly through changes to the microbiome, and promoting stability and reduced turn-over of the epithelial lining. Interestingly, some enriched networks corresponded to the activation of genes in response to virus and parasitism, defense response, as well as immune response based wholly on response to IFN-γ. Iodine and IFN-γ have been known to influence the expression of vascular adhesion molecules (ICAM-1) on the surface of antigen presenting cells, epithelial cells, and activated lymphocytes, that when up-regulated promotes cell-to-cell interactions, signals immune system, and starts homing T cells to the site of inflammation (Sharma et al., 2005). However, this was a direct effect of iodine on thyroid cells in mice that spontaneously develop autoimmune lymphocytic thyroiditis and did not increase ICAM-1 in the control BALB/c or C57BL6 strains, which suggests this is only seen in genetically predisposed mice. Our research may indicate that

the increased immune response and cell communication could be due to increased ICAM-1 of the intestinal epithelial cells, but further research is needed to confirm this idea, as ICAM-1 was not indicated in the up-regulated DEGs. As well, increased immune activation has been seen through the up-regulation of the KEGG pathway for arachidonic acid metabolism, as arachidonic acid is an Omega-6 polyunsaturated fatty acid (n-6 PUFA), a precursor to eicosanoids that are generally pro-inflammatory (Patterson et al., 2012). Lastly, iodinated water on clean litter may increase the mucus layer as the KEGG pathway associated with mucin type O-glycan biosynthesis was up-regulated and these O-glycosylations, which is the addition of GalNAc sugar to the hydroxyl group of serine or threonine residues, are abundant in intestinal mucins (Tran & Ten Hagen, 2013).

Used chicken litter had a significant effect on caecal morphology; increasing villus height, crypt depth, and the number of villi, resulting in more available surface area for nutrient absorption. To the best of our knowledge this is the first study to show the effect of re-used litter on poultry caecal morphology. This is likely due to the increased short chain fatty acid butyrate, which is a major energy source for colonocytes and acts as a gene regulator for intestinal epithelial cells by stopping histone deacetylases from condensing chromatin and making it inactive for transcription (Bedford & Gong, 2018; Nepelska et al., 2012). One study in chickens showed that adding butyrate to feed increased the villus height to crypt depth ratio (Hu & Guo, 2007). The changes in caecal morphology due to used litter may also be indicated through gene expression changes. One annotation cluster from the up-regulated DEGs of the used chicken litter on H_2O, as well as 4 clusters from the used chicken litter on 1 ppm were associated with regulation of cellular proliferation, differentiation, and survival, with the majority of GO terms associated with cell communication, cell cycle regulation, and cell recognition. Previous research in mice has shown that oral administration of SCFAs promotes the proliferation and turnover of IECs and migratory activity (Park et al., 2016). However, the most enriched networks related to the down regulated DEGs of used chicken litter on H_2O were related cytoskeleton and muscle system process, which supports the apical brush boarder surface of the intestinal epithelial cells, made up of actin filaments and other proteins in chickens (Mooseker, 1985). Overall, used litter seemed to enhance cell survival, lowering gene expression of the apical brush boarder surface of the intestinal epithelial cells, suggesting that the addition of used litter reduced cell turnover of the brush border surface, as well as increasing cell communication and cell recognition.

Overall, the effect of used chicken litter on gene expression with H_2O was similar to that on 1 ppm iodinated water on annotation clusters related to up-regulated DEGs, however used litter with 1 ppm iodinated water clusters related to down-regulated DEGs were different, with the first three clusters of enriched networks being related to phospholipase activity, eicosanoid metabolic process, and KEGG pathways of Arachidonic acid metabolism. Lipases release fatty acids, and phospholipase specifically releases arachidonic acid which is an omega-6 polyunsaturated fatty acid (n-6 PUFA), a precursor to eicosanoids that are generally pro-inflammatory in nature (Patterson et al., 2012). These results suggest that used litter mitigated the pro-inflammatory effect of iodinated water, resulting in decreased expression of pro-inflammatory genes.

2.4 Conclusion

In conclusion, this research provides convincing evidence that the exposure of newly hatched chicks to used litter can improve gastrointestinal morphology and shift SCFA profiles in the caecum of 12-day old birds. As well, the introduction of a more dynamic host microbiota to young chicks interacts with the intestinal mucosa, and initiates immune education early on, while providing protection from potentially pathogenic bacteria and increased cell homeostasis as a result. However, increased *Clostridium* in the used litter may indicate one risk to re-used litter. Iodinated water has limited effects on the chicks overall when raised on used litter, indicating that a more developed microbiome plays a significant role when it comes to dietary changes such as increased iodine. Iodine does, however, have a significant effect on both the microbiome and gene expression of the chicken caecum in the absence of used chicken litter, increasing inflammation, as well as promoting stability and reduced turn-over of the epithelial lining, and mucosal development.

Tables

Table 2.1 Effect of treatment on water disappearance. The effect of iodinated water treatment, litter treatment, and combined effect of treatment on water disappearance from bell drinkers.

	Water disappearance volume (mL)			*Repeated measures ANOVA (P-Value)*		
Day	1 ppm	10 ppm	H_2O	Effect of iodine Water	Effect of Litter	Effect of Water*Litter
D3	188.8 ± 12.74	127.5 ± 14.11	147.1 ± 11.07	0.0118	0.7490	0.4267
D5	311.3 ± 13.42	238.8 ± 12.02	258.6 ± 12.99	0.0008	0.6519	0.0411
D6	191.3 ± 9.20	155.6 ± 6.23	153.6 ± 6.79	0.0015	0.4159	0.0636
D7	206.3 ± 7.37	174.4 ± 10.11	151.4 ± 11.00	0.0015	0.0728	0.3859
D8	220.0 ± 11.02	173.8 ± 12.81	157.1 ± 9.93	0.0007	0.0058	0.9957
D9	136.3 ± 5.96	133.8 ± 6.80	138.6 ± 8.85	0.9369	0.002	0.4530
D10	160.0 ± 5.98	167.5 ± 6.48	174.3 ± 8.69	0.4603	0.2815	0.9232
D11	161.3 ± 3.98	161.3 ± 5.15	197.1 ± 7.78	0.0006	0.3279	0.8860
D12	188.8 ± 8.54	190.0 ± 7.56	180.0 ± 8.17	0.3825	0.0012	0.9897

[1] Values are means ± SEM, n = 4.

Table 2.2 Multiple comparisons on the weighted and unweighted unifrac distance matrices. Litter had the most significant effect on both the weighted and unweighted distance matrices.

	Treatment A	*Treatment B*	*Adjusted P-Value*
Unweighted Unifrac	1 ppm - Used	H_2O - Used	1.000
	1 ppm - Used	10 ppm - Used	0.420
	1 ppm - Used	H_2O - Clean	0.030
	1 ppm - Used	10 ppm - Clean	0.015
	1 ppm - Used	1 ppm - Clean	0.015
	H_2O - Used	10 ppm - Used	0.195
	H_2O - Used	H_2O - Clean	0.015
	H_2O - Used	10 ppm - Clean	0.015
	H_2O - Used	1 ppm - Clean	0.030
	10 ppm - Used	H_2O - Clean	0.015
	10 ppm - Used	10 ppm - Clean	0.015
	10 ppm - Used	1 ppm - Clean	0.015
	H_2O - Clean	10 ppm - Clean	1.000
	H_2O - Clean	1 ppm - Clean	0.015
	10 ppm - Clean	1 ppm - Clean	1.000
Weighted Unifrac	1 ppm - Used	H_2O - Used	1.000
	1 ppm - Used	10 ppm - Used	1.000
	1 ppm - Used	H_2O - Clean	0.015
	1 ppm - Used	10 ppm - Clean	0.015
	1 ppm - Used	1 ppm - Clean	0.120
	H_2O - Used	10 ppm - Used	1.000
	H_2O - Used	H_2O - Clean	0.015
	H_2O - Used	10 ppm - Clean	0.030
	H_2O - Used	1 ppm - Clean	0.135
	10 ppm - Used	H_2O - Clean	0.015
	10 ppm - Used	10 ppm - Clean	0.015
	10 ppm - Used	1 ppm - Clean	0.075
	H_2O - Clean	10 ppm - Clean	0.555
	H_2O - Clean	1 ppm - Clean	0.210
	10 ppm - Clean	1 ppm - Clean	1.000

[1]Treatment A and B indicate the comparison between the following 6 treatments: 1 ppm iodinated water with used litter (1 ppm – Used), H_2O water with used litter (H_2O – Used), 10 ppm iodinated water with used litter (10 ppm – Used), 1 ppm iodinated water with clean litter (1 ppm – Clean), H_2O water with clean litter (H_2O – Clean), and 10 ppm iodinated water with clean litter (10 ppm – Clean). *P*-values are adjusted by Bonferroni.

Table 2.3 Alpha diversity measures of Chao1, Shannon, and Inverse Simpson indices. Comparison of the 6 treatments: H_2O Used, H2O Clean, 1 ppm Used, 1 ppm Clean, 10 ppm Used and 10 ppm clean. Number indicates *P*-value.

Comparison	*Chao1*	*Shannon*	*Inverse Simpson*
10 ppm Clean - 10 ppm Used	0.0003	0.06	0.3
10 ppm Clean - 1 ppm Clean	1	1	1
10 ppm Used - 1 ppm Clean	0.002	0.003	0.006
10 ppm Clean - 1 ppm Used	0.04	0.04	0.03
10 ppm Used - 1 ppm Used	1	1	1
1 ppm Clean - 1 ppm Used	0.2	0.001	0.0003
10 ppm Clean - H_2O Clean	1	1	1
10 ppm Used - H_2O Clean	0.007	0.2	0.9
1 ppm Clean - H_2O Clean	1	1	1
1 ppm Used - H_2O Clean	0.3	0.2	0.2
10 ppm Clean - H_2O Used	0.005	0.02	0.1
10 ppm Used - H_2O Used	1	1	1
1 ppm Clean - H_2O Used	0.03	0.0007	0.002
1 ppm Used - H_2O Used	1	1	1
H_2O Clean - H_2O Used	0.06	0.1	0.5

Table 2.4 Effect of treatment the percent relative abundance of the bacterial families. Litter treatment had a more significant effect on the percent relative abundance of the bacterial families, compared to water treatment and interaction. Number indicates *P*-value.

Bacterial Family	*Water*	*Litter*	*Interaction*
Unassigned	0.1065	0.1137	0.6522
Bifidobacteriaceae	0.0071	0.0029	0.0062
Coriobacteriaceae	0.2659	0.0071	0.2659
Bacteroidaceae	0.3019	0.8159	0.0777
Prevotellaceae	0.2533	0.8019	0.0733
S24_7	0.2111	0.5255	0.8841
o_*Streptophyta*	0.1195	0.0912	0.0768
Bacillaceae	0.3212	0.3532	0.1871
Enterococcaceae	0.9975	0.0073	0.7053
Lactobacillaceae	0.3317	0.2275	0.5762
Leuconostocaceae	0.5555	0.0255	0.5182
Streptococcaceae	0.1709	0.1717	0.1644
o_*Clostridiales*_other	0.2415	0.3265	0.5853
o_*Clostridiales*_uncl	0.8776	<0.0001	0.6909
Christensenellaceae	0.7107	0.0044	0.7107
Clostridiaceae	0.6397	0.1032	0.1831
Lachnospiraceae	0.0881	0.6255	0.1667
Peptostreptococcaceae	0.1913	0.6053	0.6979
Ruminococcaceae	0.2373	0.0002	0.2435
Veillonellaceae	0.4415	0.4151	0.9207
Mogibacteriaceae	0.9146	0.0079	0.9146
Erysipelotrichaceae	0.5539	0.0172	0.7617
Campylobacteraceae	0.2230	0.0556	0.2230
Enterobacteriaceae	0.1469	<0.0001	0.0641
o_RF39	0.1674	0.0120	0.1500

Table 2.5 The phylogenetic assignment to the unique OTUs found in the used litter group. Number of unique OTUs is indicated, as well as the average percent relative abundance of the of each in the used litter treatment.

Phylogenetic assignment	Unique OTUs	Mean % relative abundance
P__Actinobacteria		
F__Coriobacteriaceae; g__; s__	1	0.001
F__Coriobacteriaceae; g__Eggerthella; s__lenta	1	0.029
P__bacteroidetes		
F__Bacteroidaceae; g__bacteroides; s__	1	0.002
P__firmicutes		
F__Lactobacillaceae; g__lactobacillus; s__	1	0.007
O__Clostridiales; f__; g__; s__	36	5.567
F__[Mogibacteriaceae]; g__; s__	2	0.028
F__Christensenellaceae; g__; s__	3	0.058
F__Clostridiaceae; g__; s__	1	0.006
F__Clostridiaceae; g__clostridium; s__	2	0.064
F__Lachnospiraceae	3	0.157
F__Lachnospiraceae; g__; s__	6	0.177
F__Lachnospiraceae; g__blautia; s__	1	0.040
F__Lachnospiraceae; g__lachnospira; s__	1	1.131
F__Ruminococcaceae; g__; s__	37	2.780
F__Ruminococcaceae; g__Faecalibacterium; s__prausnitzii	1	0.013
F__Ruminococcaceae; g__oscillospira; s__	11	0.794
F__Ruminococcaceae; g__ruminococcus; s__	11	0.509
F__Erysipelotrichaceae; g__[Eubacterium]; s__dolichum	1	0.178
F__Erysipelotrichaceae; g__coprobacillus; s__	1	0.037
F__Erysipelotrichaceae; g__holdemania; s__	1	0.045
P__Tenericutes		
C__Mollicutes; o__RF39; f__; g__; s__	8	0.065
Unassigned	19	0.084
Total	149	11.8

Figures

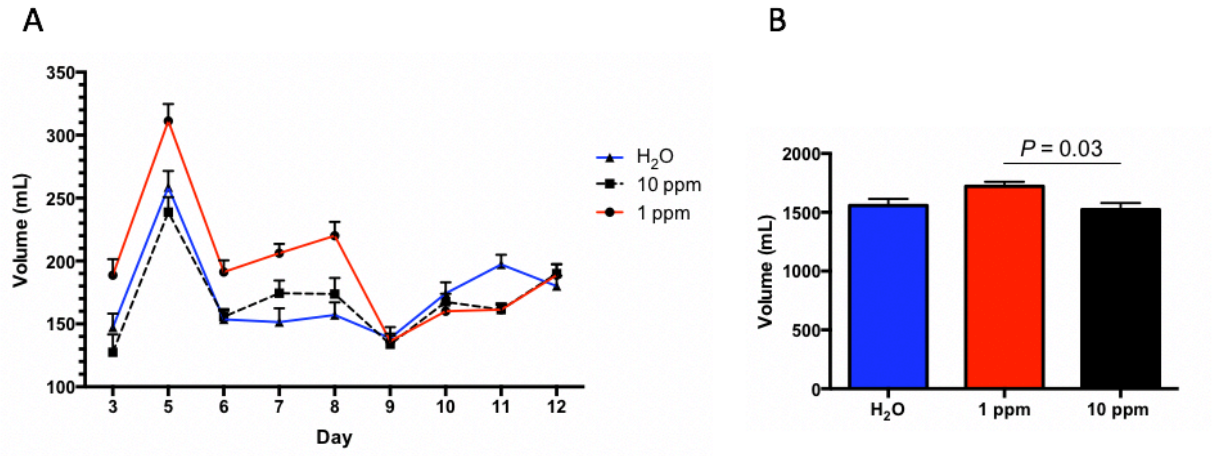

Figure 2.1 Comparison of the water disappearance between treatments. (A) Water disappearance in H_2O, 1 ppm, and 10 ppm iodinated water from day 3 to day 12 day, and (B) total water disappearance over the 12 days.

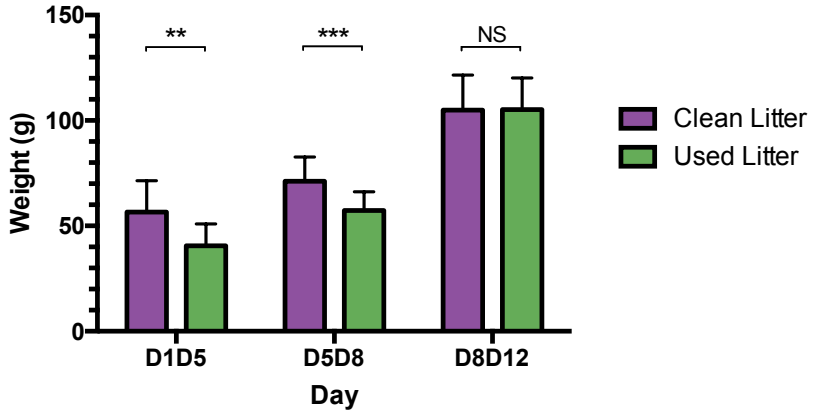

Figure 2.2 Average weight gain. Average weight gained from day 1 to 5, 5 to 8, and 8 to 12 between the clean and used litter treatments. Significance denoted by: ** = $P<0.01$, *** = $P<0.001$, NS = $P>0.05$.

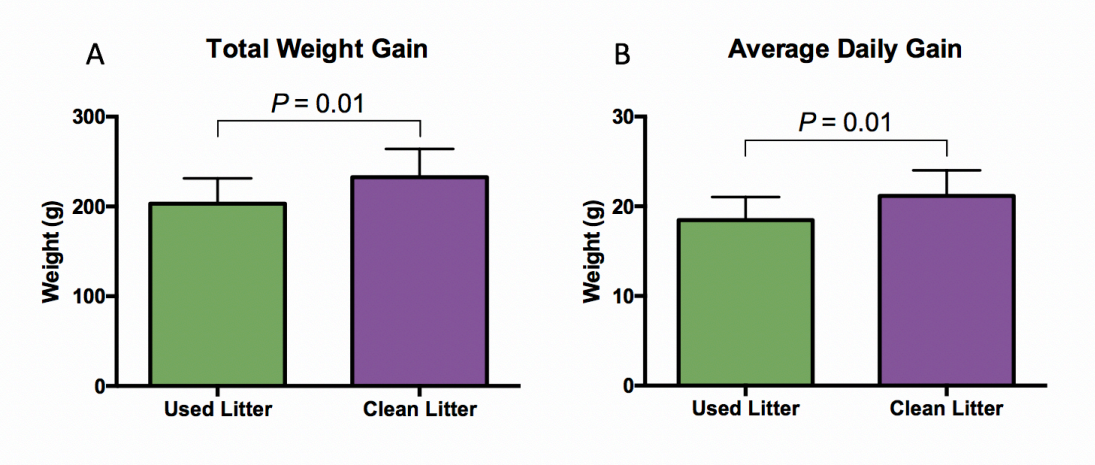

Figure 2.3 Total weight gain (A) and average daily gain (B) between the used and clean litter treatments.

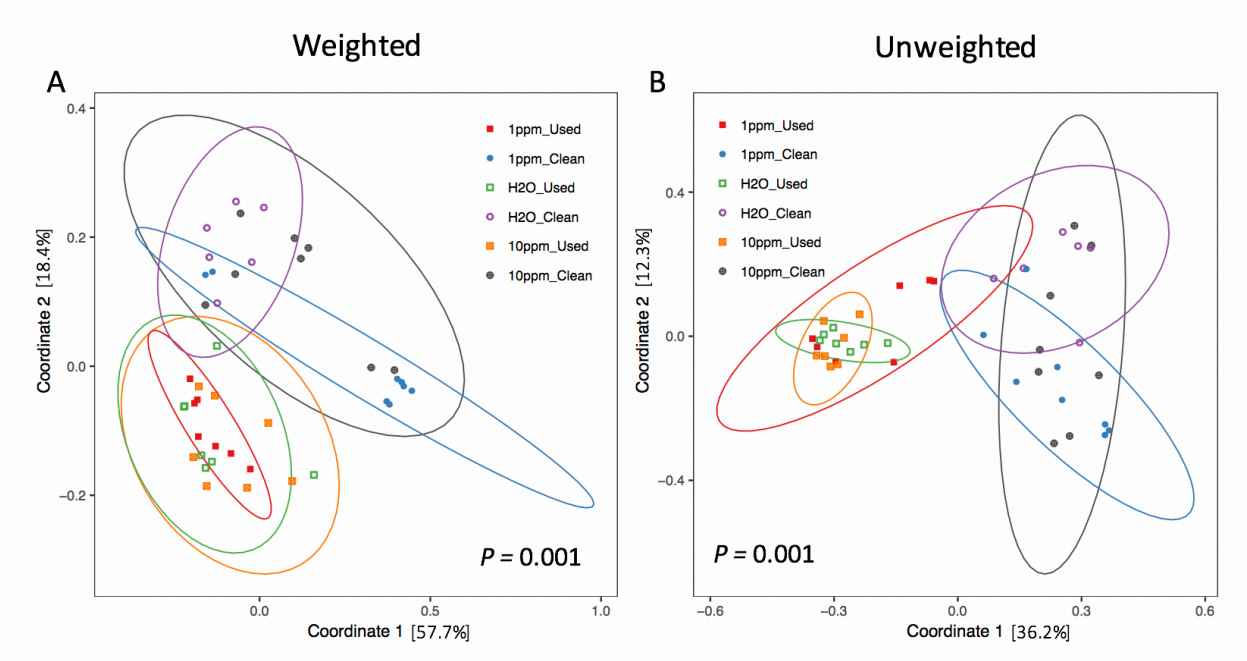

Figure 2.4 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on treatment. There was a significant effect of treatment the caecal microbial community as indicated on both the weighted (A) and unweighted (B) unifrac distance metrics (N = 6-8, Adonis, $P = 0.001$).

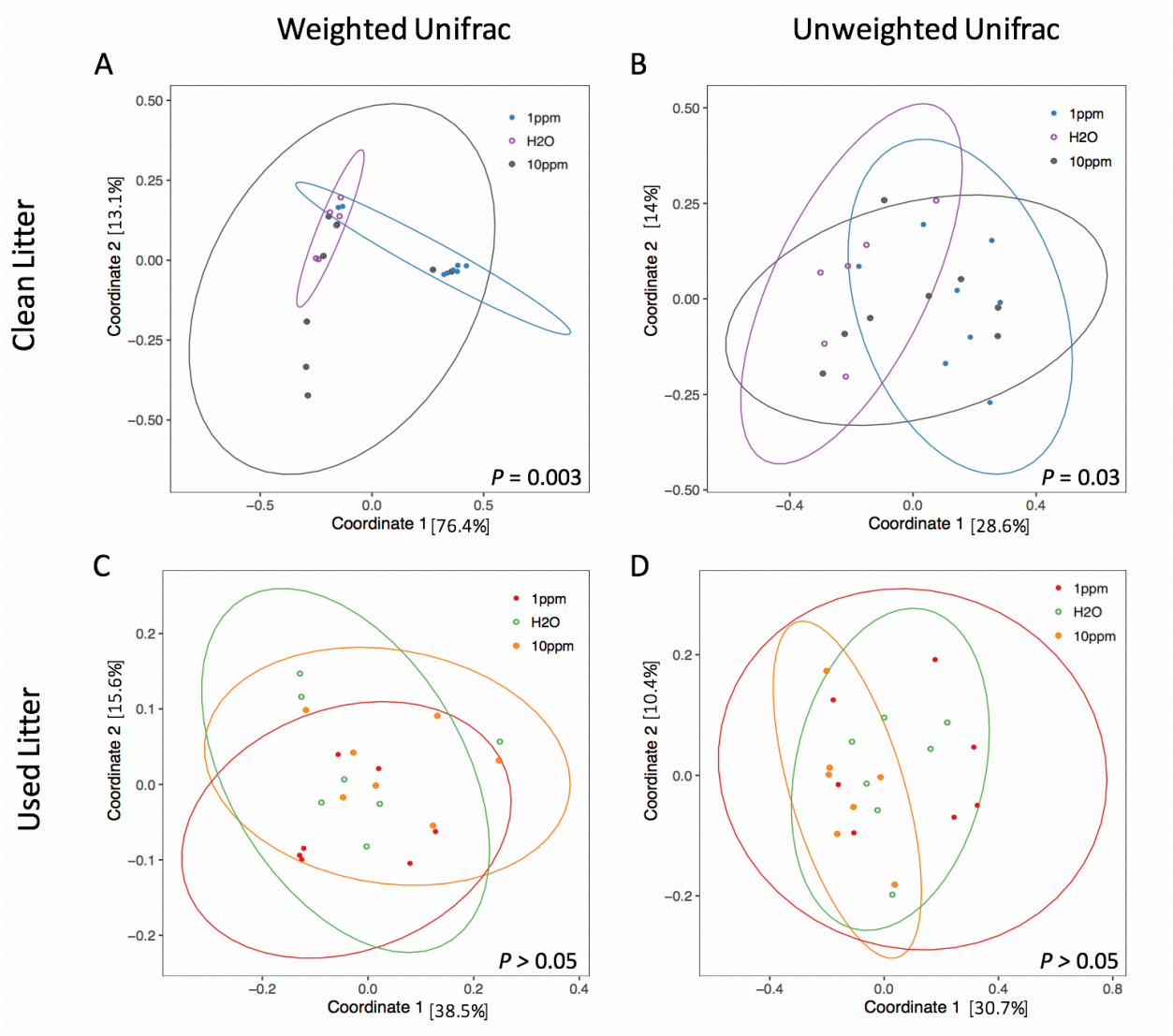

Figure 2.5 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on water treatment. Iodinated water had a significant effect on the caecal microbial community on clean litter in both the (A) weighted ($P = 0.003$) and (B) unweighted ($P = 0.03$) unifrac distances (N = 8), however not on the (C) weighted and (D) unweighted UniFrac distance metrics in the used litter treatment (N = 7, $P > 0.05$).

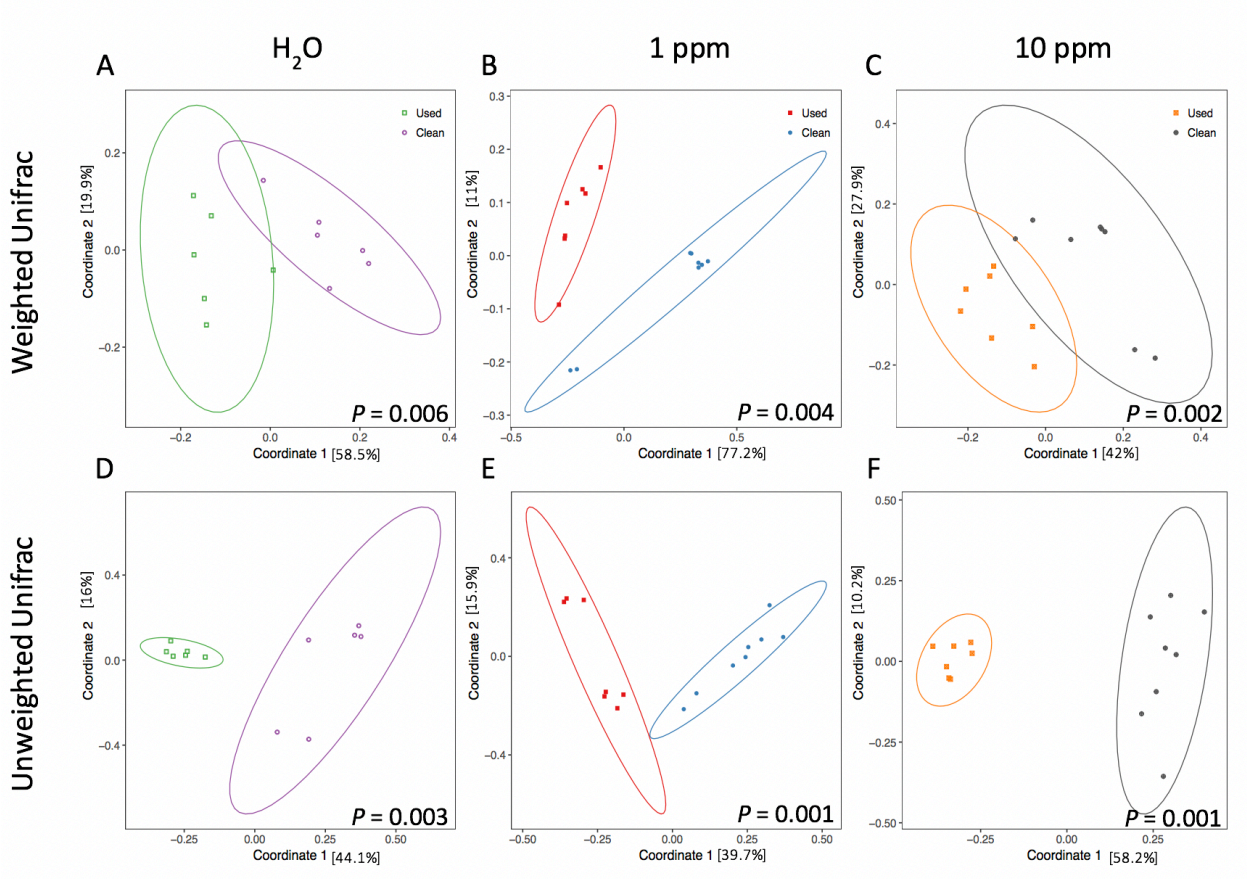

Figure 2.6 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on litter treatment. Used litter had a significant effect on the caecal microbiome on weighted UniFrac distances on (A) H_2O (N = 6, P = 0.006), (B) 1 ppm iodinated water (N = 7-8, P = 0.004), and (C) 10 ppm iodinated water (N = 7-8, P = 0.002), and unweighted UniFrac distances on (D) H_2O (N = 6, P = 0.003), (E) 1 ppm iodinated water (N = 7-8, P = 0.001), and (F) 10 ppm iodinated water (N= 7-8, P = 0.001).

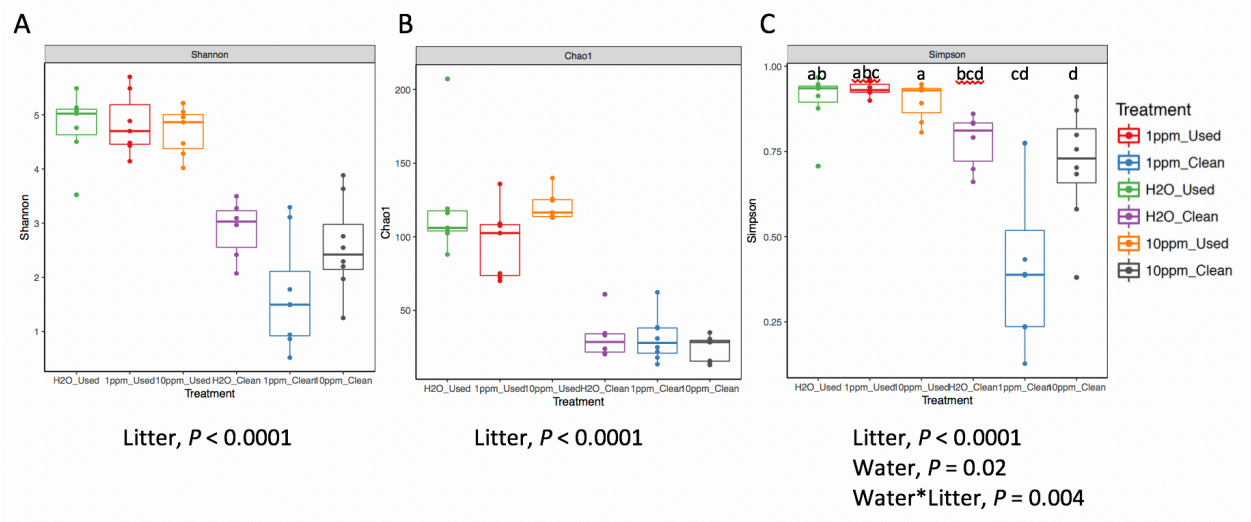

Figure 2.7 Alpha diversity indices. Caecal digesta of chickens on clean or used litter with H_2O, 1 ppm, or 10 ppm iodinated water. Used litter significantly increased Shannon (A), Chao1 (B), and inverse Simpson (C) indices (N = 6-8). There was a significant effect of water and an interaction between water and litter on the inverse Simpson index (C). Significance denoted by letter, $P < 0.05$ is considered significant.

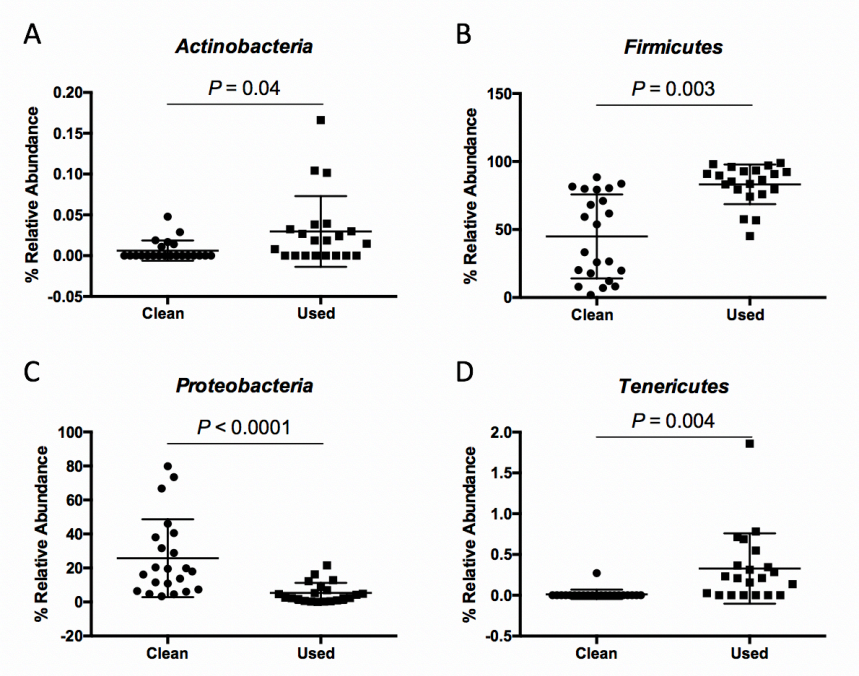

Figure 2.8 Percent relative abundance of *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Tenericutes* phyla by litter treatment. Percent relative abundance of (A) *Actinobacteria*, (B) *Firmicutes*, and (D) *Tenericutes* increased, while (C) *Proteobacteria* decreased in the used litter compared to clean litter treatment (N = 20, 22).

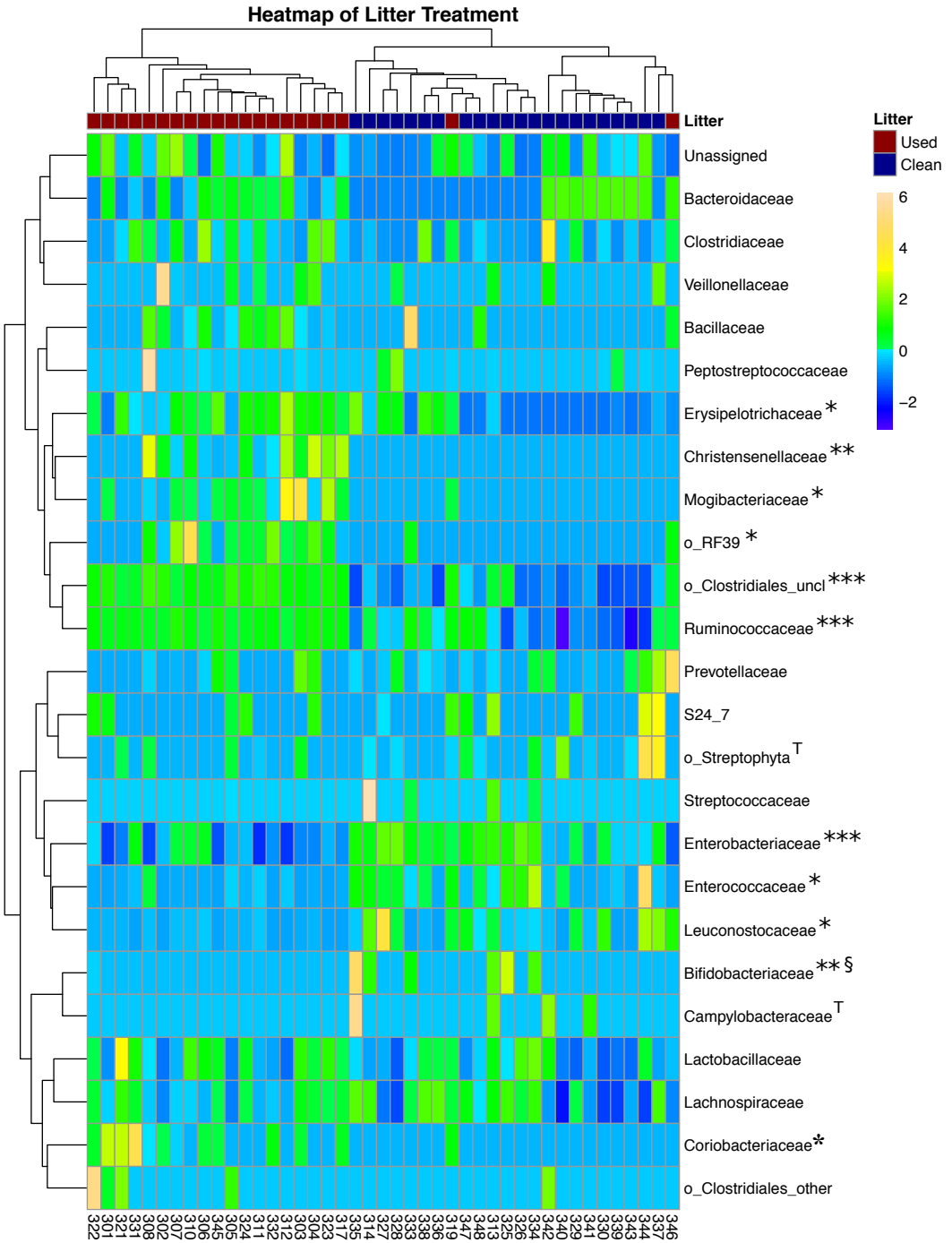

Figure 2.9 Heatmap of the percent relative abundance of bacterial families between clean and used chicken litter. Clustered significantly by litter treatment. Significance denoted as follows: * = $P < 0.05$, ** = $P < 0.005$, *** $P = < 0.0005$, T (trend) = $0.05 < P < 0.1$, § = significant interaction between water and litter treatment.

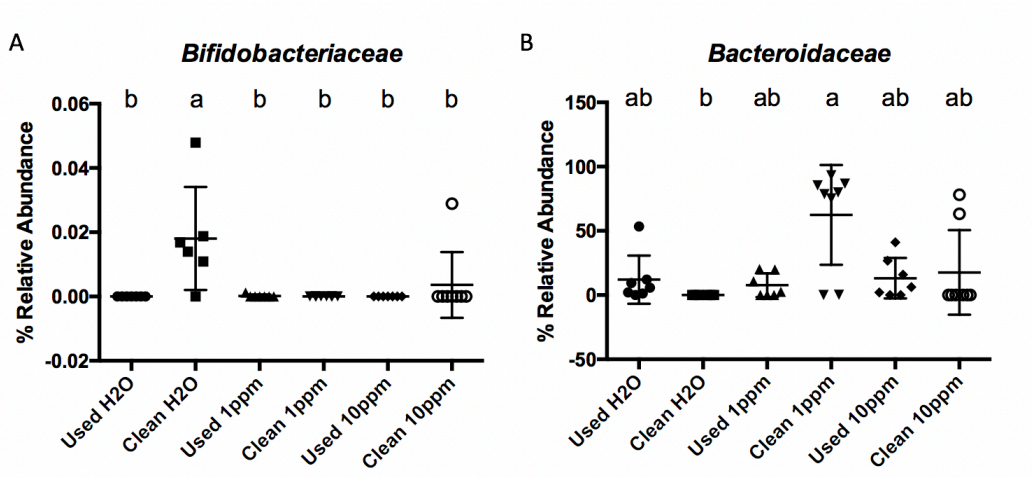

Figure 2.10 Percent relative abundances of *Bifidobacteriaceae* and *Bacteroidaceae*. Clean litter on H_2O had significantly higher *Bifidobacteriaceae* than the other 5 treatments (A). Clean 1 ppm iodinated water has significantly higher *Bacteroidaceae* than clean H_2O (B). Non-similar letters (a/b) denotes significant differences ($P < 0.05$).

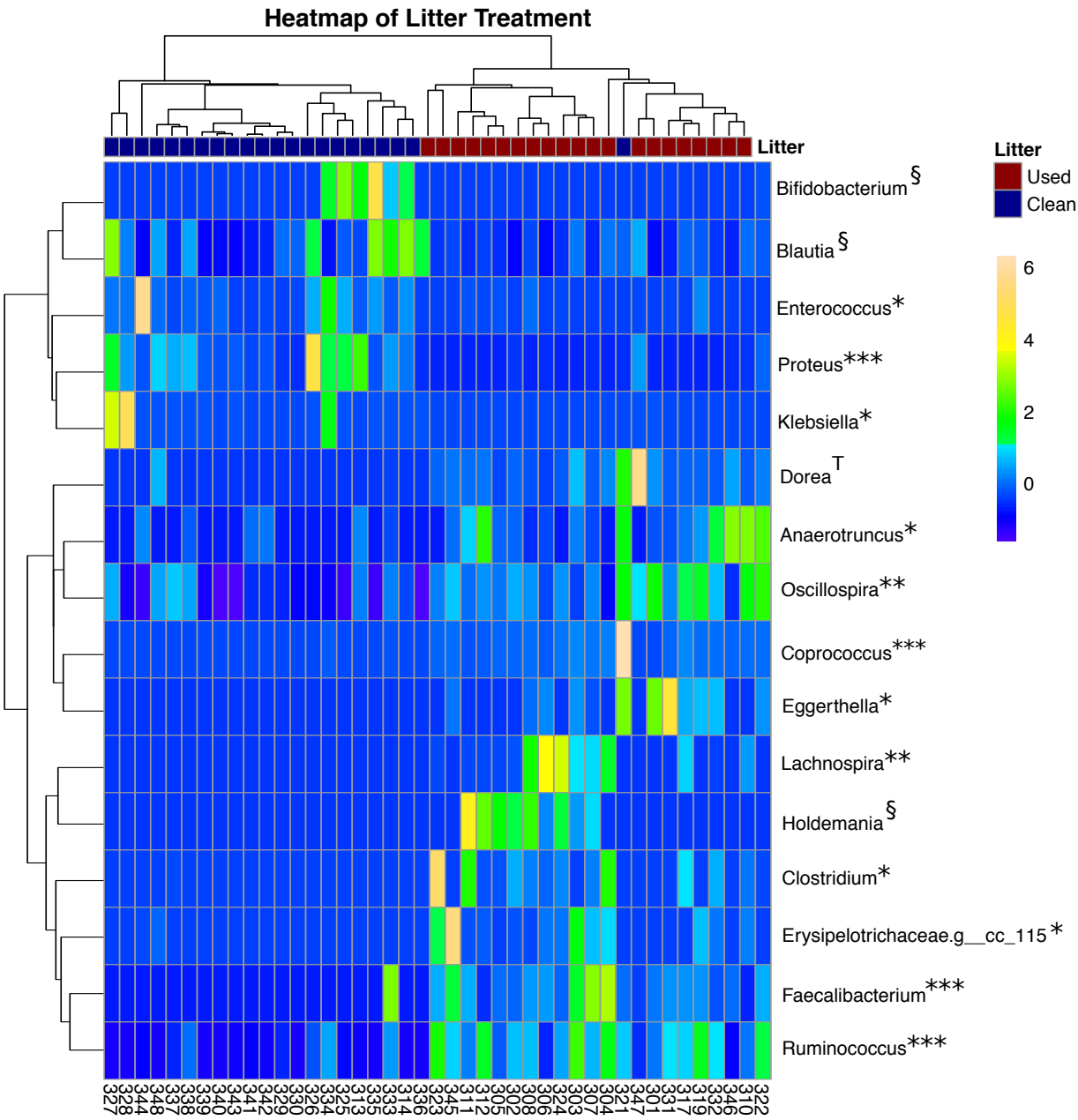

Figure 2.11 Heatmap of the percent relative abundance of the significantly different bacterial genera between clean and used chicken litter. Clustered significantly by litter treatment. Significance denoted as follows: * = $P < 0.05$, ** = $P < 0.005$, *** $P = < 0.0005$, T (trend) = $0.05 < P < 0.1$, § = significant interaction between water and litter treatment.

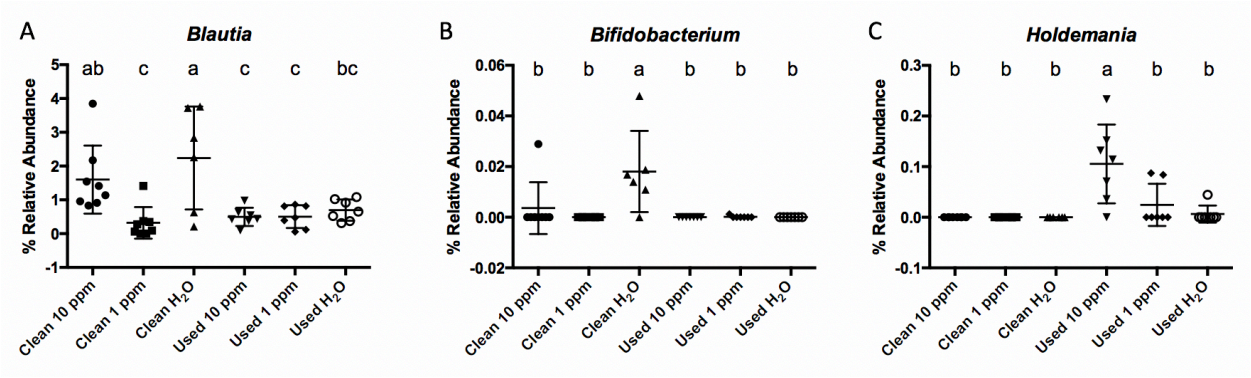

Figure 2.12 Percent relative abundance of *Blautia*, *Bifidobacterium*, and *Holdemania* genera. *Blautia* (A) and *Bifidobacterium* (B) had higher relative abundance in clean litter on H_2O, and *Holemania* (C) was highest in the used litter with 10 ppm iodinated water. Non-similar letters (a/b) denotes significant differences ($P < 0.05$).

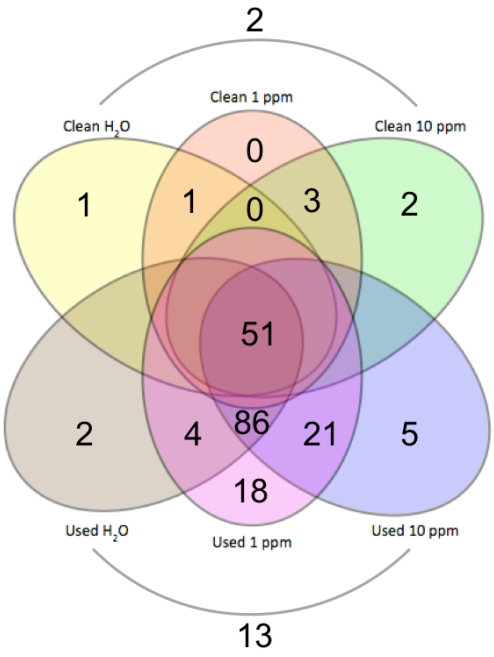

Figure 2.13 Venn diagram of the unique and shared OTUs represented in the 6 treatments. A total of 149 different OTUs were introduced by the used litter treatment.

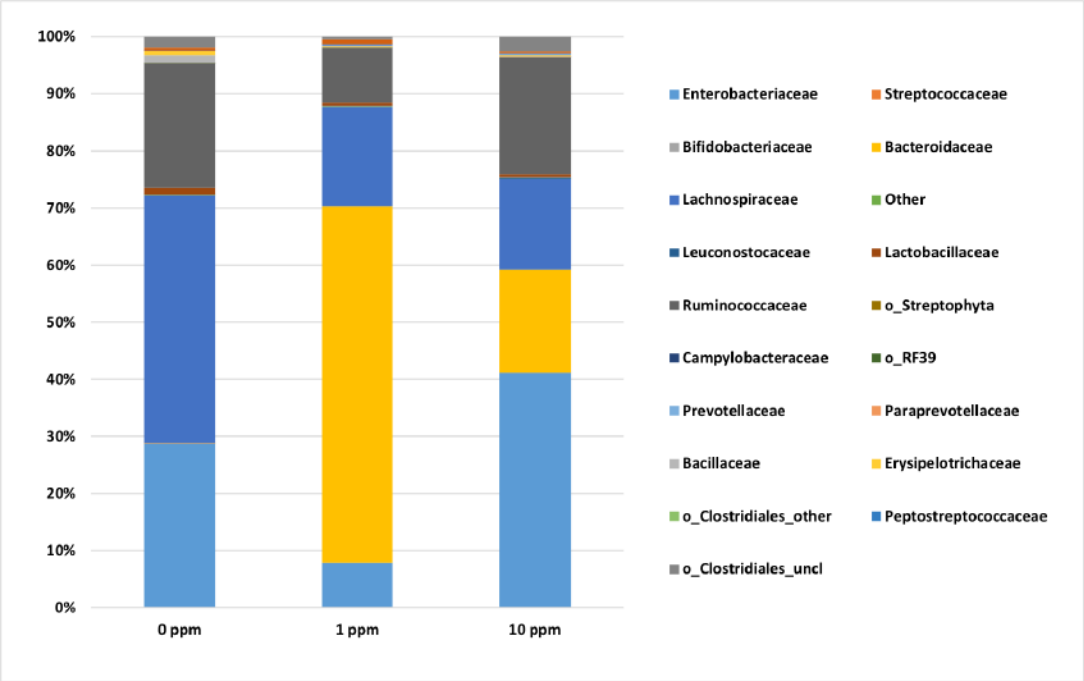

Figure 2.14 Relative abundance of bacterial families on clean litter in H_2O, 1 ppm, and 10 ppm iodinated water treatments. The relative abundance of *Enterobacteriaceae*, *Streptococcaceae*, *Bifidobacteriaceae*, and *Bacteroidaceae* families were significantly different between water treatments on clean chicken litter.

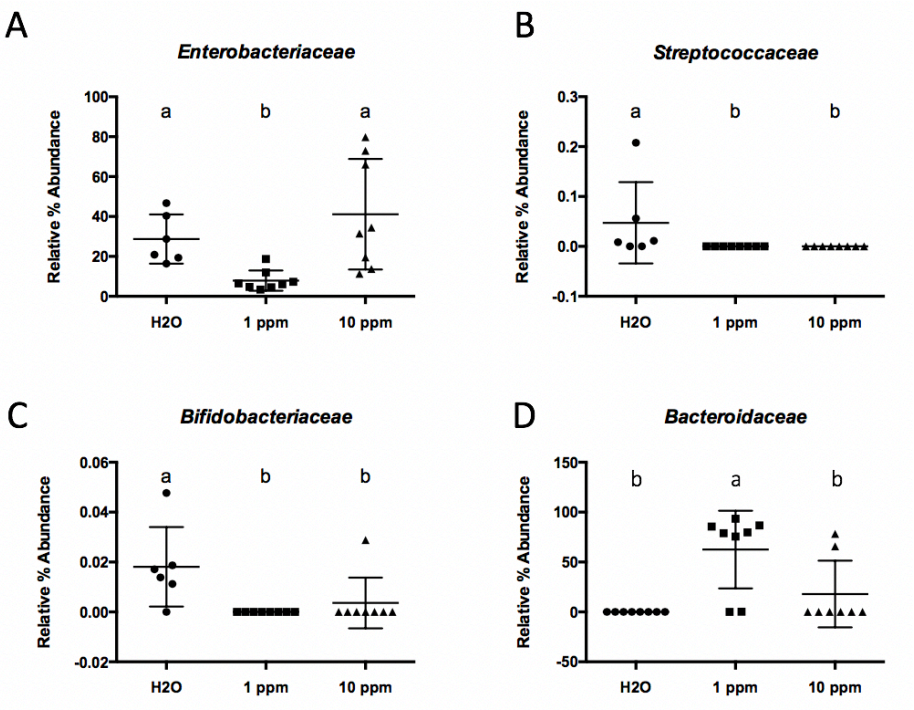

Figure 2.15 Relative abundance of *Enterobacteriaceae*, *Streptococcaceae*, *Bifidobacteriaceae*, and *Bacteroidaceae* in clean litter treatment. The relative abundance of *Enterobacteriaceae* (A) was lower on 1 ppm iodinated water. The relative abundance of *Streptococcaceae* (B) and *Bifidobacteriaceae* (C) was lower and *Bacteroidaceae* (D) was higher on both 1 ppm and 10 ppm iodinated water. Non-similar letters (a/b) denotes significant differences ($P < 0.05$).

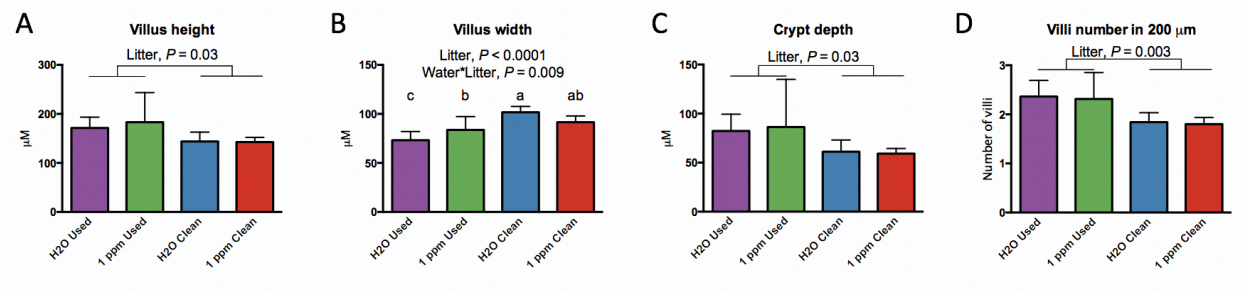

Figure 2.16 Cecal histology measurements of villus height, villus width, crypt depth, and number of villi counted in 200 micrometer section. Used litter increased villus height (A), decreased villus width (B), increased crypt depth (C) and increased number of villi in a 200 µm section (D). There was a significant interaction between litter and water treatment on the villus width (B). Non-similar letters (a/b/c) denotes significant differences ($P < 0.05$).

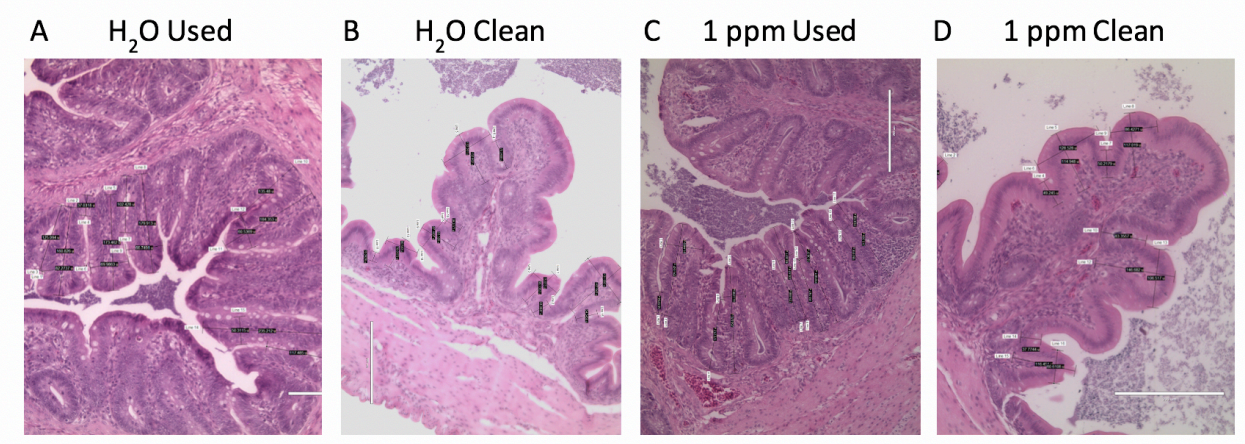

Figure 2.17 Images of caecal morphology of 12-day-old chicks at 200X. Images are of H_2O water with used litter (A), H_2O water with clean litter (B), 1 ppm iodinated water with used litter (C), and 1 ppm iodinated water with clean litter (D).

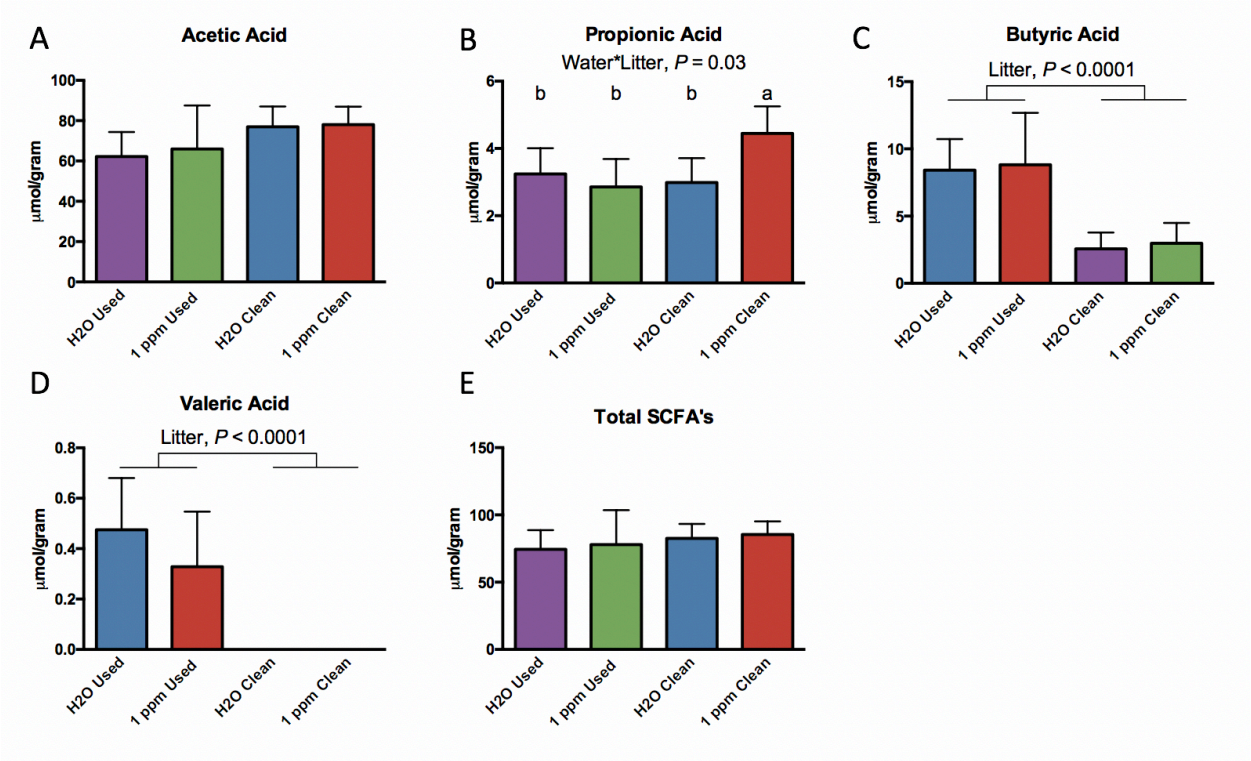

Figure 2.18 Concentration of short chain fatty acids in the chicken caecum as affected by treatment. There was no effect of treatment on Acetic acid (A), there was a significant effect of water*litter interaction on propionic acid (B), used litter significantly increased butyric acid (C) and valeric acid (D), and there was no effect of treatment on the total SCFAs. Non-similar letters (a/b) denotes significant differences ($P < 0.05$).

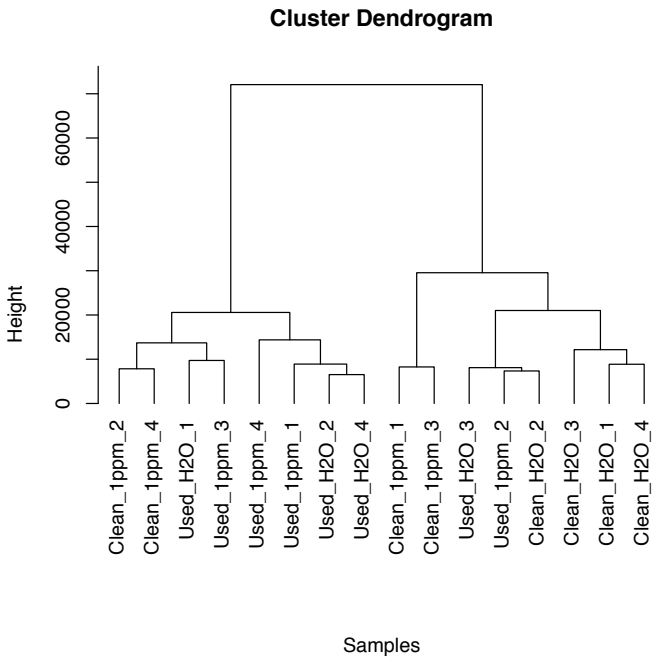

Figure 2.19 Hierarchical clustering of the samples based on gene expression. Samples clustered mostly by litter type and less by water treatment (Distance = euclidean; Method = ward.D).

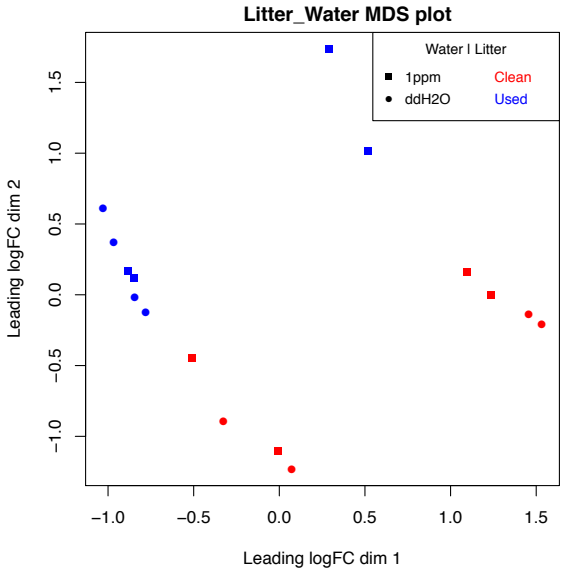

Figure 2.20 Multi-dimensional scaling plot. Plot is based on the top 500 genes with the greatest log fold change differences between groups, and indicated that samples separate mostly by litter treatment on the y-axis. Samples are labelled by colour for litter treatment, and shape for water treatment.

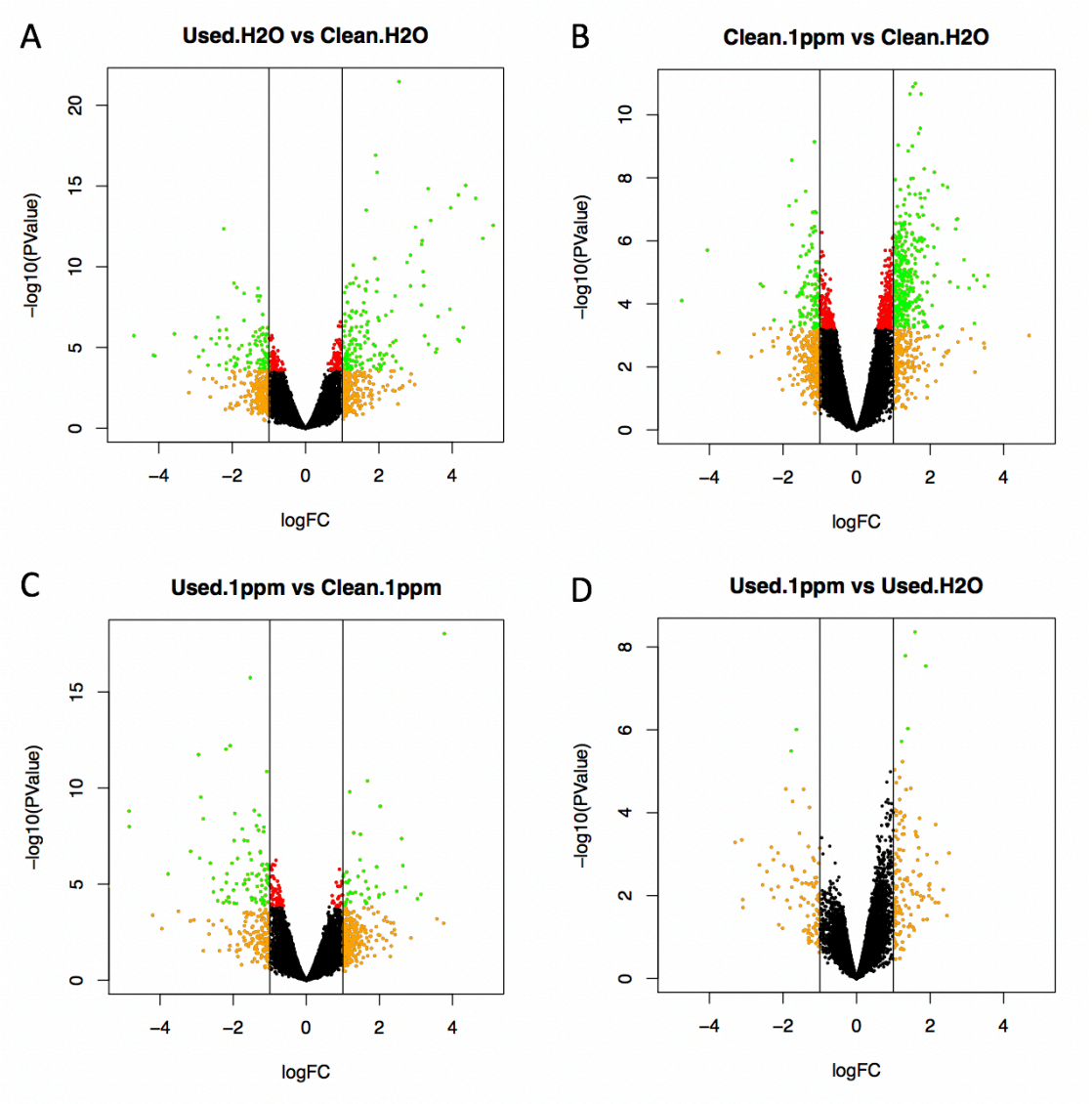

Figure 2.21 Volcano plot of DE genes in the 4 treatment contrasts. Red indicates FDR < 0.01, orange indicates FC > 2, green indicates both FDR < 0.01 and FC > 2. Black lines to indicate $\log_2$FC = 1 (FC = 2).

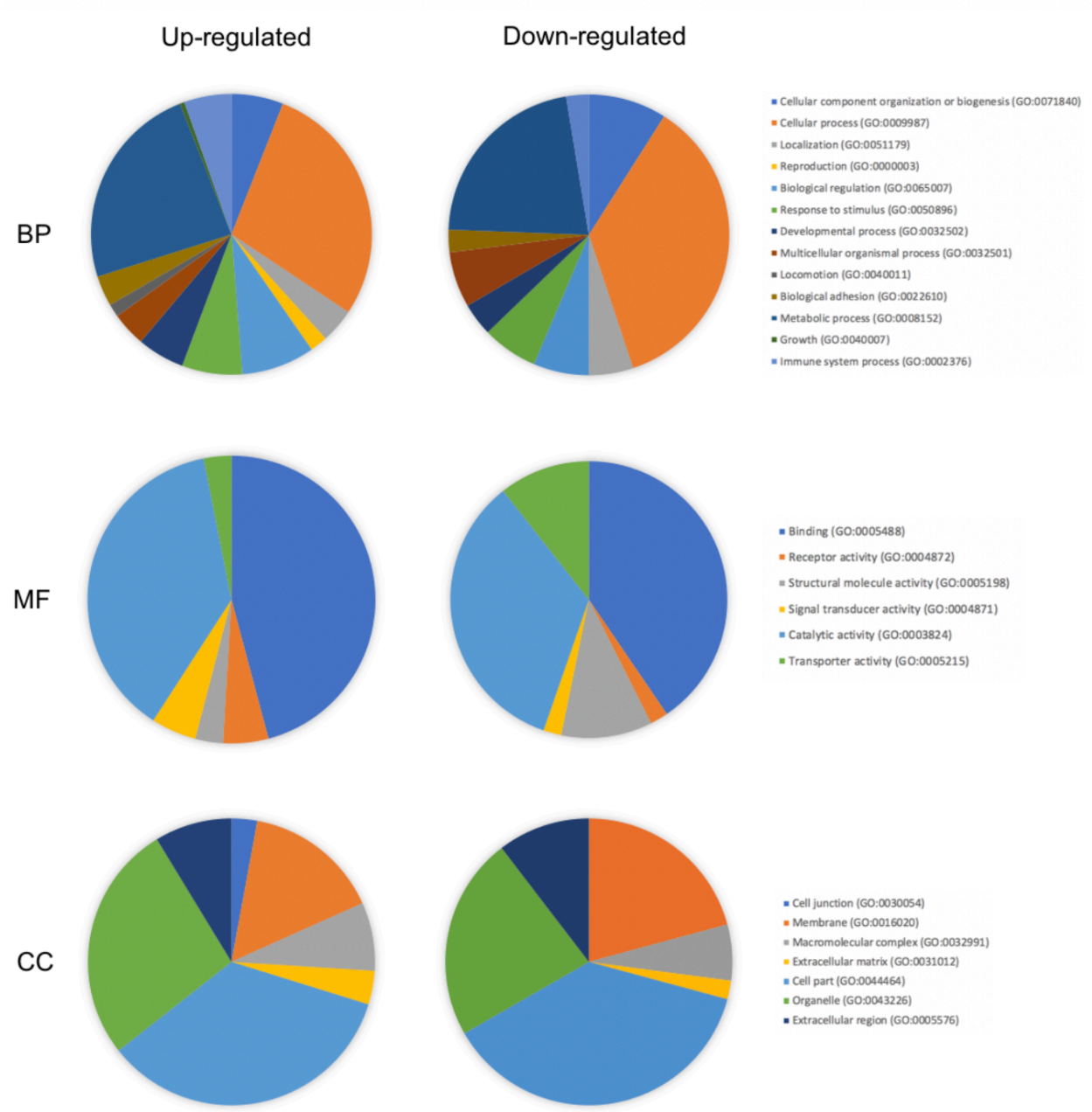

Figure 2.22 PANTHER functional classification tool pie chart of the ontology terms at the highest level based off the up-regulated and down-regulated genes in the used vs. clean litter with H_2O. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components.

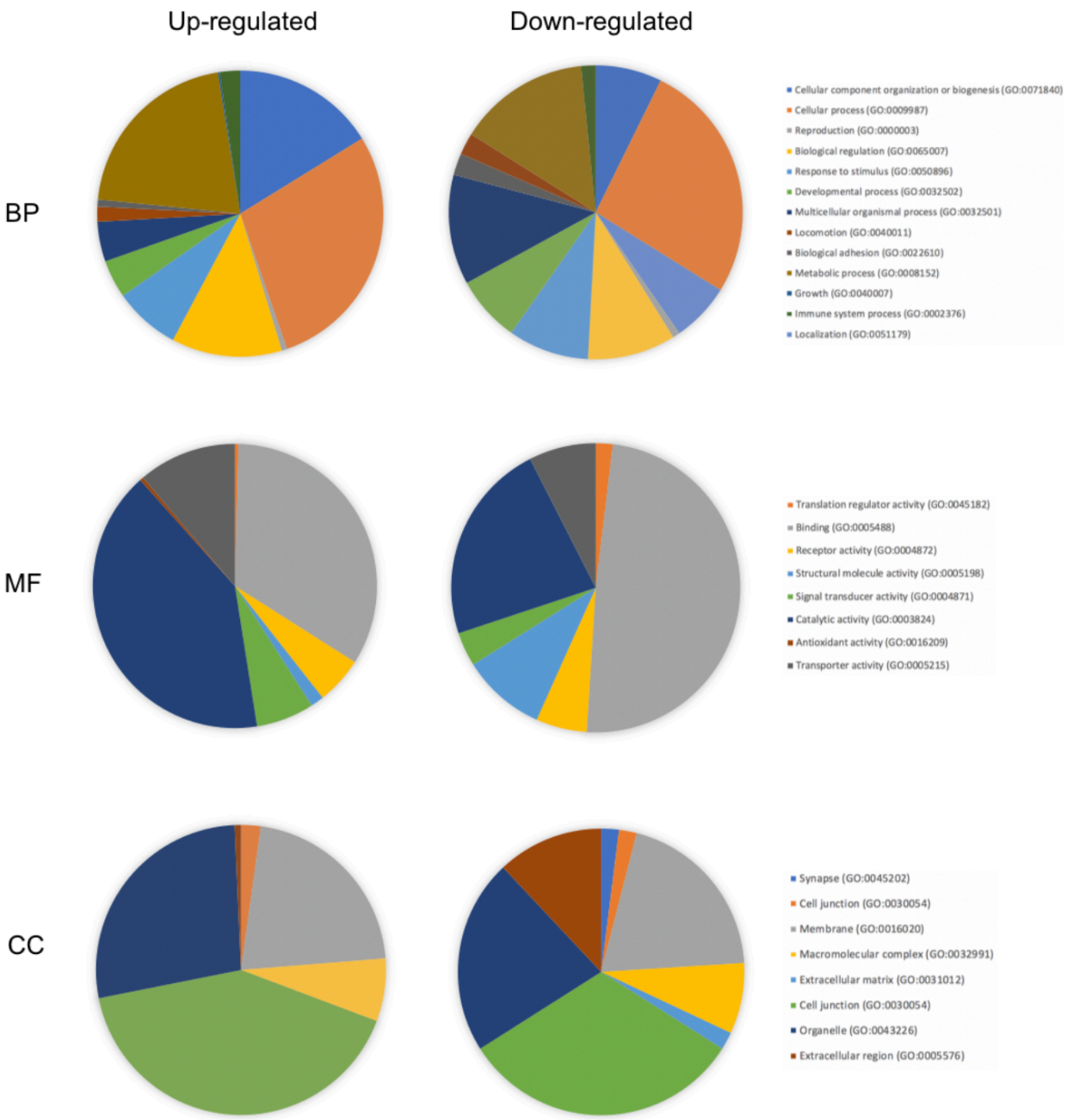

Figure 2.23 PANTHER functional classification tool pie chart of the ontology terms at the highest level based off the up-regulated and down-regulated genes in the 1 ppm vs H_2O with clean litter. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components.

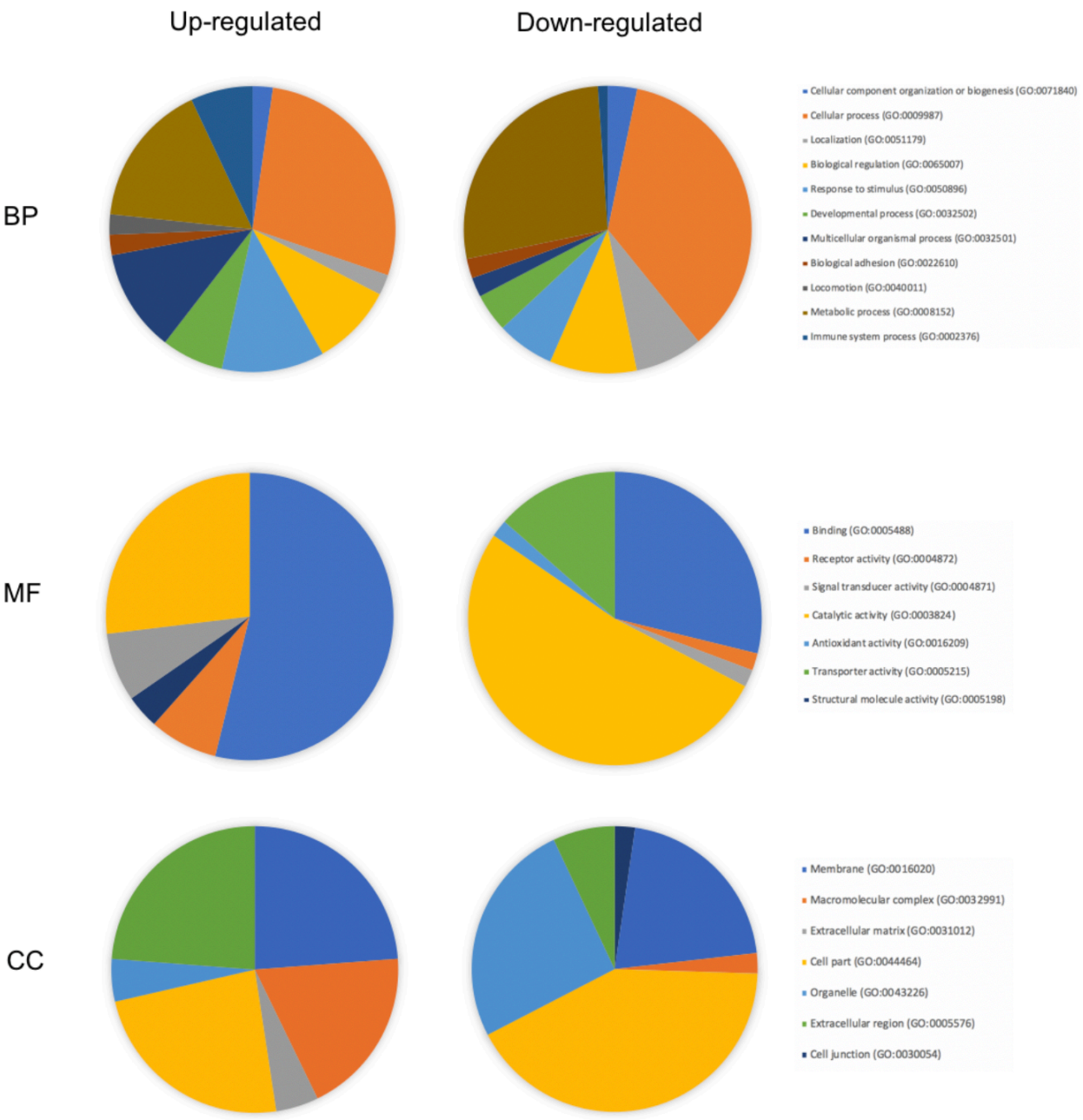

Figure 2.24 PANTHER functional classification tool pie chart of the ontology terms at the highest level based off the up-regulated and down-regulated genes between used litter and clean litter with 1 ppm iodinated water. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components.

- Chapter 3: The Effect of Iodinated Water and Used Chicken Litter on *Salmonella enterica* Colonization in Broilers.

3.0 Introduction

Salmonellosis is a world-wide problem that results in gastroenteritis, and can be fatal in very young, elderly, or immunocompromised people (Antunes et al., 2016). *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) is a common pathogen in mammals and birds, and the most common serotype causing human illness, often associated with poultry products and transmitted through poultry meat consumption (Cosby et al., 2015; Hugas & Beloeil, 2014; Suzuki, 1994). *S.* Enteritidis causes both symptomatic and asymptomatic infections in birds, often acute in young birds and asymptomatic in mature birds (Suzuki, 1994). Asymptomatic carriers are a risk for spreading *S.* Enteritidis to the entire flock causing reduced animal performance (Suzuki, 1994).

As pressure is increasing to reduce antibiotic use in animal production, new methods to maintain poultry health and production is in high demand. As well as looking at treatments for infection, mechanisms for prevention should be considered, such as maintaining water sterility. One major cause of pathogen spread in broiler barns, such as *Salmonella* and *Campylobacter*, is through contamination of the water lines and water reservoirs (Heyndrickx et al., 2002; Pearson et al., 1996). Current methods of water sterilization such as chlorine, ultraviolet radiation, and ozone have been found to be ineffective against some water-borne pathogens, and create disinfection by-products that have been associated with negative reproductive outcomes in poultry as reviewed by Shannon et al., (2008) and Reif et al., (1996). Bromide ions, which often result as a by-product of coal-fired power plants waste run-off, have been found in ground water and public water systems and have been found to decrease water and feed intake, and affect liver, kidney, and thyroid function in chickens (du Toit & Casey, 2010, 2012; Good & Vanbriesen, 2017; Hitchon et al., 1977).

Research has shown that adding iodine to water can counteract the negative effects of bromine found in well water often used in chicken production (du Toit & Casey, 2010, 2012). Iodine has also been used as a feed additive in poultry production as a means of increasing iodine in eggs, and has been shown to improve the feed efficiency and production in laying hens

(Opalinski et al., 2012; Röttger et al., 2012; Słupczyńska et al., 2014). Iodine is amongst the oldest substances used for disinfection, with current uses in human and veterinary medicine, and water treatment due to its antibacterial, antifungal, and antiviral properties (Gottardi, 1999). One study found that iodine in poultry drinking water resulted in increased body weight and feed efficiency, as well as positive behavioral changes, but failed to demonstrate the mechanisms (Emeash et al., 1994).

Another means to prevent pathogen colonization is fostering the development of a mature microbiome in young chicks. An early review on S. Enteritidis pathogenicity by Suzuki et al. (1994) proposed that the increased resistance to salmonellosis in mature birds was a result of a developed microbiome, as proposed by researchers in the early 1980's and 1990's (Corrier et al., 1991; Smith & Tucker, 1980; Suzuki, 1994). Many researchers have shown that giving chicks poultry intestinal microbiota orally works to reduce caecal pathogen colonization of *Salmonella* species through competitive exclusion of a mature microbiome (Barnes et al., 1979; Impey & Mead, 1989; Lloyd et al., 1977; Snoeyenbos et al., 1978; Stavric et al., 1985). The development of a healthy gut microbiome in young chicks is very important in infection resistance of environmental pathogens (Nurmi & Rantala, 1973). Over the first few days of life chicks are switching from an almost germ free state to the development of a mature microbiome, and they are the most susceptible for Salmonella colonization and infection at this time, so it is important to encourage this development promptly (Nurmi & Rantala, 1973). A healthy gut microbiome benefits the host by preventing infection through competitive exclusion and immune modulation (Oviedo-rondón, 2009). The microbiota primes the chicken immune system early on which can stimulate their immune defence mechanisms against pathogens such as *Salmonella* (Dibner et al., 1963). One potentially cost-effective method of introducing a diverse gut microbiome in day-old chicks is through the re-use of adult broiler litter.

Used poultry litter is a combination of bedding material and poultry fecal waste, that contains a diverse microbial community (Lovanh et al., 2007; Lu et al., 2003a). Poultry farms in the United States have the option to place new flocks on used litter, whereas in countries such as the United Kingdom, used litter is removed and houses are cleaned prior to the introduction of a new flock, as reusing litter can be a risk factor for introducing pathogenic bacteria such as *Campylobacter* (Lu et al., 2003b; Newell & Fearnley, 2003). The naïve microbiome of newly hatched chicks is low in microbial diversity and their environment in first week of life plays an

important role in the development of a diverse microbial community (Ballou et al., 2016; Oakley et al., 2014). Studies have shown that the introduction of used litter has a significant reduction on *Salmonella* colonization of species such as *S.* Typhimurium and *S.* Enteritidis in young chicks (Corrier et al., 1993; Corrier et al., 1992). However, research on the mechanism behind the benefits of used litter and how it impacts the microbiome of chicks is limited.

The benefits of iodinated water and used chicken have been demonstrated, but the underlying mechanisms have yet to be determined. In this study, we investigated the effects of iodinated water and chicken litter exposure on early establishment of intestinal microbiota, *Salmonella* resistance, and growth performance. We hypothesized that iodinated water and litter exposure would improve animal performance by reducing pathogen colonization of *Salmonella enterica* Enteritidis in young broiler chicks.

3.1 Materials and Methods

3.1.1 Ethics Statement

The animals in this study were used according to the guidelines of Canadian Council on Animal Care with approval of the University of Alberta Animal Care and Use Committee (AUP00001626). Chickens used in this study were managed by approved protocols at the Terrestrial and Aquatics Facilities at the Science Animal Support Services.

3.1.2 Animals and Experimental Design

Ninety-six 1-day-old Ross 708 broiler chicks were randomly separated into 32 micro-isolators, 3 chicks per isolator. Four isolators (n = 12) were randomly assigned to each treatment in a 2X3 factorial design with 2 levels of litter (clean or used), 2 levels of iodinated water (0 ppm or 1 ppm), and 2 levels of *Salmonella* infection (infected or uninfected). Food and water was administered *ad libitum*. Weights were taken on days 1, 5, and 10. Water was provided in bell drinkers and measured and replaced every 1-2 days. At day 3, half of the cages per treatment were infected by oral gavage with 0.15 mL of 1 x 10^6 CFU/ml *Salmonella enterica* serovar

Enteritidis PT4. Seven days post infection (day 10) the chicks were euthanized for sample collection.

3.1.3 Bacterial Strains

Birds were infected with *Salmonella enterica* serovar Enteritidis PT4 ATCC 4901 grown Luria-Bertani (LB) broth at 37°C for 16-18 hours shaking at 225 RPM. The broth culture was diluted in LB to a concentration of $1x10^6$ CFU/mL and enumerated to confirm infection dose.

3.1.4 Water treatment and measurement

Iodinated water was prepared by BioLargo Water Inc. Reverse osmosis water was pumped through the iSAN, a precision iodine dosing system, which dosed 25 ppm of iodine into water. The water was further diluted 1 in 25 to 1 ppm iodine concentration. The double distilled water (H_2O) control was supplied from a milli-q water purification system (MilliporeSigma, Burlington, MA, United States). Each cage received 500 mL of their designated water into bell drinkers. On day 3, 5, 6, 7, 8, 9, and 10 the remaining water in the bell drinkers was measured in a 500 mL graduated cylinder, discarded, and replaced with freshly made 1 ppm iodinated water or ddH_2O.

3.1.5 Litter treatment

The used pine shaving litter was acquired from the floor of adult chicken pens at the Poultry Research Centre at the University of Alberta. All 16 isolators were bedded with clean pine shaving litter, and 8 isolators were administered an additional 5 grams of used pine shaving litter day 1. The other 8 bedded isolators received only clean wood chip litter. Cages were changed when the litter was determined to wet and caked, and at each cage change 5 grams of bedding was transferred from the previous isolator to the cleaned isolator in all treatments.

3.1.6 Animal Euthanasia and Sample Collection

At 7-days post infection 48 chickens were euthanized via cervical dislocation, severing the spinal cord and separating the vertebrae so that the bird does not recover consciousness. After euthanasia, the abdominal cavity of the chickens was wetted with 70% ethanol and opened with sterile surgical scissors and forceps. The small intestine and large intestine from the Meckel's diverticulum to the cloaca was removed. The ileum was carefully dissected from the base of the Meckel's diverticulum to the top of the ileal-caecal junction. Approximately 200 μL of ileum digesta was collected into sterile 1.5 mL Eppendorf tubes and snap frozen in liquid nitrogen until storage at -80^{o}C. Five mm of the tip of the caeca was fixed for histology (10% formalin, Fisher Scientific). Approximately 200 μL of caecal digesta was collected aseptically into a sterile 1.5 mL Eppendorf tubes and snap frozen in liquid nitrogen until storage at -80^{o}C.

3.1.7 Quantitative RT-PCR for Salmonella quantification

To quantify the abundance of *Salmonella* in ileal contents, quantitative RT-PCR was used. Total DNA was extracted from the snap frozen ileal digesta with the QIAamp DNA Mini Stool Kit (Qiagen, Inc. Mississauga, ON, Canada) following the manufacture protocol with an additional bead-beading step using a FastPrep-24 (MP Biomedicals) at 6.0 meters per second for 30 seconds with garnet beads (BioSpec Products, Bartlesville, OK). Each samples DNA concentration was measured by Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific) and diluted to 10 ng/uL. *Salmonella* enterotoxin gene (Stn) was chosen for amplification with Stn specific forward and reverse primers (5'-CTT TGG TCG TAA AAT AAG GCG-3', 5'-TGC CCA AAG CAG AGA GAT TC-3') (Ziemer & Steadham, 2003). A standard curve was generated from a pure culture using a single colony of *Salmonella enterica* Enteritidis PT4 added to LB broth and incubated for 16-18 hours at 37^{o}C shaking. The broth culture was centrifuged to pellet the cells and supernatant was removed. The pelleted Salmonella was extracted with the QIAamp DNA Mini Stool Kit (Qiagen, Inc. Mississauga, ON, Canada). The quantity of DNA was determined using a Qubit Fluorometer (ThermoFisher Scientific). The concentration of DNA and *Salmonella enterica* Enteritidis PT4 genome length (4, 804, 382 base pairs) was entered into the URI Genomics & Sequencing Center website (http://cels.uri.edu/gsc/cndna.html) to calculate the double stranded copy number of Stn gene. A 10-fold serial dilution was performed to create a standard curve. Each reaction mixture was a

total of 20 uL and contained 10 uL sybr green mix, 1 ul forward primer, 1 ul reverse primer, 3.5 uL nuclease free water, and 4.5 uL DNA. The real time PCR was performed on an ABI StepOne^{TM} real-time System (Applied Biosystems, Foster City, CA) at 94^{o}c for 5 min, followed by 35 cycles at 94^{o}C for 30 seconds, 56^{o}C for 30 seconds, and 72^{o}C for 30 seconds for the cycling stage, with a melt curve stage of 72^{o}C for 7 minutes, 94^{o}C for 15 seconds, 65^{o}C for 1 minute, and 94^{o}C for 15 seconds.

3.1.8 Histology

The first 5 mm of the tip of the caeca was fixed in 10% formalin at room temperature. After 24 hours, the formalin was replaced with 70% ethanol. Caecal tissues were embedded in paraffin and sectioned into 5 um slices and stained with Haematoxylin and Eosin (H&E) staining. Using the EVOS FL Auto Imaging System (Thermo 215 Scientific, Nepean, ON) images of the tissues were taken and assessed for pathology. Three images were taken from each caecum, with 5 measurements per image, equaling 15 measurements per chick. Villus height was measured top of the villus surface epithelium to the top of the lamina propria, crypt depth measured from base upward to the region of transition between the crypt and villus, and villus width was measured within the top 3rd of the villus height.

3.1.9 Microbial Analysis

3.1.9.1 16S rRNA gene amplification and sequencing

Total DNA was extracted from caecal contents using the QIA stool extraction kit (Qiagen Inc., Valencia, CA), where contents were added into 2mL tubes containing 2.0 mm diameter garnet beads (BioSpec Products, Bartlesville, OK) with the addition of a 60 second bead-beating step at 6.0 meters per section (FastPrep instrument, MP Biomedicals, Solon, OH, USA) to physically disrupt cell walls of gram-positive bacteria. The V3V4 region of the 16s rRNA gene was amplified as indicated in the Illumina protocol (16S Metagenomic Sequencing Library Preparation) with the following primers: (Forward 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’ and Reverse 5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). A paired-end sequencing run with 2 x 300 cycles was run on the Illumina MiSeq Platform (Illumina Inc. San Diego, CA).

3.1.9.2 Microbial composition analysis

The raw sequencing data obtained was checked for quality using FastQC 0.11.5 (Babraham Bioinformatics) and primers were removed and the paired end reads were merged using PANDAseq algorithm (Masella et al., 2012). The resulting merged reads were dereplicated and singletons were discarded using Vsearch 2.7.1 (Rognes et al., 2016). The reads were clustered into operation taxonomic units (OTUs) that were greater than 97% similar using USEARCH 7 (Navas-Molina et al., 2013). Reads were mapped against the Usearch 7.1 database and chimeras were removed using the gold database in Usearch 7.1 (Navas-Molina et al., 2013). OTUs were labelled using the Usearch 7.1 python script (Navas-Molina et al., 2013). Reads were mapped back to OTUs using the Vsearch 2.7.1 (Rognes et al., 2016), and tab-delimited OTU table was generate using USEARCH 7.1 python uc2otutab.py (source). Using the QIIME (Quantitative Insight into Microbial Ecology) 1.9.1 pipeline, taxonomy was assigned using QIIME Ribosomal Database Project (RDP) classifier (Wang, Garrity, Tiedje, & Cole, 2007), and alpha and beta diversity were determined using the QIIME core_diversity_analyses.py. OTUs that were present in more that 30% of at least one treatment were considered to be true and kept for analysis. Any present in less that 30% of only one treatment group were deleted and assumed artifacts of sequencing. OTU Venn diagram was created at http://bioinformatics.psb.ugent.be/webtools/Venn/ (VIB/UGent Bioinformatics & Evolutionary Genomics, Gent, BE).

3.1.10 Statistical Analysis

The data is expressed as mean +/- SEM. The data from the weight gain, water consumption, quantitative real time-PCR for *Salmonella* quantification, alpha diversity and histology measurements were analyzed for outliers analyzed in Prism using the Robust regression and Outlier removal (ROUT) method in Prism (Motulsky & Brown, 2006). The

weight gain, water consumption, quantitative real time-PCR for Salmonella quantification, alpha diversity and histology measurements were tested for normality using the Shapiro-Wilk W statistical measures (SAS University Edition). Any non-normal distributed data as indicated by a Shapiro-Wilk P-value less than 0.05 was log transformed for further statistical analysis. The data was first analyzed as a 2 by 2 factorial design comparing litter and water treatment on the data (Proc mixed ANOVA, Bonferroni adjusted P value for multiple comparisons). If no combined effects were observed between water and litter on the *Salmonella* quantification, alpha diversity and histology measures, the main effects of treatment were shown. If no combined effects were observed between water and litter on weight gain or water consumption, the main effects of treatment were further analyzed by a repeated measures ANOVA (Proc mixed ANOVA, repeated measures, Bonferroni adjusted P value). The beta diversity analysis was calculated using Adonis. The beta diversity within treatment distances between clean and used litter was analyzed with a Bonferroni corrected student's t-test. The relative phylum, family, and genus abundances were log transformed plus 1 to create normality and remove zeros from the data set, and analyzed as a 2 by 2 factorial design comparing litter and water treatment on the data (Proc mixed ANOVA, Satterthwaite, Bonferroni adjusted P value for multiple comparisons).

3.2 Results

3.2.1 1 ppm iodinated water was consumed more than ddH_2O

To determine if the birds were averse to consuming the iodinated water, water disappearance was measured through the course of the study. There was no combined effect of water and litter treatment on the consumption, and water type did not have a significant effect on water disappearance. Overall, water disappearance was not significantly different on any of the days (Figure 3.1).

3.2.2 Used litter but not iodinated water had a significant impact on early life weight gain

To determine whether these treatments had an effect on the weight gain of both the infected and uninfected birds, we recorded weights on day 1, 5, and 10 and analysed total weight

gain (TWG) and average daily gain (ADG). There was no effect of water type, litter type, or infection on the TWG and ADG of the birds over the 10 days. Weight gain from day 1 to day 5, and day 5 to day 10 were analyzed in a 2X3 factorial design and no effect of water type or salmonella infection, and no combined effects of treatment were found, however there was a significant effect of litter type. For more statistical power, this data was analyzed as repeated measures, comparing the effect of litter treatment on the weight gain from day 1 to day 5, and day 5 to day 10. At the second timepoint, used litter group gained significantly more weight than the clean litter group from day 5 to day 10 (Figure 3.2).

3.2.3 Litter significantly reduced Salmonella colonization in the ileum

To determine if used chicken litter or iodinated water had a significant impact on infection resistance to *Salmonella*, birds were infected by oral gavage on day 3. It was found that the used litter had a significant impact on decreasing Salmonella colonization in the ileum, and that iodinated water on clean litter increased colonization compared to the other 3 treatments (Table 3.1, Figure 3.3).

3.2.4 Litter treatment but not iodinated water significantly increased the villus height and crypt depth in the challenged birds

Figure 3.4 demonstrates the visible differences in caecal morphology between the used litter treatment with *Salmonella* compared to clean litter with *Salmonella*, 1 ppm iodinated water with *Salmonella* and uninfected control, showing longer thinner villi with more villi present. *Salmonella* infection decreased villus width, but did not affect villus height, crypt depth, and number of villi in 200µm comparing the infected to uninfected control (Figure 3.5). Iodine with *Salmonella* infection was not significantly different than the infected control in all four measurements. Used chicken litter treatment group with *Salmonella* infection significantly increased the villus height, crypt depth, and number of villi in 200µm (Figure 3.5 A, C, & D). All three infection treatments had decreased villi width compared to the uninfected control (Figure 3.5 B).

3.2.5 Litter treatment altered caecal microbial community structure in the uninfected control birds

There was an overall effect of treatment on the microbial community structure of the caeca based on the unweighted UniFrac and weighted UniFrac distances (Figure 3.6, Adonis P = 0.001). The 4 treatment groups were analyzed separately to determine the effect of iodinated water and litter treatments independently on the microbial composition in the caeca. Iodinated water had a significant impact on the microbiome of the clean litter treatments only with unweighted unifrac, (Adonis $P < 0.05$), but had no significant impact in the presence of used chicken litter (Figure 3.7). Used litter had a significant effect on the composition of the microbiome on both 1 ppm iodinated and ddH_2O water, both weighted and unweighted unifrac distance matrices (Figure 3.8, Adonis $P = 0.001$). The alpha diversity of the microbiomes was analyzed using Shannon, Chao1, and Simpson indices. Used litter increased all alpha diversity measures, while iodinated water did not have an impact on alpha diversity in clean or used litter treatment (Table 3.2, Figure 3.9, $P < 0.0001$). Next, we looked more specifically at the impact that used litter had on the microbiome in the presence of regular water and 1 ppm iodinated water. We compared the beta diversity, looking at the within treatment distances, and found the clean litter group to be significantly more distant within treatment than the used litter using the unweighted unifrac distance metric (Bonferroni corrected student's t-test, $P < 0.001$, Figure 3.10). Comparing the percent relative abundance at the phylum level it was found that iodinated water had no effect on the relative abundance, however, iodinated water had a significant impact on the percent relative abundance of genus *Blautia* in both the clean and used litter treatments (Figure 3.11). The relative abundance of *Blautia* was 20 times higher in the clean litter group (10.6 percent relative abundance), to that of the used litter group (0.53 percent relative abundance).

There was no combined effect of litter and iodinated water treatment on phylum level, so the main effects of litter were analyzed (Table 3.3). The phylum *Bacteroidetes* was significantly increased in the presence of used litter, and the phylum *Proteobacteria* was significantly reduced by used litter (Figure 3.12). Interestingly, unclassified *Enterobacteriaceae* family and specifically the *Klebsiella* genus were significantly higher percent relative abundance in the clean litter treatment (Figure 3.13 and 3.14). The introduction of used litter also significantly

increased the relative abundance of 9 bacterial taxa including *Ruminococcaceae*, unclassified *Clostridiales*, *Christensenellaceae*, *Mogibacteriaceae*, unclassified *RF39*, *Rikenellaceae*, *Bacillaceae*, *Bacteroidaceae*, and Unassigned (Figure 3.13). At the genus level shown in figure 3.14, used litter increased *Faecalibacterium* ($P < 0.0001$), *Coprococcus* ($P = 0.002$), *Lachnospira* ($P = 0.003$), *Clostridium* ($P < 0.0001$), *Dorea* ($P < 0.0001$), *Holdemania* ($P = 0.01$), *Bacteroides* ($P = 0.02$), *Bacillus* ($P = 0.03$), and *Eubacterium* ($P = 0.03$). Used litter decreased the relative abundance of 4 bacterial taxa at the family level, including *Leuconostocaceae*, *Lachnospiraceae* (specifically *Blautia* genus), *Enterococcaceae* (specifically *Enterococcus* genus), and *Enterobacteriaceae* (specifically *Klebsiella* genus) (Figure 3.13 and 3.14). There was a significant combined effect of litter and iodinated water treatment on two families *Leuconostocaceae* and *Clostridiaceae* (Figure 3.13). At the genus level with *Klebsiella*, *Erysipelotrichaceae cc 115*, and *Blautia*, there was a combined effect of treatment (Figure 3.15).

A total of 203 unique OTUs were identified in all treatments combined. Used litter introduced 125 unique OTUs to the caecal microbiome that were otherwise absent in the clean litter group (Figure 3.16). Interestingly, 2 unique OTUs were present in the used litter plus iodinated water group that were not present in the used litter and H_2O, an unclassified member of the *Clostridales* order, and unclassified member of the *Ruminococcaceae* family. The 125 unique OTUs introduced by the used litter were assigned to 28 taxonomic phylogenies (Table 3.4).

3.3 Discussion

The re-use of poultry litter and use of iodinated water in poultry production has shown to be beneficial in growth and disease resistance in birds, however current research on the mechanisms behind their conferred benefits is very limited. Our research shows that used litter can significantly reduce *Salmonella* colonization, modify the composition of the caecal microbial community and impact gut development. As well, we show that iodinated water has a very limited effect on the caecal microbiome on used litter. However, on clean litter iodine changes the microbial community and increases *Salmonella* colonization.

Contrary to our hypothesis, 1 ppm iodinated water had a limited effect on the intestinal microbial community, *Salmonella* colonization, intestinal morphology and growth in 10-day old chickens.. Interestingly, 1 ppm iodinated water did not reduce *Salmonella* colonization in the

chick ileum, and when given to chicks on clean litter it increased colonization compared to regular water. As we previously showed, iodinated water in the absence of a diverse microbial community increased genes associated to inflammation in the chicken cecum. This activation of inflammation in this treatment may prove to be a benefit to *Salmonella* colonization, as inflammation increases *Salmonella*'s ability to compete with other gut commensals (Thiennimitr et al., 2011). There was no difference in water consumption between the two groups, indicating that the chicks were not averse to consuming the 1 ppm iodinated water, and iodinated water may still have a role in reducing the spread of pathogens among birds as it is actively antimicrobial (Gottardi, 1999).

Iodinated water had a limited effect on the caecal microbiota on the chicks, as it had no effect on the alpha diversity in either litter treatments, and there were no differences in the community composition on the weighted unifrac, which considers the abundance of OTUs, in either litter treatment. However, it did affect the community composition on the unweighted unifrac of the chickens on clean litter, which weighs all OTUs the same, suggesting that it affected the presence or absence of low abundant bacteria. Iodinated water significantly decreased unclassified *Clostridiaceae* and *Ruminococcaceae*, which are members of the *Clostridiales* order, specifically known for their health benefits as large contributors to short chain fatty acid production (Jumpertz et al., 2011). Iodinated water increased *Leuconostocaceae*, a member of the *Firmicutes* phylum, that has genus level members used as a probiotic in the poultry industry (Seo et al., 2012). Unclassified *Blautia* was also increased in iodinated water, and is a genus commonly found in both humans and animals, including chickens, and is from the family *Lachnospiraceae* which is known for breaking down complex polysaccharides into short chain fatty acids that are beneficial to the host (Biddle et al., 2013; Eren et al., 2015). Interestingly, although iodinated water seemed to increase *Salmonella* as indicated by qPCR, it decreased the relative abundance of *Klebsiella*, another member of the *Enterobacteriaceae* family. Iodinated water in the clean litter treatment had fewer unique OTUs, and fewer total OTUs compared to the distilled water treatment groups. This reduction in species richness in a sterile environment may explain the increase in *Salmonella* colonization during infection, by reducing the competition of the host microbiome (Lloyd et al., 1977; Nurmi & Rantala, 1973). However, one limitation of our study is that the chicks were raised in sterile microisolators,

which do not allow for the introduction of microbes from there environment that would be introduced in a barn setting (Ballou et al., 2016; Oakley et al., 2014).

Used chicken litter did not have a significant impact on the total weight gain or average daily gain of the birds compared to clean litter. Previous research has indicated higher growth rate in chickens reared on re-used litter (Kennard & Chamberlin, 1951). However, other studies have shown that used litter does not have a significant impact on growth rate or feed conversion over longer term growth (Jones & Hagler, 1983; Mccartney, 1971; Yamak et al., 2015). We have previously shown that used litter activates the expression of genes involved in response to virus and biotic stimulus, and it is likely that the used litter not only introduced commensal microbes, but also viruses, promoting inflammation and stunting the initial growth of the birds. Although there was no impact on the total weight gain, in the present study used litter significantly increased weight gain from day 5 to day 10 which was similar to research conducted by Vieira & Moran (1999), that showed that although used litter reduced weight gain in the first 21 days, from days 21 to 42 their growth was higher than clean litter, compensating for the initial stunting (Vieira & Moran, 1999). The introduction of used litter dynamically changed the microbiome, and as a developed microbiome plays important roles in host digestion and nutrient absorption, this suggests that after the initial shock of new species, the rate of weight gain increased from days 5 to 10 as a result.

Similar to previous research, the present study showed that *Salmonella* colonization was significantly decreased in the chicks as a result of used litter (Corrier et al., 1992; Corrier et al., 1993). When analyzing the caecal microbiome of the uninfected birds, our research indicated that used litter has a significant effect on both the relative abundance and presence or absence of bacteria in the microbiota, regardless of water treatment. As well, used chicken litter increased the richness and evenness of the caecal microbiota. The addition of used litter created tighter clustering of samples compared to clean litter, increasing the stability of the microbiome within treatment, showing that succession is less chaotic with litter exposure. The significant change to microbial community on the unweighted unifrac distance was likely due to the introduction of 125 unique OTUs, not present in the clean litter group, that were able to fill more niches within the caecum. It is possible that increased diversity of bacteria and species richness in dirty litter exposed birds may act to competitively exclude pathogenic bacteria such as *Salmonella*. Used litter significantly increased the relative abundance of *Ruminococcaceae*, *Christensenellaceae*,

Mogibacteriaceae, along with other unclassified members of the *Clostridiales* order, which have been shown to be the majority of sequences in the chicken microbiome, and are important contributors to SCFA metabolism (Oakley et al., 2014). However, one genus increased within the *Clostridiales* order was *Clostridium*, which contains some pathogenic species such as *Clostridium* perfringens, and well know chicken pathogen for causing necrotic enteritis in chickens (Hofacre et al., 2002). However, we were unable to detect to the species level, and so are not sure if this is a detrimental organism. There are numerous commensal *Clostridium* species that play important roles in resistance to infection along with immune system development and modulating immunological tolerance in humans (Lopetuso et al., 2013). Used litter also increase *Bacillaceae*, a member of the *Firmicutes* phylum, along with the *Clostridiales* order, which have been associated with an increase in nutrient absorption in humans (Jumpertz et al., 2011). Used litter also increased in *Rikenellaceae* and *Bacteroidaceae*, both members of the *Bacteroidetes* phylum, which makes up the second largest phylum, as well as unclassified *RF39*, a member of the *Tenericutes* phylum, which makes up a very small proportion of the chicken microbiota as reviewed by Oakley et al., 2014. When looking at the specific genus level changes, used litter increased *Bacteroides*, a genus known for their commensal activity and modulation of the host immune system (Oviedo-rondón, 2009). *Bacteroides*, a key member of the microbiome of most animal species, were minimally detected in non-litter treatments, suggesting that *Bacteroides* do not survive the incubation and hatching process indicating that birds arriving from the hatchery are not sufficiently microbially exposed.

Used litter also decreased certain bacterial families, such as *Leuconostocaceae*, *Lachnospiraceae*, *Enterococcaceae*, and *Enterobaceriaceae*, which is congruent with our previous findings. Members of both the *Leuconostocaceae* and *Enterococcaceae* families have been used in poultry for probiotics (Pourakbari et al., 2016; Seo et al., 2012), while members of the *Lachnospiraceae* family are butyrate producers in the intestine of chickens (Rinttilä & Apajalahti, 2013). Although these families contain beneficial microbes, it is likely that the introduction of other commensals within the *Clostridiales* order were more suited for the niche, and reduced the relative abundance of these bacteria. Importantly, used litter reduced total *Proteobacteria*, specifically *Klebsiella* and unclassified *Enterobacteriaceae*. The *Enterobacteriaceae* family includes common pathogenic genera including *E. coli*, *Salmonella*, *Proteus*, and *Klebsiella*; food borne pathogens known to cause disease in humans and animals

(Wu et al., 2016). Previous studies have shown that reduced *Salmonella* as a result of re-used litter is likely due to the introduction of normal microbiota, indicated by significant increases in volatile fatty acids in the caecum (Corrier et al., 1992; Corrier et al., 1993), and through our research we were able to identify the specific changes and addition of bacteria to the cecal microbial community achieved by the addition of used litter.

Used litter with *Salmonella* infection significantly modified caecal morphology, increasing villus height, crypt depth, and increasing the number of villi present, therefore increasing the available surface area for absorptive capacity. This is likely due to higher abundance of bacterial fermentation products, SCFA's, which have been shown to promote the development of the mucosal epithelium through increasing the intestinal epithelial cell turnover and migratory activity (Park et al., 2016), and are key energy sources for the colonocytes, specifically butyrate, which acts as a gene regulator for intestinal epithelial cells (Bedford & Gong, 2018; Nepelska et al., 2012). Other studies investigating the effect of direct fed microbials and probiotics in chickens during *Salmonella* infection have also showed increased villus height, width, villus height to crypt depth ratio, as well as total surface area (Biloni et al., 2013; Salim et al., 2013). Iodine with *Salmonella* infection did not have any effect on the caecal morphology, measuring the same as the infected control, likely because it did not reduce *Salmonella* colonization, and had limited effects on the microbiome. Previous research has indicated that *Salmonella* infection decreases villus height, villus height to crypt depth ratio, and villus surface area was higher in non-infected birds (Rajani et al., 2016), however in our trial we found that width decreased as a result of infection, while villus height, crypt depth and number of villi did not change. One reason we may not see the same effect of *Salmonella* on these measures compared to our controls is because our controls were on clean litter in sterile microisolators, while this other study was conducted in floor pens in barns, a less sterile environment with more microbial interaction.

3.4 Conclusion

The present study provided evidence that re-used chicken litter has a significant impact on the caecal morphology and microbial ecology in the chick, resulting in suppressed *Salmonella enterica* Enteritidis colonization. Increasing gastrointestinal health and reducing infection

provides strong evidence that re-using chicken litter can be beneficial for chicken rearing. This may suggest that there needs to be a paradigm shift, where increased microbial exposure to a mature microbiome is seen as a pathogen prevention strategy rather than a source of pathogens. A better approach may be to allow for litter re-use unless there was a disease outbreak or high pathogen loads in the previous flock. Future research will be to identify microbes responsible for increasing this stability so that they can be introduced in a controlled manner. The minimal effects of iodinated water on the microbiome would suggest that this is not the mechanism supporting improved performance observed in other studies and may indeed increase *Salmonella* colonization in infected birds in the absence of a developed microbiome. However, in our experiment we compared clean sterile water with iodinated water, and therefore different effects may be seen if we compared iodinated water to farm well water. Further research is needed to determine if iodinated water reduces the transmission of infection through contaminated water.

Tables

Table 3.1 Average log copy number of *Salmonella* enterotoxin gene in the ileal contents of *Salmonella* challenged chickens. *P*-value indicates the significant between the comparisons of the 4 treatments, H_2O used, H_2O clean, 1 ppm used, and 1 ppm clean. *P*-value < 0.05 is considered significant

Comparison	*Mean ± SEM of 1*	*Mean ± SEM of 2*	*Adjusted P-value*
H_2O/Used vs. H_2O/Clean	2.500 ± 0.00	3.366 ± 0.23	0.0005
H_2O/Used vs. 1 ppm/Used	2.500 ± 0.00	2.500 ± 0.00	> 0.9999
H_2O/Used vs. 1 ppm/Clean	2.500 ± 0.00	3.967 ± 0.17	< 0.0001
H_2O/Clean vs. 1 ppm/Used	3.366 ± 0.23	2.500 ± 0.00	0.0009
H_2O/Clean vs. 1 ppm/Clean	3.366 ± 0.23	3.967 ± 0.17	0.0300
1 ppm/Used vs. 1 ppm/Clean	2.500 ± 0.00	3.967 ± 0.17	< 0.0001

Table 3.2 Alpha diversity measures of Shannon, Inverse Simpson, and Chao1 indices. Numbers indicate *P*-value for each comparison treatment groups. *P*-value < 0.05 is considered significant.

Comparison	Shannon	Chao1	Inverse Simpson
1ppm clean - 1ppm used	5.67E-06	1.79E-05	1.79E-05
1ppm clean - H_2O clean	1.00E+00	1.00E+00	1.00E+00
1ppm used - H_2O clean	2.32E-04	8.02E-05	8.02E-05
1ppm clean - H_2O used	8.26E-04	1.04E-02	1.04E-02
1ppm used - H_2O used	1.00E+00	1.00E+00	1.00E+00
H_2O clean - H_2O used	1.35E-02	3.06E-02	3.06E-02

Table 3.3 Relative percent abundance at phylum level. P-value < 0.05 indicates significant effect of treatment.

OTU	H_2O_clean	1ppm_clean	H_2O_used	1ppm_used	FDR P-value
Proteobacteria	20.273	17.090	3.195	1.175	<0.0001
Tenericutes	0.000	0.000	0.546	3.300	< 0.0001
Bacteroidetes	0.054	2.510	17.977	7.359	0.0003
Actinobacteria	0.026	0.000	0.374	0.240	<0.0001
Unassigned	0.037	0.049	0.102	0.345	0.15
Firmicutes	79.593	79.628	74.120	87.522	0.24
Verrucomicrobia	0.015	0.717	1.885	0.009	0.32

Table 3.4 Unique OTUs and assigned taxonomy. Number of OTUs assigned to each taxonomy including relative percent abundance as introduced by used chicken litter.

Phylogenetic assignment	Unique OTUs	Percent relative abundance
p_Actinobacteria		
f__Bifidobacteriaceae; g__Bifidobacterium	1	0.410
p_Bacteroidetes		
f__Porphyromonadaceae; g__Parabacteroides; s__distasonis	1	0.536
p_Firmicutes		
f__Bacillaceae; g__Bacillus	1	0.174
o__Clostridiales; f__[1]	29	8.906
f__[Mogibacteriaceae]; g__[1]	2	0.027
f__Christensenellaceae; g__[1]	1	0.041
f__Clostridiaceae; g__[1]	1	0.021
f__Clostridiaceae; g__Candidatus Arthromitus	1	0.110
f__Clostridiaceae; g__Clostridium	2	0.153
f__Lachnospiraceae	16	2.451
f__Lachnospiraceae; g__Anaerostipes	1	0.005
f__Lachnospiraceae; g__Blautia	1	0.275
f__Lachnospiraceae; g__Coprococcus	1	0.014
f__Lachnospiraceae; g__Dorea	1	0.091
f__Lachnospiraceae; g__Lachnospira	1	0.529
f__Ruminococcaceae; g__[1]	34	5.551
f__Ruminococcaceae; g__Anaerotruncus	1	0.051
f__Ruminococcaceae; g__Faecalibacterium; s__prausnitzii	1	0.090
f__Ruminococcaceae; g__Oscillospira	8	0.845
f__Ruminococcaceae; g__Ruminococcus	9	0.530
f__Erysipelotrichaceae; g__[Eubacterium]; s__dolichum	1	0.169
f__Erysipelotrichaceae; g__cc_115	1	0.073
f__Erysipelotrichaceae; g__Coprobacillus	1	0.101
f__Erysipelotrichaceae; g__Holdemania	1	0.036
p_Proteobacteria		
f__Desulfovibrionaceae; g__Bilophila	1	0.119
p_Tenericutes		
f__Anaeroplasmataceae; g__Anaeroplasma	1	1.644
o__RF39; f__;[1]	4	0.314
Unassigned	2	0.208

[1]Unclassified order or family;

Figures

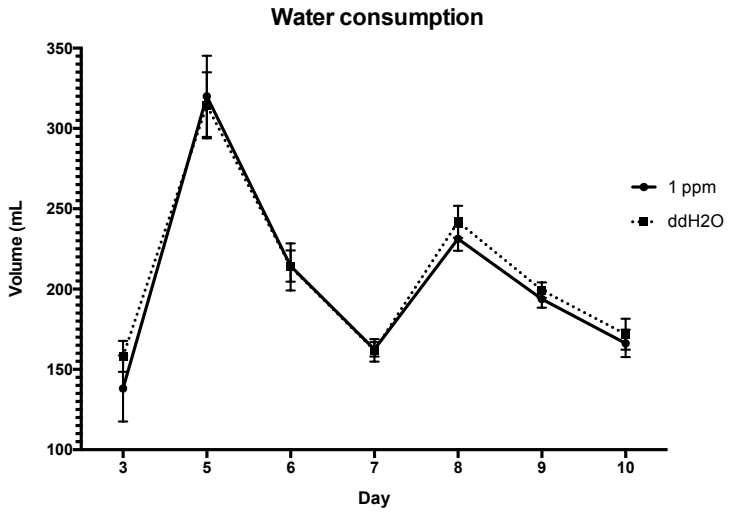

Figure 3.1 Effect of water type on water disappearance. Water volume remaining in the bell drinkers was not different between the 1 ppm iodinated water and distilled water (N = 12) ($P > 0.05$).

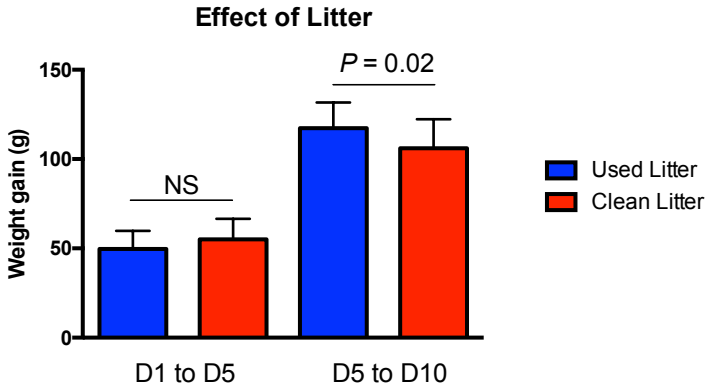

Figure 3.2 Effect of litter treatment on weight gain. There was no effect on weight gain from day 1 to 5 ($P > 0.05$). However, chicks reared on used litter gained significantly more weight between day 5 and day 10 of growth (N = 12, $P = 0.02$).

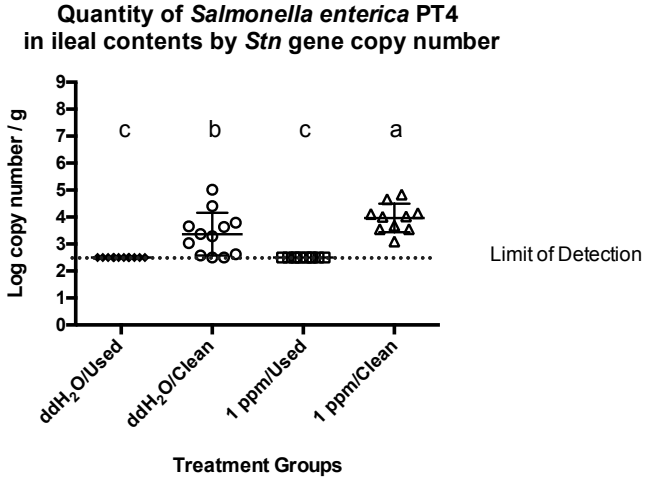

Figure 3.3 Effect of water and litter on *Salmonella enterica* Enteritidis PT4 colonization. Regardless of water treatment, used litter had the most significant effect on reducing *Salmonella enterica* colonization. On clean litter, 1 ppm iodinated water increased *Salmonella enterica* colonization. Non-similar letters (a/b/c) denotes significant differences ($P < 0.05$).

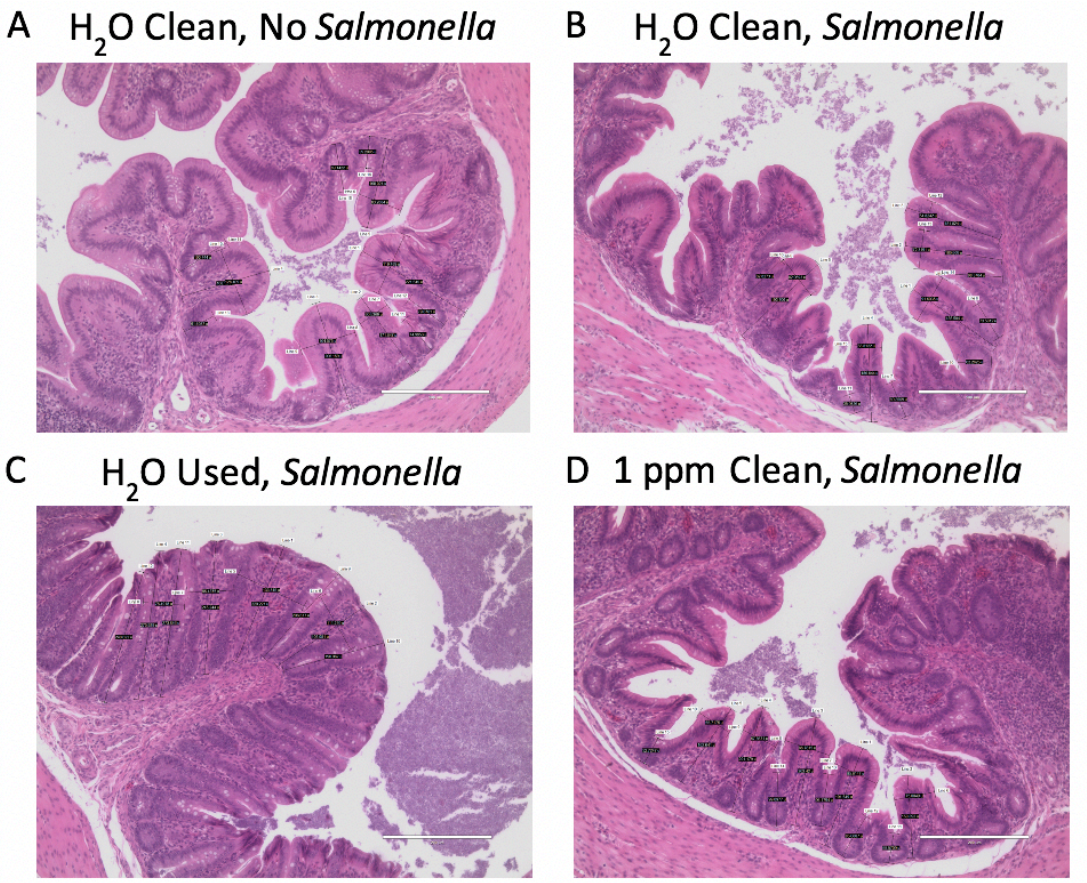

Figure 3.4 Images of caecal morphology of chicks at 10 days. (A) uninfected control birds on clean litter with H_2O, (B) *Salmonella* infected with H_2O on clean litter, (C) *Salmonella* infected with H_2O on used litter, and (D) *Salmonella* infected with 1 ppm iodinated water on clean litter.

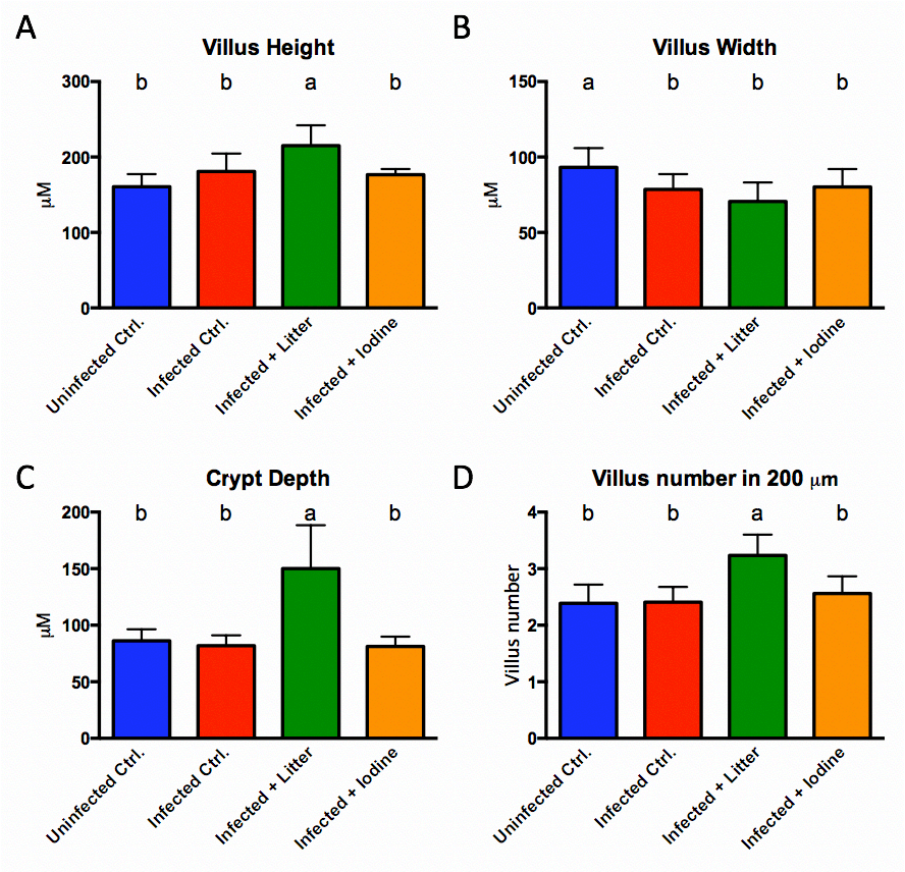

Figure 3.5 Cecal histology measurements comparing uninfected to infected chicks with used litter or 1 ppm iodinated water treatments. Used litter significantly increased villus height (A), *Salmonella* infection overall decreased villus width (B), used litter increased crypt depth (C), and used litter increased number of villi in 200um (D). Non-similar letters (a/b/c/d) denotes significant differences ($P < 0.05$).

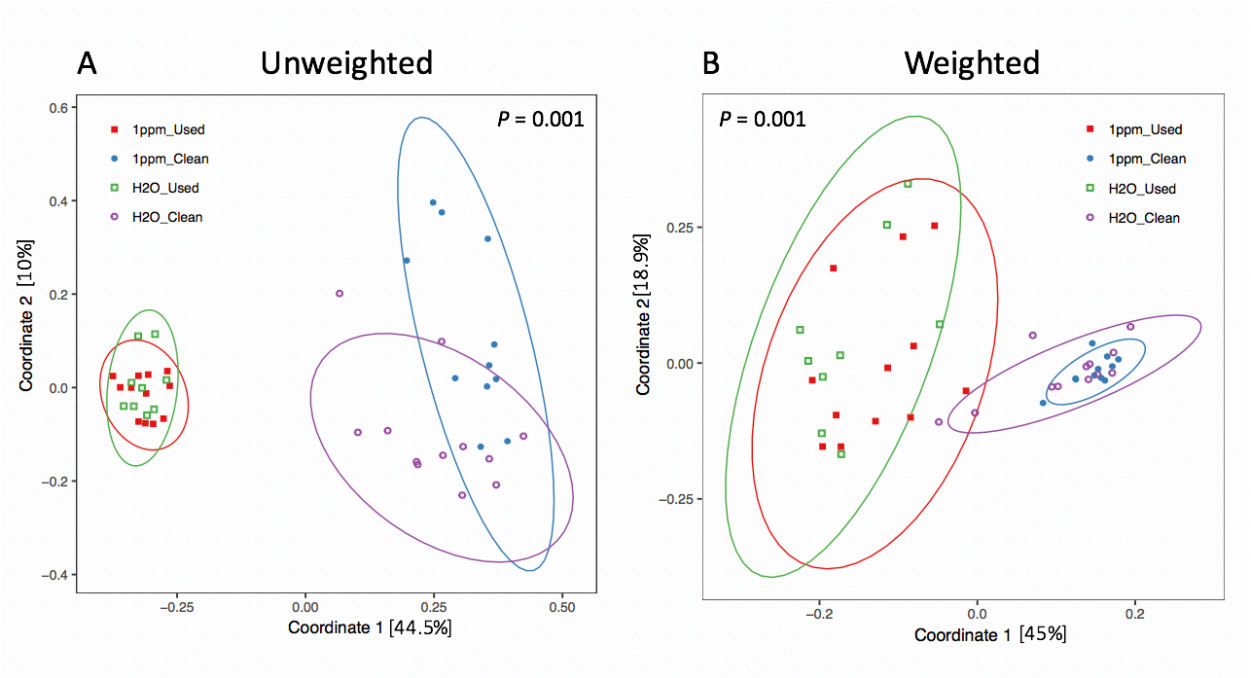

Figure 3.6 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on treatment. There was a significant effect of treatment on the caecal microbial community indicated in both the (A) weighted UniFrac distance metric (N = 9-12, Adonis P = 0.001), and (B) unweighted UniFrac distance metric (N = 9-12, Adonis P = 0.001).

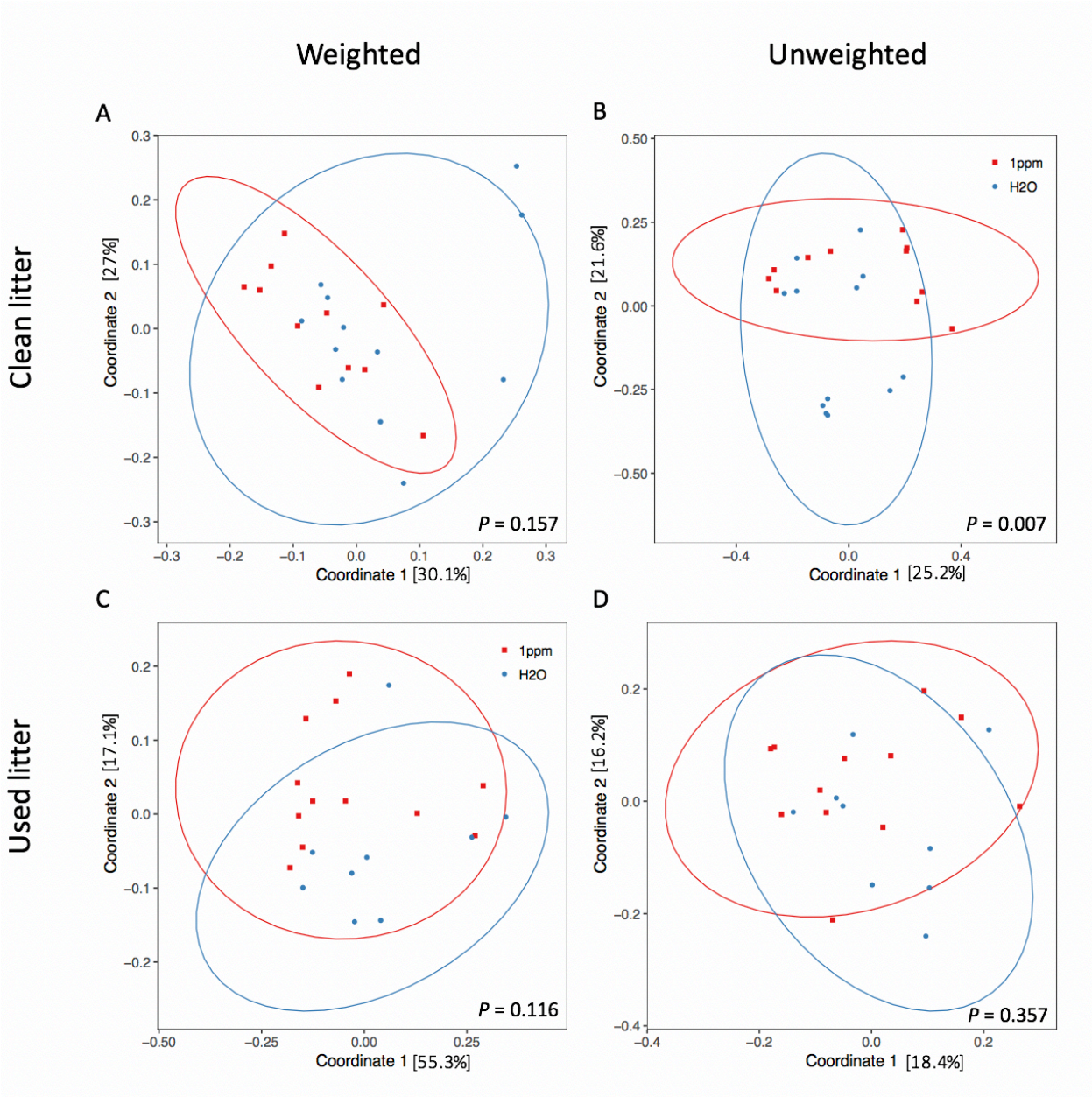

Figure 3.7 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on water treatment. Iodinated water had a significant effect on the caecal microbial community on (B) clean litter in unweighted unifrac distance metric (N = 11-12, $P = 0.007$), however not on the (A) weighted clean (N = 11-12, $P = 0.157$), (C) weighted used (N = 9-12, $P = 0.116$), and (D) unweighted used litter (N = 9-12, $P = 0.357$).

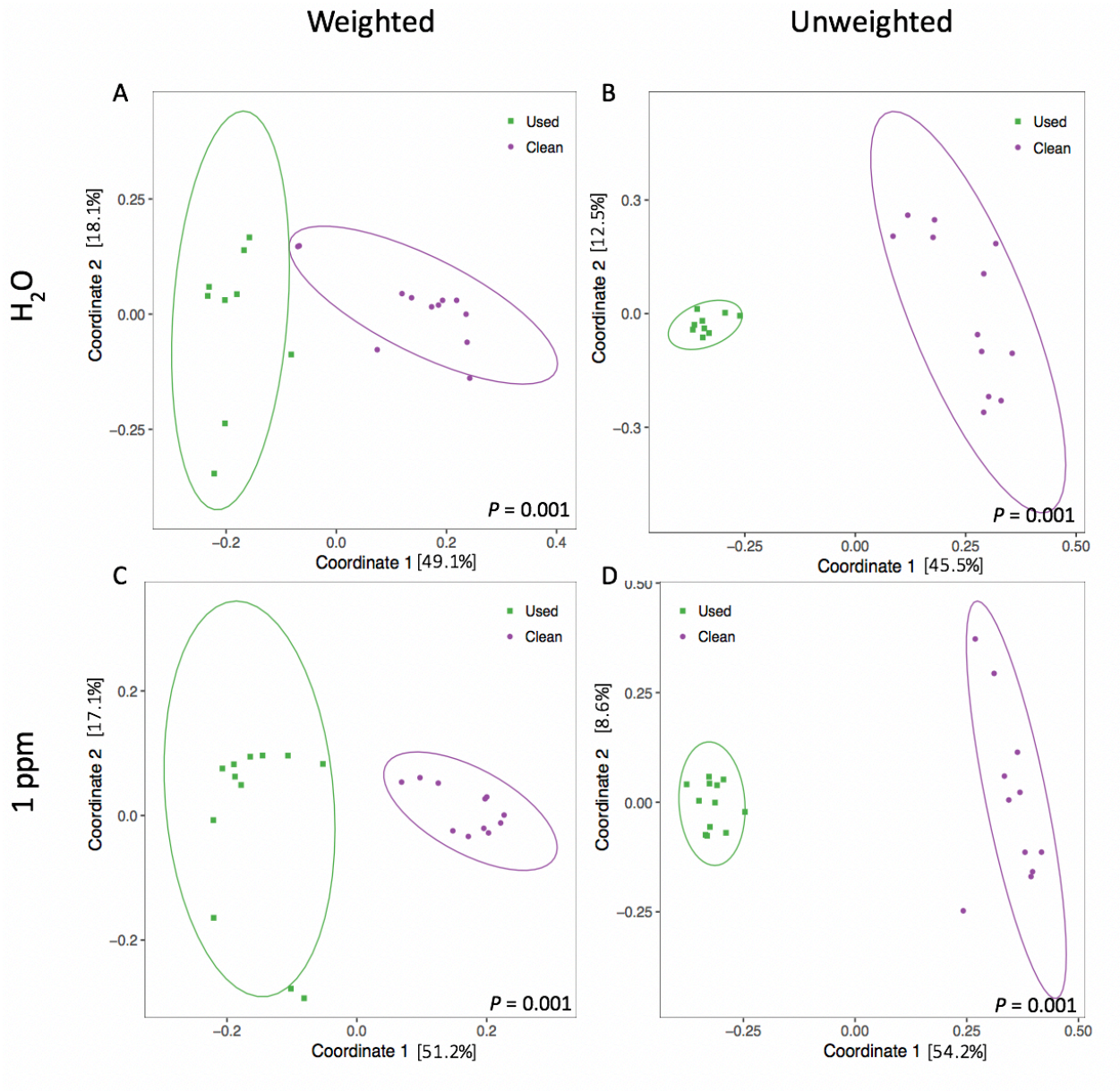

Figure 3.8 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on litter treatment. Used litter had a significant effect on the caecal microbial community on weighted UniFrac distances on (A) H_2O (N = 9-12, P = 0.001) and (C) 1 ppm iodinated water (N = 11-12, P = 0.001), as well as on unweighted UniFrac distances (B) H_2O (N = 11-12, P = 0.001), and (D) 1 ppm iodinated water (N = 11-12, P = 0.001).

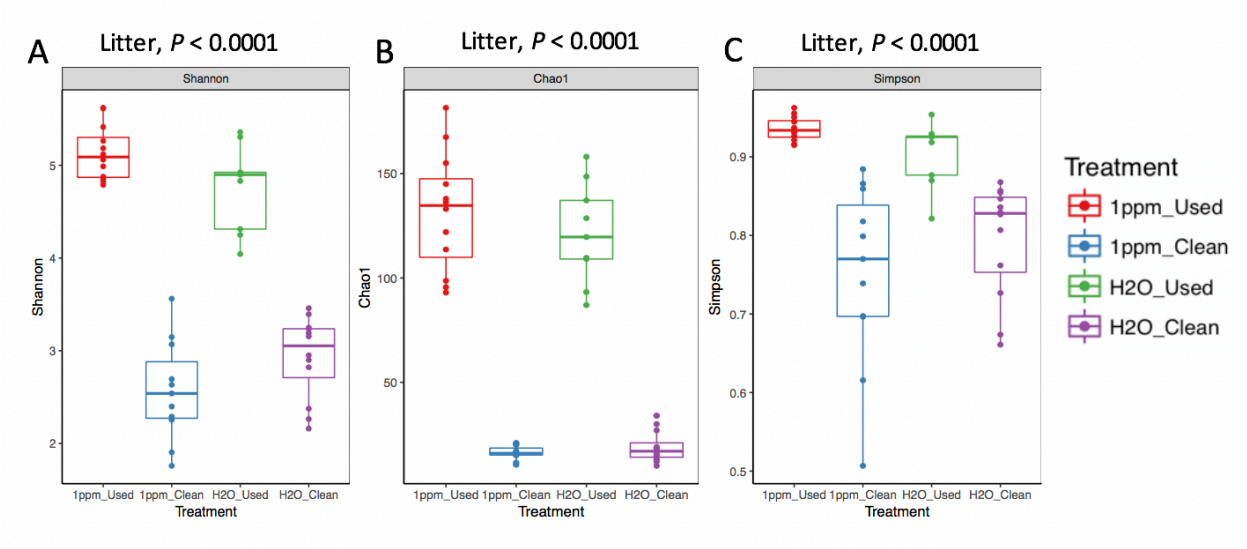

Figure 3.9 Alpha Diversity Measures. Used litter treatment, but not iodinated water treatment had a significant effect on alpha diversity, increasing Shannon index (A), Chao1 index (B) and Inverse Simpson index (C), $P < 0.0001$.

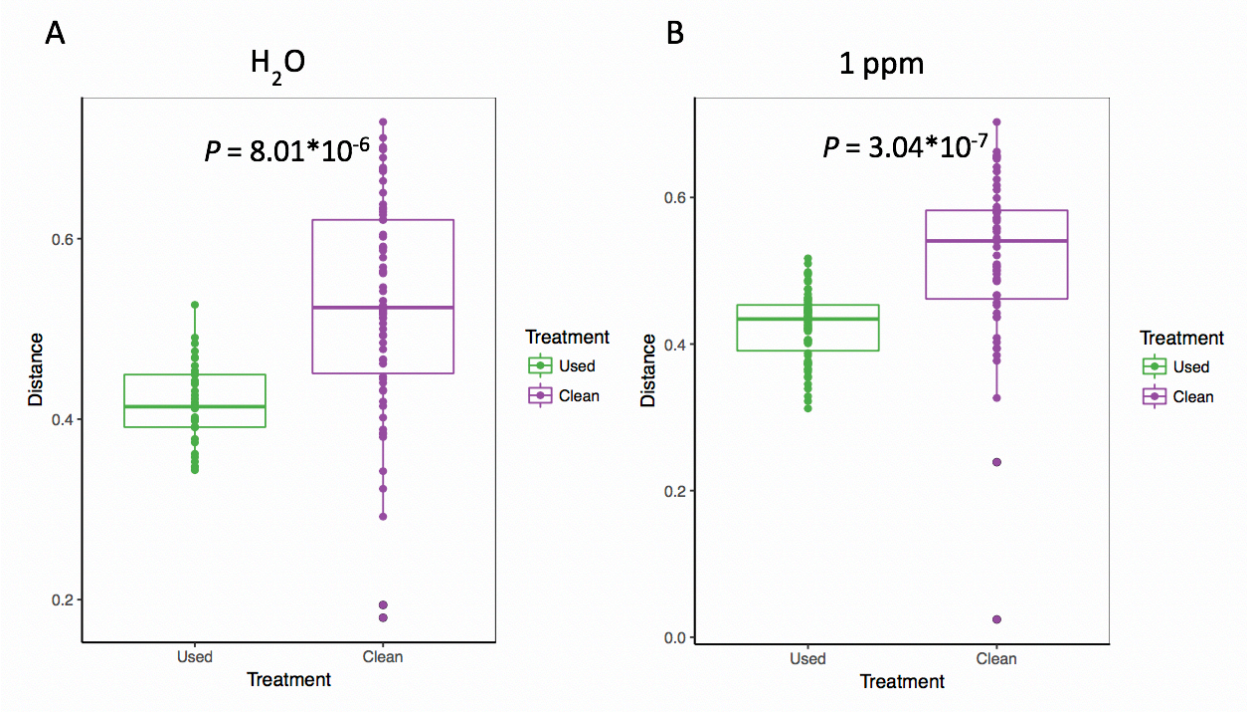

Figure 3.10 Effect of used litter treatment on beta diversity within treatment distances. Used litter reduces the within treatment distances on both (A) H_2O (Student's t-test P = 8.01E-06) and (B) 1 ppm iodinated water (Student's t-test P = 3.04E-07).

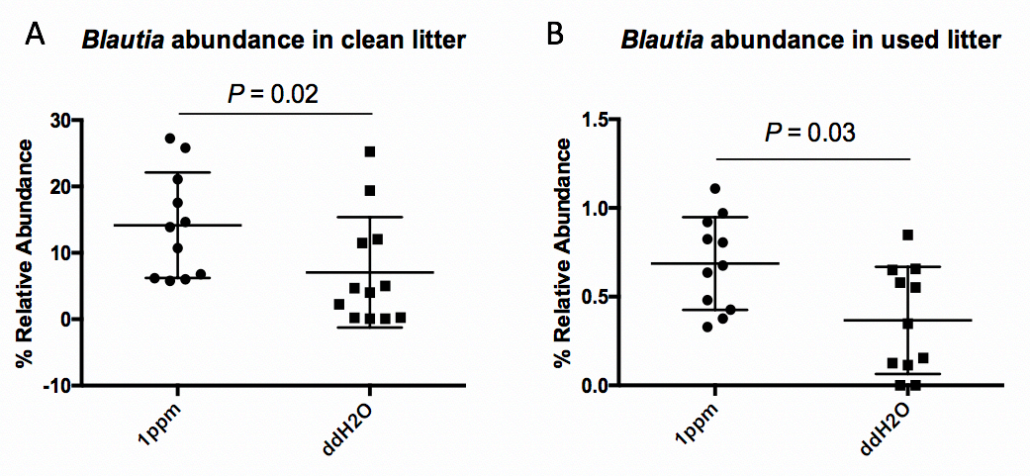

Figure 3.11 Effect of iodinated water on the percent relative abundance of genus *Blautia.* Iodinated water increased *Blautia* in both the (A) clean ($P = 0.02$) and (B) used ($P = 0.03$) litter treatments.

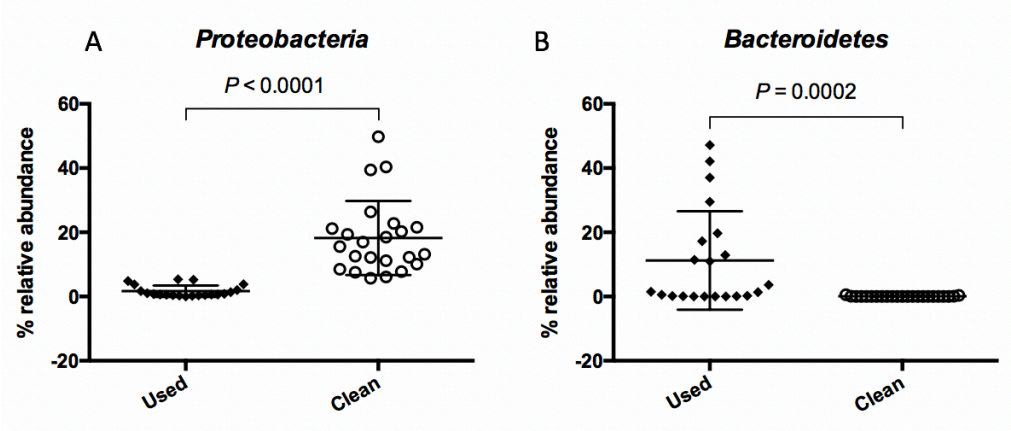

Figure 3.12 Percent relative abundance of *Proteobacteria* and *Bacteroidetes.* Used litter treatment contained significantly lower relative abundance of (A) *Proteobacteria* ($P < 0.0001$) and higher relative abundance of (B) *Bacteroides* ($P < 0.0002$) than the clean litter treatment.

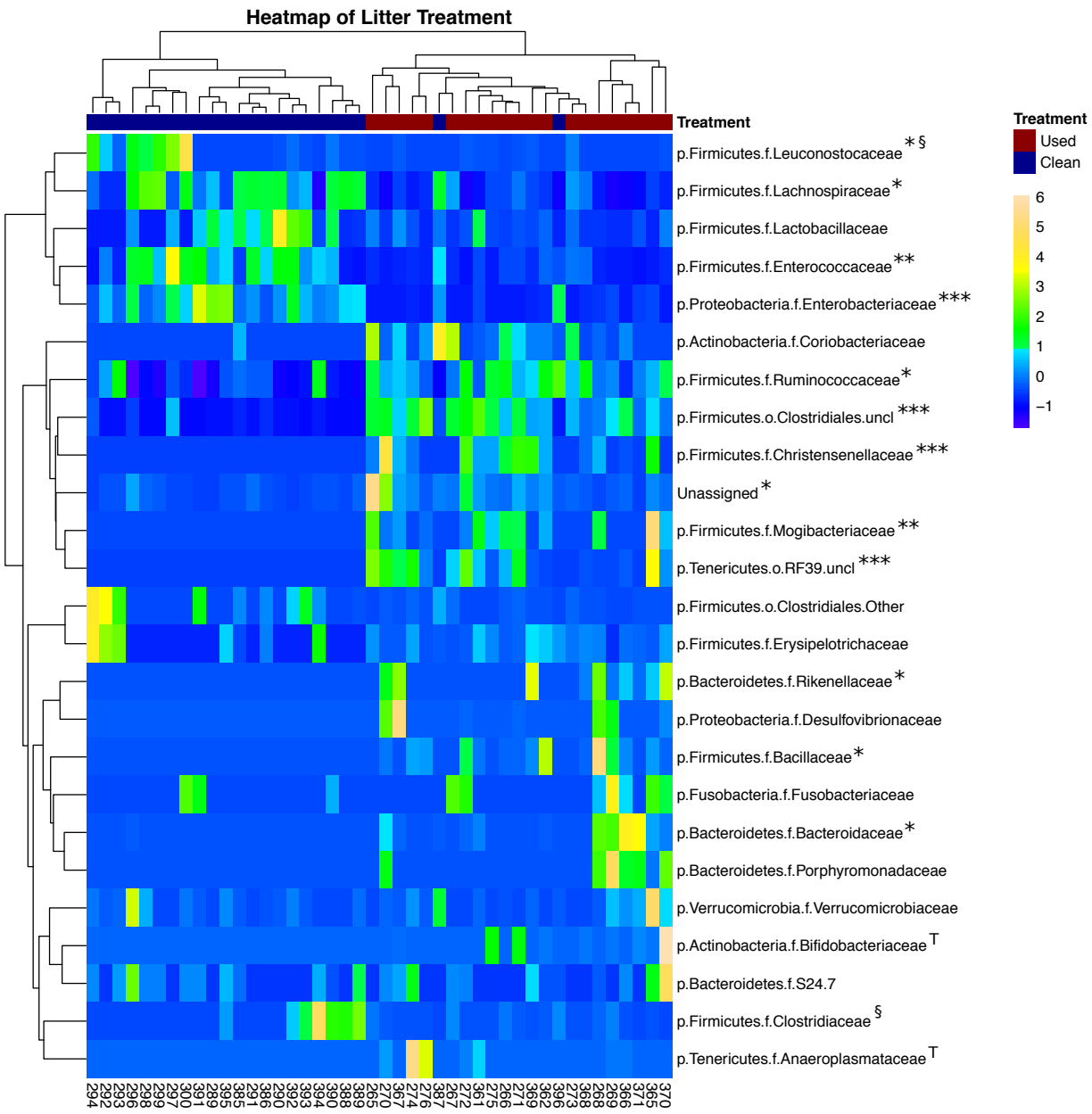

Figure 3.13 Heatmap of the percent relative abundance of bacterial families between clean and used chicken litter. Clustered significantly by litter treatment. * = P < 0.05, ** = P < 0.005, *** P = < 0.0005, T (trend) = P < 0.1, § = significant interaction between water and litter treatment.

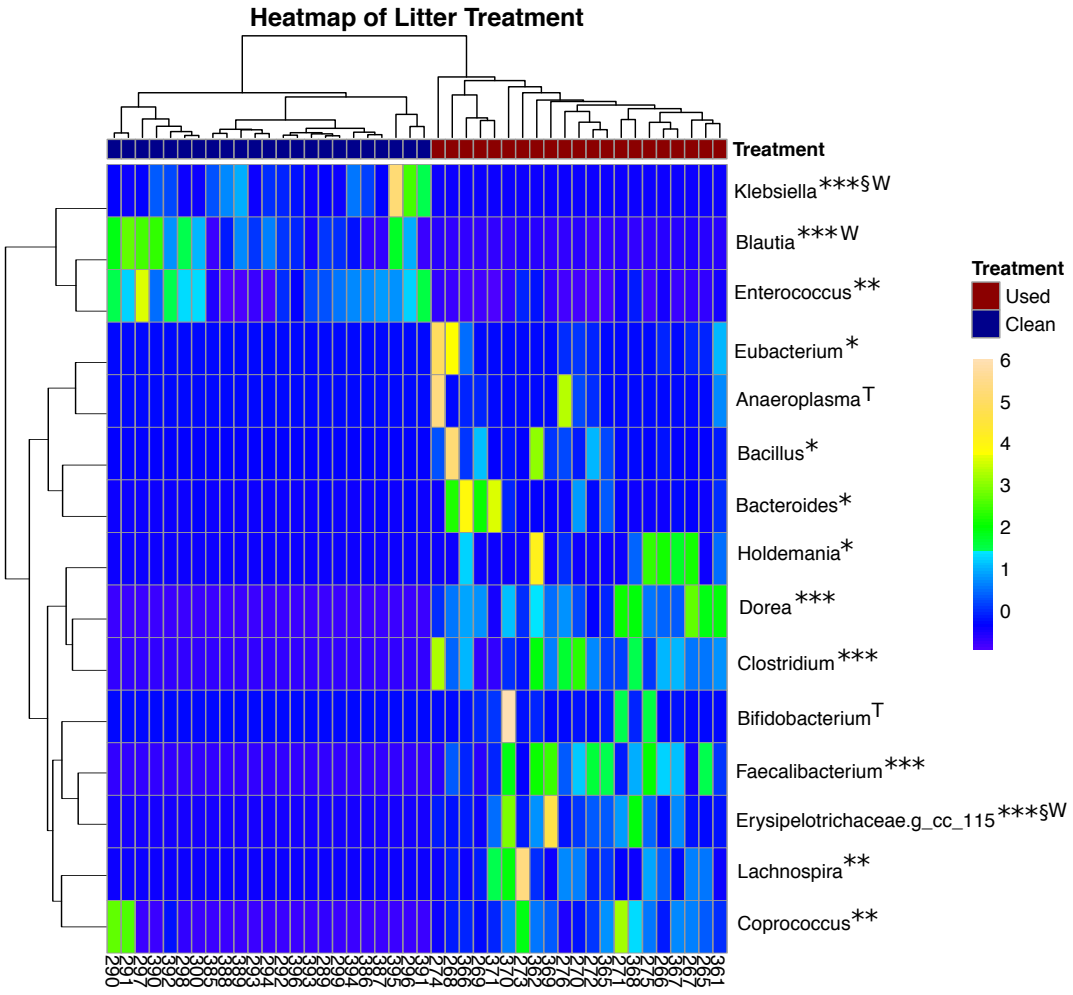

Figure 3.14 Heatmap of the percent relative abundance of the significantly different bacterial genera between clean and used chicken litter. Clustered significantly by litter treatment. * = P < 0.05, ** = P < 0.005, *** P = < 0.0005, T (trend) = P < 0.1, W = significant effect of water, § = significant interaction between water and litter treatment.

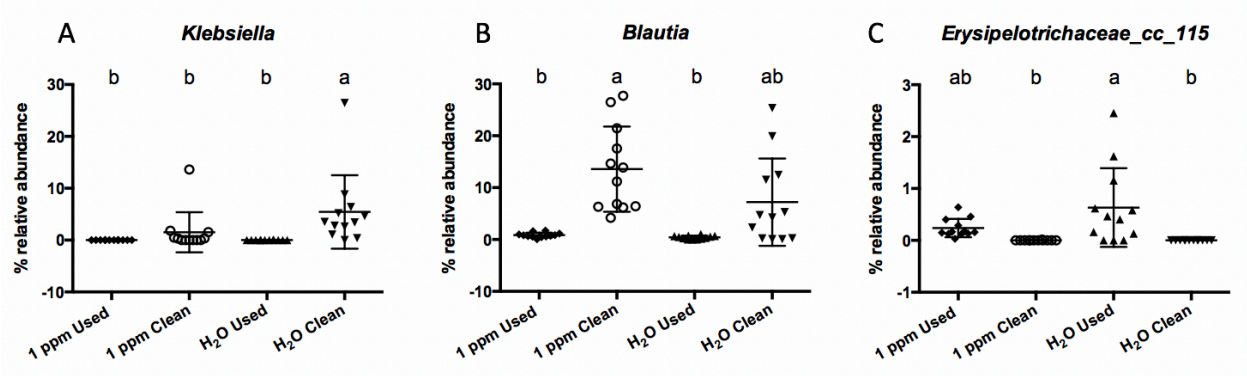

Figure 3.15 Effect of treatment on *Klebsiella, Blautia,* and *Erysipelotrichaceae cc 155.* The relative abundance of *Klebsiella* was lower in clean litter on H2O than the other three treatments. The relative abundance of *Blautia* was higher in 1 ppm clean than in both used litter groups. *Erysipelotrichaceae cc 155* was significantly higher in H2O used than both clean litter groups. Non-similar letters (a/b) denotes significant differences ($P < 0.05$).

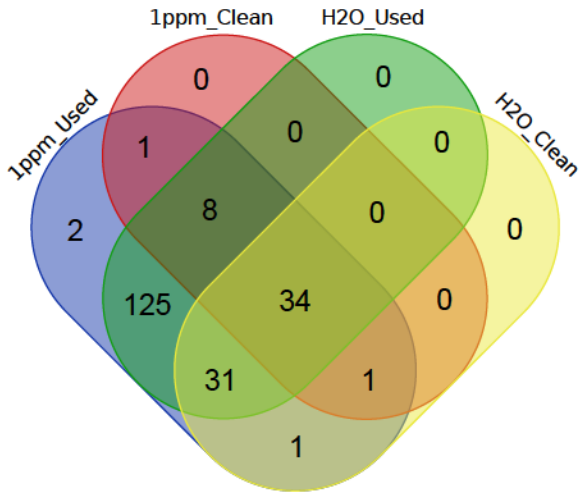

Figure 3.16 Venn diagram showing unique OTUs within and between treatments in chicken caecum. The used litter treatment contains 125 unique OTUs out of the total 203 identified.

- Chapter 4: Impact of Iodinated Water on the Growth Performance and Microbiome in Adult Broiler Chickens

4.0 Introduction

The need to reduce antibiotic use in animal production is of high importance, as it is a major cause of antibiotic resistant pathogens (Koch et al., 2017). Preventing the introduction and spread of pathogens within a flock is one means to reducing the need for antibiotic use in poultry production. Water lines have been found as one way that pathogens such as *Salmonella* and *Campylobacter* can spread from animal to animal (Pearson et al., 1996), therefore, finding new methods of water sterilization would prove to be beneficial.

Iodine has been recorded for use in water disinfection since the early 1900's (Backer & Hollowell, 2000), and is known for its antibacterial, antifungal, and antiviral properties (Gottardi, 1999). Ground water is commonly used in animal production and frequently contains bromine, which causes negative health outcomes in poultry production, however adding iodine to the water can mitigate these effects (du Toit & Casey, 2010, 2012). Iodine has been added to poultry feed and has shown to improve growth rate of broilers (Stanley et al., 1989), as well as iodine added to poultry water has also seen positive effect on feed efficiency and body weight (Emeash et al., 1994), however, the mechanisms involved are not well understood.

We hypothesize that the effects of iodinated water in poultry performance are due to changes in the gastrointestinal microbiome. In this study, we provided 1-day-old broiler chicks with 1 ppm iodinated water for 5 weeks to determine if iodinated water could improve animal performance and change the intestinal microbial community in the crop, ileum and caecum of adult broilers.

4.1 Materials and Methods

4.1.1 Ethics Statement

This study was performed according to the guidelines provided by Canadian Council on Animal Care and with approval of the University of Alberta Animal Care and Use Committee (AUP00001626). Chickens used in this study were managed by approved protocols at Poultry Research Centre at the University of Alberta.

4.1.2 Animals and Experimental Design

A total of 144-day-old ross 308 broiler chicks were assigned to 1 of 2 treatments, either receiving 1 ppm iodinated water, or reverse osmosis (RO) water control. For each treatment, 11 chicks were placed into 7 floor pens onto pine shavings and were fed a standard starter diet from day 0 to 14 and then switched to a grower diet from day 15 to 35, *ad libitum*. Both diets contained coccidiostats and antibiotic growth promoters. The water treatments were supplied through water lines to nipple drinkers. Floor pens were all along one side of the poultry house, placing and treatment pens alternated along the length of the house. Entire cages of birds were weighed on day 0 and then every week after for 5 weeks. Feed was weighed back on day 0 and then every week thereafter for 5 weeks.

4.1.3 Water Treatment and Measurements

Iodinated water was made and supplied by BioLargo Water Inc. using their iSAN precision iodine dosing system, using reverse osmosis water at a 1 ppm concentration using RO water. Both the iodinated water and RO water was replaced every 1-2 days.

4.1.4 Animal Euthanasia and Sample Collection

Birds were terminated after 5 weeks of growth by cervical dislocation. A total of 1 mL of contents from the crop, ileum (from the ileo-caecal junction to the Meckel's Diverticulum), and a caeca were collected into 2 mL microcentrifuge tubes (Eppendorf, Mississauga, ON, Canada), snap frozen in liquid nitrogen and stored at -80ºC for later use for microbial analysis by 16s rRNA gene sequencing.

4.1.5 Microbial Analysis

4.1.5.1 16S rRNA gene amplification and sequencing

Total DNA was extracted from the crop, ileum, and caecum contents using the QIA stool extraction kit (Qiagen Inc., Valencia, CA), following the manufacturers protocol with the addition of 2.0 mm diameter garnet beads (BioSpec Products, Bartlesville, OK) and 60 second bead-beating step at 6.0 meters per second (FastPrep instrument, MP Biomedicals, Solon, OH, USA). Amplicon libraries were constructed using primers to amplify the V3-V4 region of the bacterial 16S rRNA gene following the Illumina protocol (16S Metagenomic Sequencing Library Preparation). The primers for amplification were as follows: (Forward:5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; and Reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). Using the Illumina MiSeq Platform (Illumina Inc. San Diego, CA), paired-end sequencing was run with 2 x 300 cycles.

4.1.5.2 Microbial composition analysis

The sequence reads obtained were first filtered for quality control with the FastQC 0.11.5 (Babraham Bioinformatics). The primers were removed and the paired end reads were merged using PANDAseq algorithm. From these merged reads, Vsearch 2.7.1 was used to dereplicate and remove singletons (Rognes et al., 2016). Using Usearch version 7.1 (Edgar, 2010), the reads were clustered into operational taxonomic units (OTUs) based on 97% similarity and mapped against the Usearch 7.1 gold database to remove any chimeras (Rognes et al., 2016). The OTUs were labelled using Usearch 7.1 python script and reads were mapped back to OTUs via Vsearch 2.7.1 (Rognes et al., 2016). The uc2otutab.py in Usearch 7.1 was used to generate tab-delimited OTU tables (Rognes et al., 2016). The QIIME (Quantitative Insight into Microbial Ecology) version 1.9.1 (Caporaso et al., 2010) was used to assign taxonomy with the Ribosomal Database Project (RDP) classifier V2 (Wang et al., 2007). Alpha and beta diversity was analyzed using the QIIME core_diversity_analyses.py (Caporaso et al., 2010). Any OTUs that were present in less

than 30% of the samples in only one treatment group, and below 0.01% relative abundance were deleted and assumed to be sequencing artifacts.

4.1.6 Statistical Analysis

The weight gain, feed intake, and feed conversation ratio were analyzed for outliers using boxplot function in SAS (SAS University Edition) and Robust regression and outlier removal (ROUT) method of regression in Prism (Motulsky & Brown, 2006). The overall distribution of each data set was analyzed for normality using Shapiro-Wilk W statistical measures (SAS University Edition). The effect of water treatment on weight gain, feed intake, and feed conversion ratio was analyzed using a repeated measures analysis of variance (SAS University Edition, bonferroni adjusted P value). Beta diversity significance was calculated using ADONIS. The alpha diversity was checked for normality and was compared for significance by students T-test. The comparison of bacterial relative abundances was done using a non-parametric Mann Whitney test.

4.2 Results

4.2.1 Iodinated water had a limited effect on weight gain, feed intake, but not feed conversion

Iodinated water has a significant effect on the average weight gained per chicken showing higher weight than the control group at day 21 (Figure 4.1). However, there was no effect on the average weight gain on any other days (Figure 4.1). From day 21 to 28, chickens on 1 ppm iodinated water consumed less feed than those in the control group, but there was no effect on feed intake on any other days of measurement (Figure 4.2). Lastly, 1 ppm iodinated water did not have a significant impact on the feed conversion ratio (Figure 4.3).

4.2.2 Iodinated water had a limited impact on the overall microbiota composition in the crop, ileum and caeca, however did affect the relative abundance of some members.

When the tissues were analyzed separately and compared between water treatments, the samples did not cluster significantly based on water type, in either weighted or unweighted unifrac distances (Figure 4.5). As well, there was no significant effect of iodinated water on the alpha diversity measures of richness and evenness shown by Chao1 index, Shannon index, and inverse Simpson index (Table 4.1). Iodinated water had some impact on the percent relative abundance in the crop, increasing *Bacteroidetes* phylum ($P = 0.048$), decreasing the *Flavobacteriaceae* family ($P = 0.023$), and increasing the *Sanguibacter* genus ($P = 0.046$) (Figure 4.7). Iodinated water did not have an impact at the phylum level in the ileum, but did impact 12 families, significantly increasing *Mycobacterium*, *Collinsella*, *Virgibacillus*, unclassified *Lactobacillales*, *Allobaculum*, *Parabateroides*, *Anaerobacillus*, *Arcanobacterium*, *Bulleidia*, and *Brevibacterium*, $P < 0.05$ (Figure 4.8). It also decreased unclassified *Clostridiales* and *Faecalibacterium*, $P < 0.05$ (Figure 4.8). Iodinated water had no impact on the relative abundance at the phylum level in the caeca, however it reduced unclassified *Dehalobacteriaceae* family ($P = 0.013$).

4.2.2 Type of tissue had a significant impact on the overall microbiota composition in the crop, ileum and caeca in chickens at 35 days of age.

The type of tissue had a significant effect on the composition of the microbiota. The samples clustered separately based on crop, ileum, and caeca, for both weighted and unweighted unifrac distances (Figure 4.4). The relative percent abundance at the phylum level was different between tissue locations (Figure 4.6). The crop contained the following 6 phylum from most abundant to least: *Firmicutes*, *Proteobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Verrucomicrobia*. Within the *Firmicutes* phylum, the *Lactobacillus* genus made up 70% in the control and 79% in the iodinated water, however this was not significant. The ileum had more diversity with a total of 9 phylum, listed from most abundant to least: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Cyanobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Spirochaetes*, *Fusobacteria*, and *Tenericutes*. Within the *Proteobacteria* phylum, unclassified *Enterobacteriaceae* make up the majority of the sequences in the ileum, with 48% in the iodinated water and 50% in the control. The caecum contained 8 phylum, from most abundant to least: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Tenericutes*, *Verrucomicrobia*,

and *Cyanobacteria*. From the *Firmicutes* phylum, unclassified *Clostridiales* made up 25% and 26% of the total sequences in the control and iodinated water groups respectively.

4.3 Discussion

Providing poultry clean water is important to prevent pathogen introduction, and iodinated water may further prevent by maintaining sterility longer. Prior to this study, there was limited information on the impact of iodinated water on the health and development of chickens. Overall we were unable to reproduce the positive result of iodinated water on poultry growth performance seen by Emeash et al., (1994). However, our control group was given sterile RO water, and using a more standard farm well water may result in different findings.

Overall, 1 ppm iodinated water had a limited impact on the broiler chicken's performance over the five weeks. Even though by week 5 the weight gain of the birds was the same, the iodinated water group had higher weight at 21 days. One potential reason for this change in weight may be that the control birds were challenged at 21 days causing a reduction in weight, and from which the iodinated group was protected. As iodine is has antimicrobial properties (Gottardi, 1999), and water is one means of spreading pathogens through a flock (Heyndrickx et al., 2002; Pearson et al., 1996), it is possible the iodinated water could prevent pathogen spreading within that treatment. However, this is speculative as no samples were collected for pathogen testing over the course of the trial. The iodinated water group showed reduced feed intake on day 28 of the trial, but overall had no effect on feed efficiency. As the control group was given sterile reverse osmosis water, a study using farm well water may indicate a stronger effect of iodinated water on these production parameters.

Iodinated water had no effect on the overall beta diversity as seen by unifrac distance measures, although it did influence specific percent relative abundance at the phylum, family and genus level in crop, ileum and caeca. In the crop, iodinated water increased phylum *Bacteroidetes*, a common phylum in the chicken microbiota that has been associated with improved performance (Oakley et al., 2014; Torok et al., 2011). Not all members of the *Bacteroidetes* are beneficial, as unclassified members of the *Flavobacteriaceae* family were increased in the crop of iodinated water birds, which contains many genera, however some are pathogenic in chickens, such as Ornithobacterium (Morales-Erasto et al., 2016; Szabó et al.,

2017). *Sanguibacter* genus in the *Actinobacteria* phylum was also increased in the crop of iodinated water group. *Sanguibacter* are soil and plant bacteria, and are not likely standard members of the crop microbiota, but transiently introduced through the feed, as they are known to be ligonocellulose degraders, which is found in wheat straw, corn stover, and switchgrass (de Lima Brossi et al., 2016).

Iodinated water had a much larger impact on the percent relative abundance of the bacterial families and genera in the ileum. Out of the 10 bacteria increased, 5 classify under the *Firmicutes* phylum, a major group in the poultry microbiome (Oakley et al., 2014) also associated with beneficial performance in chickens (Torok et al., 2011). One of these was unclassified *Lactobacillales*, which are lactic acid bacteria commonly used as a probiotic in poultry rearing (García-Hernández et al., 2016). Four out of the 10 increased bacteria classify under the *Actinobacteria* phylum, which typically makes up a small proportion of the chicken microbiota (Oakley et al., 2014). Three were members of the *Actinomycetales* order, and the last one a *Collinsinella* genus, a member of *Coriobacteriales*. *Collinsinella* in chickens has shown to be capable of transforming deoxynivalenol (mycotoxin from fungi) into deepoxy-4-deoxynivalenol (DOM-1), a product much less toxic (Yu et al., 2010). Iodinated water also reduced the relative abundance of two members typically beneficial members of the *Firmicutes* phylum, *Faecalibacterium*, an common member of the chicken microbiota (Rehman et al., 2007), and unclassified *Clostridiales*, known for their contribution to short chain fatty acid production (Oakley et al., 2014).

An increase in *Mycobacterium* genus in the iodinated water group may not prove to be beneficial. The *Mycobacterium* genus contains some pathogenic species such as *M. paratuberculosis*, known to cause chronic granulomatous inflammation of the gastrointestinal tract of ruminants, as well as focal lesions in the gut of chickens (Arrazuria et al., 2016; Van Kruiningen et al., 1991). In one study looking at rabbits infected with *Mycobacterium avium* subspecies *paratuberculosis*, *Dehalobacteriaceae* was positively associated with *Mycobacterium avium* abundance in the gut (Arrazuria et al., 2016). Interestingly in our study unclassified *Dehalobacteriaceae* family had increased relative expression in the chicken caeca on 1 ppm iodinated water.

The biggest effect on the relative abundance of bacteria was the tissue location. In the crop, the *Lactobacillus* genus being the most abundant is not surprising, as digestion in the crop

is mostly starch breakdown and lactate fermentation which is facilitated by *Lactobacillus* species (Rehman et al., 2007; Stanley et al., 2014; Van der Wielen et al., 2000). The *Proteobacteria* phylum make up the largest portion of the ileum microbiota, specifically the *Enterobacteriaceae* family, which contains some pathogenic bacteria such as *Salmonella*, *Klebsiella*, *Proteus*, and *E. coli*. The largest phylum in the caeca was *Firmicutes*, more specifically the unclassified *Clostridiales*, which are known for their short chain fatty acid production in the chicken caeca (Oakley et al., 2014), especially butyrate, which is a major energy source for the colonocytes (Bedford & Gong, 2018; Nepelska et al., 2012).

4.4 Conclusion

Iodinated water may have potential to reduce the spread or introduction of pathogens, but has limited effects on weight gain, feed intake, and no effect on feed efficiency in boilers when added to high quality water. Its impact on the microbiome is most pronounced in the ileum, where it encouraged the growth of some bacteria that are known to be beneficial such as *Lactobacillales*, however decreased other beneficial members such as *Faecalibacterium* and unclassified *Clostridiales*. The increase the abundance of the *Mycobacterium* genus in the ileum and *Flavobacteriaceae* family in the crop may prove to be a disadvantage as these groups contain potentially pathogenic members. However, by targeting the 16s rRNA gene in bacteria for sequencing we were unable to identify at the species level in *Mycobacterium* and *Flavobacteriaceae*, so attributing their increase as a detriment is not appropriate. As the iodinated water did not have a negative effect on poultry performance, it should still be considered as a means for water sterilization, and further research is needed to determine if it is able to reduce pathogen spread throughout a flock. As well, further research comparing farm well water to iodinated well water may indicate additional benefits not seen with our sterile RO water control.

Tables

4.1 Alpha diversity indices in crop, ileal, and caecal digesta of chickens fed 1 ppm iodinated water

Tissue	α diversity test	1 ppm (mean ± SEM)	Control (mean ± SEM)	*P* values
Crop				
	Chao1	163.4 ± 9.180	164.9 ± 15.81	0.937
	Shannon	2.307 ± 0.093	2.343 ± 0.162	0.851
	Simpson	0.665 ± 0.017	0.678 ± 0.027	0.669
Ileum				
	Chao1	188.2 ± 19.34	172.7 ± 17.27	0.557
	Shannon	2.138 ± 0.208	2.540 ± 0.359	0.351
	Simpson	0.546 ± 0.056	0.594 ± 0.735	0.582
Caeca				
	Chao1	150.4 ± 8.625	153.6 ± 4.882	0.754
	Shannon	4.516 ± 0.145	4.450 ± 0.160	0.763
	Simpson	0.904 ± 0.009	0.888 ± 0.016	0.408

Figures

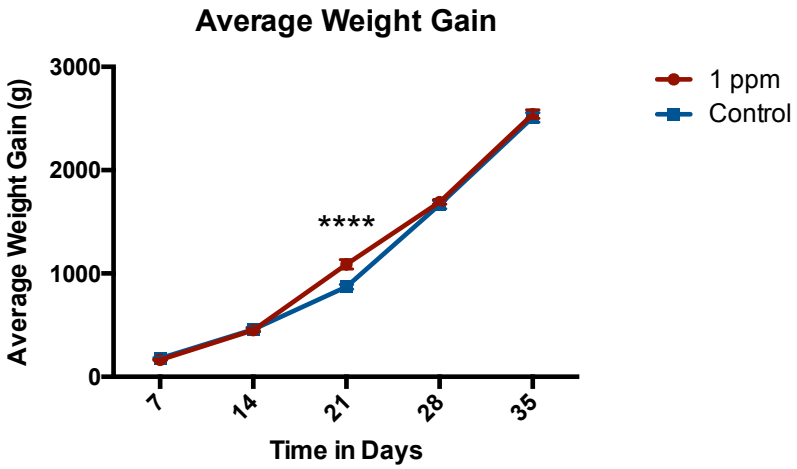

Figure 4.1 Effect of 1 ppm iodinated water on the average weight gained per chicken from day 7 to day 35. On day 21, chickens on the 1 ppm iodinated diet gained significantly more weight than the control (repeated measures ANOVA, Tukey adjusted P-value < 0.0001).

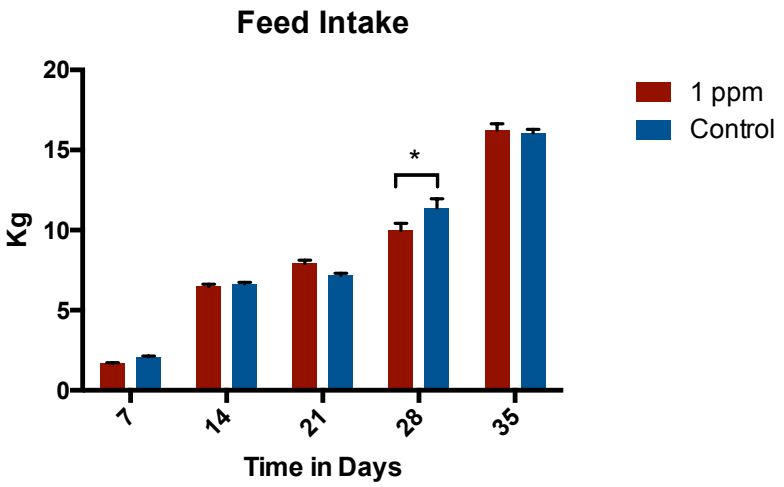

Figure 4.2 Effect of 1 ppm iodinated water on total feed intake per cage from day 7 to day 35. On day 28, chickens on the 1 ppm iodinated consumed significantly less feed than the control (repeated measures ANOVA, Tukey adjusted P-value = 0.02).

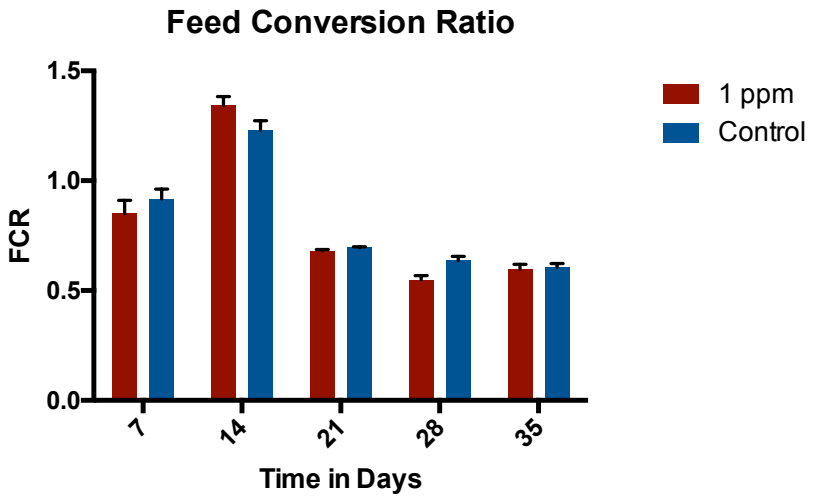

Figure 4.3 Effect of 1 ppm iodinated water on the feed conversion ratio from day 7 to day 35. There was no significant effect on treatment on the FCR (repeated measures ANOVA, Tukey adjusted P-value > 0.05).

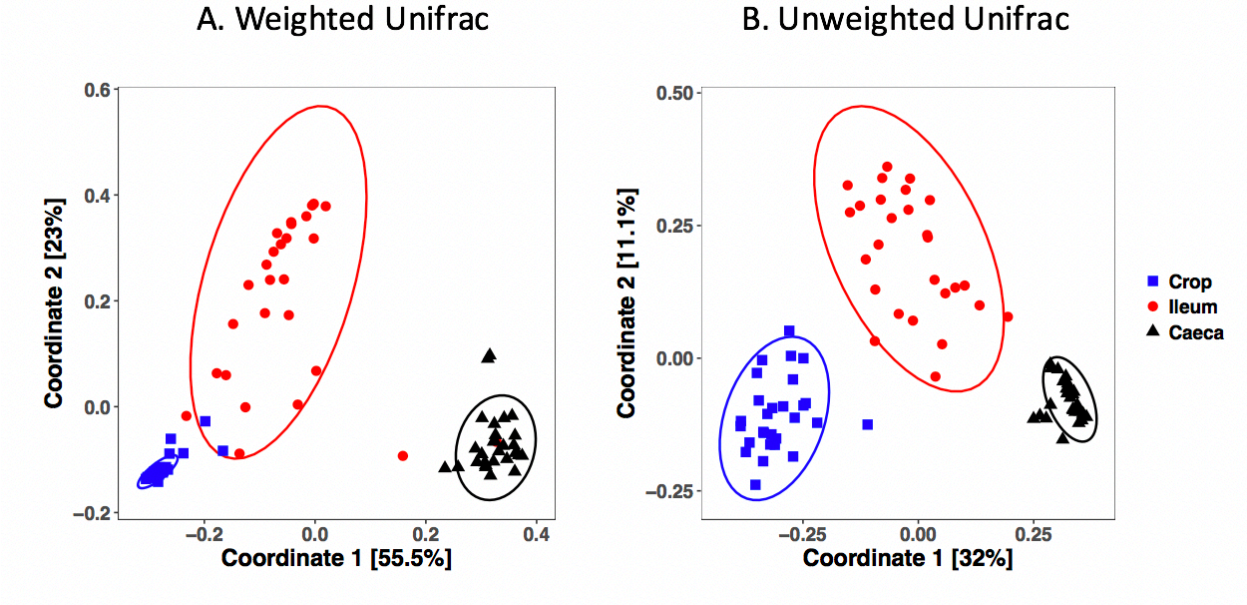

Figure 4.4 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on tissue. Tissue type had a significant effect on the (A) weighted and (B) unweighted UniFrac distances ($P < 0.001$, Adonis).

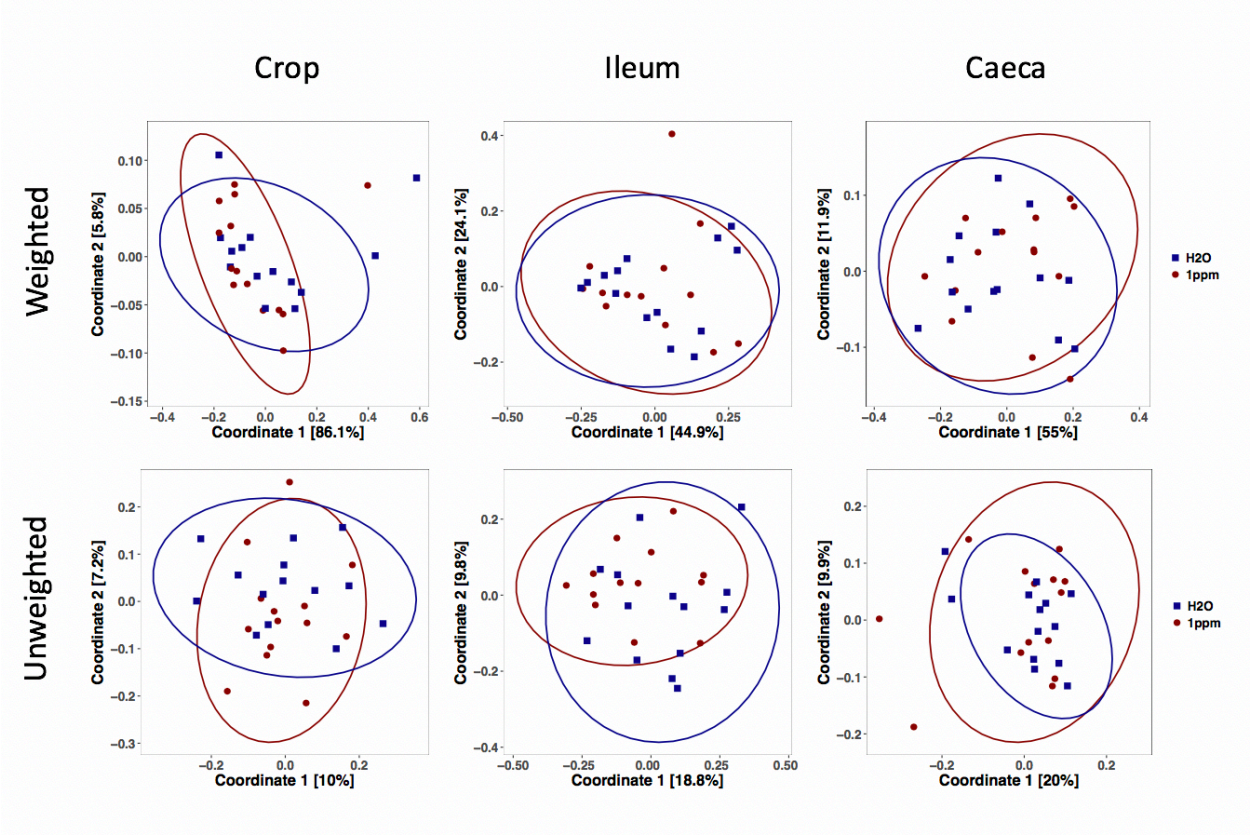

Figure 4.5 Principle coordinate analysis of weighted and unweighted UniFrac distance metrics based on treatment. Iodinated water had no significant effect on the weighted and unweighted unifrac distances of the microbiota in crop, ileum, and caeca ($P > 0.05$, Adonis).

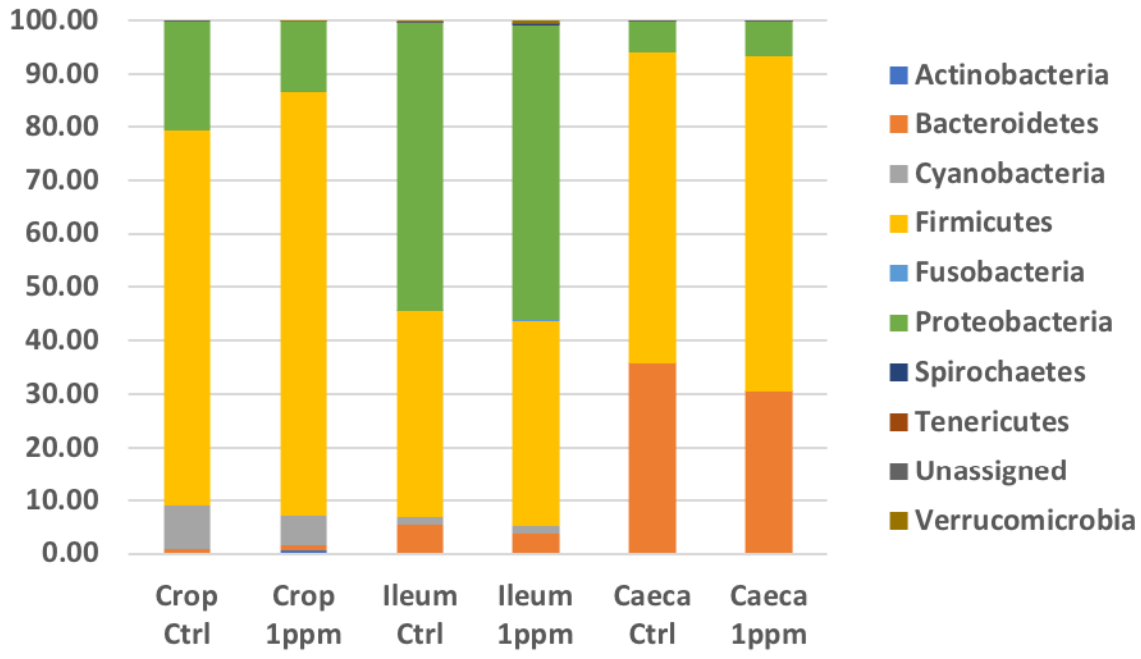

Figure 4.6 Percent relative abundance of bacterial phylum in the crop, ileum, and caeca based on treatment. 1 ppm iodinated water (1ppm) compared to water control (Ctrl) did not have a significant impact on the relative abundance at the bacterial phylum level ($P > 0.05$).

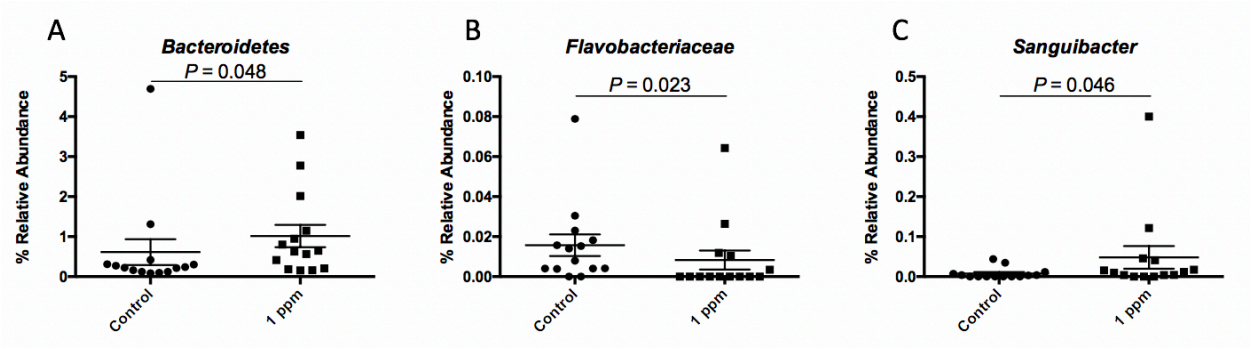

Figure 4.7 The effect of iodinated water on phylum *Bacteroidetes,* family *Flavobacteriaceae*, and genus *Sanguibacter* in the crop. Iodinated water treatment had higher relative abundance of (A) *Bacteroidetes* ($P = 0.048$), lower relative abundance of (B) *Flavobacteriaceae* ($P = 0.023$), and higher relative abundance of (C) *Sanguibacter* ($P = 0.046$).

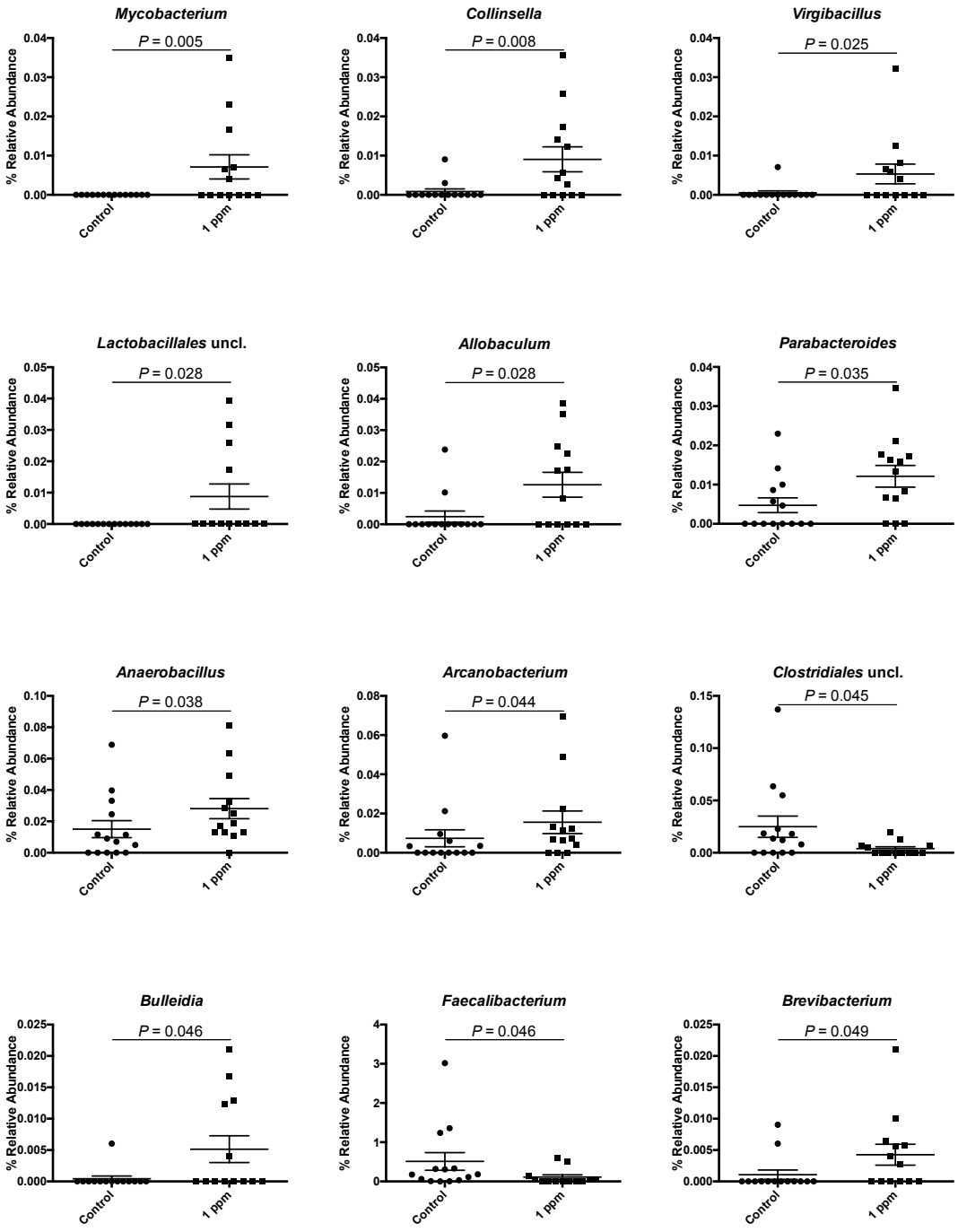

Figure 4.8 The effect of iodinated water on relative abundance of Family level bacteria in the ileal contents. Iodinated water treatment had higher percent relative abundance of *Mycobacterium*, *Collinsella*, *Virgibacillus*, unclassified *Lactobacillus*, *Allobaculum*, *Parabacteroides*, *Anaerobacillus*, *Arcanobacterium*, *Bulleidia*, and *Brevibacterium*, and lower percent relative abundance of unclassified *Clostridiales* and *Faecalibacterium*. ($P < 0.05$).

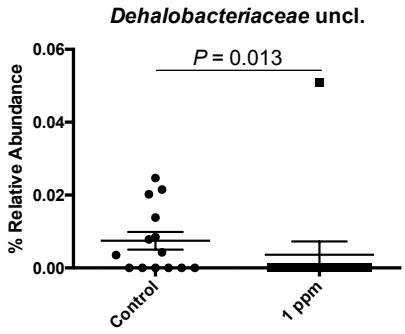

Figure 4.9 Effect of iodinated water on the relative abundance of unclassified *Dehalobacteriaceae* family in the caecal contents. Iodinated water treatment had lower relative abundance of *Dehalobacteriaceae* family ($P = 0.013$).

- Chapter 5: General Discussion

5.1 Summary and conclusions

It has long been known that antibiotic use in animal production is a major contributor to the development of antibiotic resistant pathogens. There is great need to reduce antibiotic use in poultry production, however we need to find ways of doing so without risking the health of the birds as well as keeping in mind the financial barriers to the farmer. By preventing infection as well as encouraging healthy development of chickens we can reduce the need for antibiotic use. Creating sterile water using iodine may prove to prevent pathogen spreading, and has been shown to improve poultry performance, however the underlying mechanisms are not known. As well, promoting the development of a healthy microbiome results in many benefits in chickens, such as improved growth and pathogen resistance. One inexpensive way to introduce and develop the chicken microbiome is through exposure to re-used chicken litter. Experimental evidence for the relationship between used litter and changes in the microbiome have been shown, yet microbiome changes have not been sufficiently characterized and indirect mechanisms through modulation of host intestinal and immune development have not been explored. Overall, the objective of this study was to explore the association between re-used litter and iodinated water on poultry production performance, microbial ecology, pathogen resistance, and impact on the intestinal and immune development.

5.1.1 Discovering the effects of re-used litter and iodinated water on the microbiome, intestinal development and gene expression in chicks

The use of iodinated water to prevent pathogens from spreading within a flock may prove to be useful because of iodine's antimicrobial properties (Gottardi, 1999). As well, both iodine added to poultry feed and water has shown positive effects on their growth performance (Emeash et al., 2014; Stanley et al., 1989). Given their antimicrobial properties it was expected that iodine would impact the microbiota and intestinal development in chickens, however, this had not been previously studied. Re-using chicken litter in new flocks has been shown to reduce mortality and

improve animal performance (Kennard & Chamberlin, 1951; Yamak et al., 2015). Studies have indicated that the re-use of litter changes the microbial communities in the chicken intestinal tract (Cressman et al., 2010; Cressman, 2014), and the development of the microbiome has many conferred health benefits to the host (Sommer & Bäckhed, 2013). However, the impact of these changes on poultry gastrointestinal development is not well understood. We hypothesized that both use litter and 1 ppm and 10 ppm iodinated water would provide positive health benefits to the host by modifying the caecal microbial community, resulting in a reduction of pathogenic bacteria and modifying gene regulation and tissue morphology in the caecum. In chapter 2, our data revealed that used litter had a significant impact on the development of the caecal microbiome in the first ten days of life, introducing 149 unique OTUs, increasing the relative abundance of beneficial bacteria from the *Clostridiales* order, therefore changing the short chain fatty acid profile and tissue morphology in the caecum. As well, used litter significantly reduced potentially pathogenic bacteria in the *Enterobacteriaceae* family, and shifted the gene expression in the caecum towards cell homeostasis and immune modulation, therefore promoting healthy intestinal development. These results provide evidence that rearing on re-used litter changes the intestinal microbial community, and increases the number of different bacterial OTU's at 10 days of age compared to clean litter. The increased growth performance of chickens reared on re-used litter as indicated by other studies may be in part due to the increased gut homeostasis as seen through gene expression changes, as well as the increase in butyrate, a SCFA that is a major energy source for colonocytes. Iodinated water had a very limited impact on the microbiome and transcriptome of chicks on used litter, however did significantly alter caecal communities and gene expression on the clean litter towards increased inflammatory responses, decreased cell turnover, and mucosal development. These results indicate that the effect of iodinated water is only seen in a microbially depleted environment, and that there are very limited effects when chicks have a developed microbiome. As the iodinated water had a limited impact on the microbial community and gene expression, it is unlikely that this is the cause of the improved animal performance seen in previous research.

5.1.2 Achievement in determining the efficacy of re-used litter and iodinated water on pathogen reduction in chicks

The re-use of poultry litter has proven to be effective in reducing *Salmonella* infection and increasing short chain fatty acid production (Corrier et al., 1993; Corrier et al., 1992). It is known that re-used litter plays a role in the development of the chick microbiome, however previous research has not looked at the change in microbiome and *Salmonella* infection. Iodinated water is antimicrobial, and it is believed that production benefits in poultry rearing may be due to its ability to reduce the spread of pathogens, however the impact of iodinated water on *Salmonella* infection in birds has not been studied. We hypothesized that both used litter and iodinated water would be able to decrease *Salmonella enterica* serovar Enteritidis challenge through the alterations in the intestinal microbial community and morphology. In chapter 3 our results indicated that used litter significantly reduced *Salmonella* infection, likely due to the increase in OTUs not present in the clean litter group that were able to fill the niche. For the second time used litter showed to decreased family *Enterobacteriaceae* like in chapter 2, and potentially pathogenic genus *Klebsiella*. A total of 8 bacterial families increased due to used chicken litter, 5 of which were the same as see in chapter 2, specifically increases in *Christensenellaceae*, *Mogibacteriaceae*, *Ruminococcaeae*, and unclassified RF39 and *Clostridiales* orders. At the genus level, 12 bacterial groups increased in used litter, 5 of which were the same as in chapter 2, those being *Clostridium*, *Faecalibacterium*, *Erysipelotrichaceae* Cc 115, *Lachnospira*, and *Coprococcus*. Similar to chapter 2, the majority of the unique OTUs introduced were members of the *Clostridiales* order, known for their contribution to SCFA production. However, iodinated water again had very limited effect on the microbiome of used litter chicks, and only had an effect on the clean litter birds, as was seen in chapter 2 and chapter 3. On clean litter, 1 ppm iodinated water increased *Salmonella* colonization, which could have been a result of the activation of inflammation by iodine on clean litter as inflammation benefits Salmonella colonization, allowing it to become competitive with commensals (Thiennimitr et al., 2011) or because iodine turned to iodide in the gut may supported Salmonella growth, as iodide is used for Salmonella selective media (Palumbo & Alford, 1970). Again, these results point to the positive effects of developing a mature microbiome in chicks with re-used litter, and indicate that in the presence of this microbiome iodinated water has limited effects.

5.1.3 Achievement in defining the effect of iodinated water on the growth performance and microbiome in adult broilers

Previous research has indicated that the iodine added to poultry feed and water can improve feed efficiency, growth performance, and behavioral changes with increased sleeping and body preening (Emeash et al., 1994; Słupczyńska et al., 2014; Stanley et al., 1989). The mechanisms involved with these positive results are not indicated currently in the research. We hypothesized that iodinated water may improve poultry performance in adult broilers raised in a conventional setting by changing the microbiome and reducing pathogenic bacteria in the intestines. In chapter 4 we found that the effect of iodinated water on poultry performance was very limited compared to the control water, and the changes in the microbiome were minute, having no significant effect on the overall microbial structure, however modifying the expression of a few groups within the ileum. The use of iodinated water may prevent pathogen challenges from spreading, as at day 21 the iodinated water birds had higher growth compared to the control, which may indicate a challenge faced by the control birds but not the iodinated birds. However, the weight at week 5 was the same between the two treatments, and there was no effect on the feed efficiency. We accept that iodinated of clean water does not have the health promoting effects in poultry production, however it did not have any detriment on development, however may prove to have a positive impact in cases where birds receive a lower quality water, and in the event of an infection may reduce spread throughout a flock.

5.2 Limitations

Overall, the research conducted provided evidence on how iodinated water and used chicken litter affect the intestinal microbial community, the gene expression in the caecum, effect on pathogen colonization, and the growth performance in chicken production. However, there are a few limitations of the present study. Water disappearance was measured in both chapter 2 and chapter 3, but the results varied greatly between the two experiments. The chicks were supplied the water in bell drinkers which allowed them to play in the water and splash water out of the bell drinkers, resulting in greater variation, and making this measurement less accurate. The water measurements do not necessarily indicate if either water was consumed more or less than the other, but help to suggest that the birds were not averse to consuming the iodinated water.

In both chapter 2 and 3, the effect of litter increased possibly commensal bacteria from the *Clostridiales* order, and reduced potentially pathogenic bacteria in the *Enterobacteriaceae* family, however with the 16S rRNA gene amplicon sequencing approach, we were unable to determine the identity of many of the specific genera and species of bacteria that change, and therefore cannot be sure that these bacteria were responsible for the positive effects. Bacterial identification through 16S rRNA gene amplification has other limitations, as it does not identifiy if the bacteria are live contributors to the gut community, as sequencing runs may be biased and have the potential to artificially increase perceived diversity. Further research using a more dynamic sequencing method such as metatranscriptomics of the microbial community may provide more information on the physiological roles of the individual species that increased within the context of the entire microbiome (Yeoman et al., 2012). Another limitation was the source of litter. In both chapter 2 and 3 used boiler litter was collected from the same poultry production facility. Examining litter from more than one location on the effects on poultry performance would add important information on the overall impact of litter on poultry performance.

The effect of both water and litter treatments on the gene expression in the caecum in chapter 3 was analyzed with high-throughput sequencing on a small sample size of 4 chicks per treatment. Using a small sample size has its limitations, but can be fine with further verification of the results. The genes determined to be changed between treatments could be compared in more samples using real-time quantitative PCR techniques to determine if the effect is found in more than the 4 samples sequenced. As well, using proteomics may be useful in determining what proteins are actively being transcribed from the genes, in addition to increased gene expression.

In both chapter 2 and 3, the experiments were conducted in micro-isolator chambers, limiting our ability to see the effects of treatments over a longer course of time. As well, using microisolators may not be equivalent to the environment introduced to chicks in a cleaned barn, as it may not allow for as much bacterial introduction through the water, feed, and environment. Conducting these trials over a longer period in a barn setting could provide further information into the effects of used litter on the growth and performance of adult chickens. As well, it would be beneficial to see if the change in microbiome is seen over a longer period of time, or if the two microbial communities begin to look similar, and the effects of litter are temporal.

Lastly, as the iodinated water was made with sterile reverse osmosis water, we compared the effect of iodinated water against the same RO water to determine the effect of iodine alone in chapter 4. However, this type of sterile water would not typically be used in a barn setting, which would likely contain other impurities, as well and farm waters have been shown to contain disinfection by-products that may have negative effects on poultry health (Reif et al., 1996). Therefore, our results, although informative on the direct effect of iodine, do not necessarily replicate the type of husbandry practice found in a barn, and using well water for both the development of the iodinated water and control would likely provide different results.

5.3 Future Directions

Our study contributed important information regarding the benefits associated with the re-use of poultry litter and chicken health, as well as the potential for using iodinated water as a means of pathogen prevention in poultry husbandry. However, used litter has been known to introduce pathogens to new flocks (Michael D. Cressman et al., 2010; Newell & Fearnley, 2003), and this risk needs to be mitigated. This research indicated the importance for the development of the stable microbiome in young chicks, and future research is needed to identify the microbes responsible for increasing this stability so that they can be introduced in a controlled manner. Work needs to be done to identify what may be called “missing microbes” between wild and conventionally raised chickens, in the hopes that we can tailor a mixture of missing bacteria to be given back to conventionally raised birds. As litter re-use has demonstrated to be an effective way to introduce microbes and increase the relative abundance of some bacteria, the generation of specific pathogen free flocks that do not have pathogenic bacteria could produce litter that would be safe to introduce to newly hatched chicks to initiate the development of a commensal microbiome without risk of infection.

Although our research indicated little effect of iodine on the growth performance and intestinal microbial community, there is still further research to determine its benefit compared to farm well water. As water is a source of pathogen spread in flocks (Pearson et al., 1996), future studies where pathogens are introduced through the water systems in a poultry house could indicate iodinated waters efficacy in preventing pathogen spread. As well, trials comparing

iodinated farm water compared to regular farm water or well water may distinguish iodine's efficacy in improving poultry health and production.

References

Abrams, G. D., Bauer, H., & Sprinz, H. (1963). Influence of the normal flora on mucosal morphology and cellular renewal in the ileum: A comparison of germ-free and conventional mice. *Lab. Invest*, (12), 355–364.

Andersen, S., Guan, H., Teng, W., & Laurberg, P. (2009). Speciation of iodine in high iodine groundwater in China associated with goitre and hypothyroidism. *Biological Trace Element Research*, *128*(2), 95–103. https://doi.org/10.1007/s12011-008-8257-x

Antunes, P., Mourão, J., Campos, J., & Peixe, L. (2016). Salmonellosis: The role of poultry meat. *Clinical Microbiology and Infection*, *22*(2), 110–121. https://doi.org/10.1016/j.cmi.2015.12.004

Ardissone, A. N., De La Cruz, D. M., Davis-Richardson, A. G., Rechcigl, K. T., Li, N., Drew, J. C., … Neu, J. (2014). Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS ONE*, *9*(3). https://doi.org/10.1371/journal.pone.0090784

Arrazuria, R., Elguezabal, N., Juste, R. A., Derakhshani, H., & Khafipour, E. (2016). Mycobacterium avium subspecies paratuberculosis infection modifies gut microbiota under different dietary conditions in a rabbit model. *Frontiers in Microbiology*, *7*(MAR), 1–14. https://doi.org/10.3389/fmicb.2016.00446

Backer, H., & Hollowell, J. (2000). Use of Iodine for Water Disinfection: Iodine Toxicity and Maximum Recommended Dose. *Environmental Health Perspectives*, *108*(8), 679–684. https://doi.org/10.1289/ehp.00108679

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V, Koh, G. Y., Nagy, A., … Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718–23. https://doi.org/10.1073/pnas.0407076101

Bagchi, N., Brown, T. R., Urdanivia, E., & Sundick, R. S. (1985). Induction of autoimmune thyroiditis in chickens by dietary iodine. *Science (New York, N.Y.)*, *230*(4723), 325–7. https://doi.org/10.1126/science.4048936

Ballou, A. L., Ali, R. A., Mendoza, M. A., Ellis, J. C., Hassan, H. M., Croom, W. J., & Koci, M. D. (2016). Development of the Chick Microbiome: How Early Exposure Influences Future Microbial Diversity. *Frontiers in Veterinary Science*, *3*(January), 1–12. https://doi.org/10.3389/fvets.2016.00002

Bar-Shira, E., & Friedman, A. (2006). Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick. *Developmental and Comparative Immunology*, *30*(10), 930–941. https://doi.org/10.1016/j.dci.2005.12.002

Bar-Shira, E., Sklan, D., & Friedman, A. (2003). Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Developmental and Comparative Immunology*, *27*(2), 147–157. https://doi.org/10.1016/S0145-305X(02)00076-9

Barnes, E. M., Impey, C. S., & Stevens, B. J. H. (1979). Factors affecting the incidence and anti-salmonella activity of the anaerobic caecal flora of the young chick. *Journal of Hygiene*, *82*(2), 263–283. https://doi.org/10.1017/S0022172400025687

Barrow, P. A. (1991). Experimental infection of chickens with Salmonella enteritidis. *Avian Pathology*, *20*(1), 145–153. https://doi.org/10.1080/03079459108418749

Bedford, A., & Gong, J. (2018). Implications of butyrate and its derivatives for gut health and animal production. *Animal Nutrition*, 1–9. https://doi.org/10.1016/j.aninu.2017.08.010

Bhogoju, S., Nahashon, S., Wang, X., Darris, C., & Kilonzo-Nthenge, A. (2018). A comparative analysis of microbial profile of Guinea fowl and chicken using metagenomic approach. *PLoS ONE*, *13*(3), 1–18. https://doi.org/10.1371/journal.pone.0191029

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bouguen, G., Dubuquoy, L., Desreumaux, P., Brunner, T., & Bertin, B. (2015). Intestinal steroidogenesis. *Steroids*, *103*, 64–71. https://doi.org/10.1016/j.steroids.2014.12.022

Burek, C. L., & Talor, M. V. (2009). Environmental triggers of autoimmune thyroiditis. *Journal of Autoimmunity*, *33*(3–4), 183–189. https://doi.org/10.1016/j.jaut.2009.09.001

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nature Publishing Group*, *7*(5), 335–336. https://doi.org/10.1038/nmeth0510-335

Chowdhury, S. R., King, D. E., Willing, B. P., Band, M. R., Beever, J. E., Lane, A. B., … Rex, H. R. (2007). Transcriptome profiling of the small intestinal epithelium in germfree versus conventional piglets. *BMC Genomics*, *8*. https://doi.org/10.1186/1471-2164-8-215

Coates, M. E., Davies, M. K., & Kon, S. K. (1955). The Effect of Antibiotics on the Intestine of

the Chick. *British Journal of Nutrition*, *9*(01), 110. https://doi.org/10.1079/BJN19550016

Coker, A. O., Isokpehi, R. D., Thomas, B. N., Amisu, K. O., & Larry Obi, C. (2002). Human campylobacteriosis in developing countries. *Emerging Infectious Diseases*, *8*(3), 237–243. https://doi.org/10.3201/eid0803.010233

Cook, R. H., & Bird, F. H. (1973). Duodenal Villus Area and Epithelial Cellular Migration in Conventional and Germ-Free Chicks,. *Poultry Science*, *52*(6), 2276–2280. https://doi.org/10.3382/ps.0522276

Corrier, D. E., Hargis, B., Hinton Jr., A., Lindsey, D., Caldwell, D., Manning, J., … DeLoach, J. (1991). Effect of anaerobic cecal microflora and dietary lactose on colonization resistance of layer chicks to invasive Salmonella enteritidis. *Avian Dis*, *35*(2), 337–343. https://doi.org/10.2307/1591186

Corrier, D. E., Hargis, B. M., Hinton, A., & DeLoach, J. R. (1993). Protective Effect of Used Poultry Litter and Lactose in the Feed Ration on Salmonella enteritidis Colonization of Leghorn Chicks and Hens. *Avian Diseases*, *37*(1), 47. https://doi.org/10.2307/1591456

Corrier, D. E., Hinton, A., Hargis, B., & DeLoach, J. R. (1992). Effect of Used Litter from Floor Pens of Adult Broilers on Salmonella Colonization of Broiler Chicks. *Avian Diseases*, *36*(4), 897. https://doi.org/10.2307/1591548

Cosby, D. E., Cox, N. A., Harrison, M. A., Wilson, J. L., Buhr, R. J., & Fedorka-Cray, P. J. (2015). *Salmonella* and antimicrobial resistance in broilers: A review: Table 1. *The Journal of Applied Poultry Research*, *24*(3), 408–426. https://doi.org/10.3382/japr/pfv038

Craven, S. E., Stern, N. J., Cox, N. A., Bailey, J. S., & Berrang, M. (1999). Cecal carriage of Clostridium perfringens in broiler chickens given Mucosal Starter Culture. *Avian Diseases*, *43*(3), 484–90. https://doi.org/10.2307/1592646

Cressman, M. D., Yu, Z., Nelson, M. C., Moeller, S. J., Lilburn, M. S., & Zerby, H. N. (2010). Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Applied and Environmental Microbiology*, *76*(19), 6572–6582. https://doi.org/10.1128/AEM.00180-10

Cressman, M. D., Zerby, H. N., Moeller, S. J., Walkden-Brown, S. W., & Yu, Z. (2014). Effects of Litter Reuse on Performance, Welfare, and the Microbiome of the Litter and Gastrointestinal Tract of Commercial Broiler Chickens DISSERTATION Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the.

Dahiya, J. P., Wilkie, D. C., Van Kessel, A. G., & Drew, M. D. (2006). Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Animal Feed Science and Technology*, *129*(1–2), 60–88. https://doi.org/10.1016/j.anifeedsci.2005.12.003

De Carli, S., Ikuta, N., Lehmann, F. K. M., Da Silveira, V. P., De Melo Predebon, G., Fonseca, A. S. K., & Lunge, V. R. (2015). Virulence gene content in Escherichia coli isolates from poultry flocks with clinical signs of colibacillosis in Brazil. *Poultry Science*, *94*(11), 2635–2640. https://doi.org/10.3382/ps/pev256

de Lima Brossi, M. J., Jiménez, D. J., Cortes-Tolalpa, L., & van Elsas, J. D. (2016). Soil-Derived Microbial Consortia Enriched with Different Plant Biomass Reveal Distinct Players Acting in Lignocellulose Degradation. *Microbial Ecology*, *71*(3), 616–627. https://doi.org/10.1007/s00248-015-0683-7

Degen, W. G. J., Van Daal, N., Rothwell, L., Kaiser, P., & Schijns, V. E. J. C. (2005). Th1/Th2 polarization by viral and helminth infection in birds. *Veterinary Microbiology*, *105*(3–4), 163–167. https://doi.org/10.1016/j.vetmic.2004.12.001

Delange, F., de Benoist, B., & Alnwick, D. (1999). Risks of iodine-induced hyperthyroidism after correction of iodine deficiency by iodized salt. *Thyroid: Official Journal Of The American Thyroid Association*, *9*(6), 545–556. Retrieved from http://login.ezproxy.library.ualberta.ca/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=cmedm&AN=10411116&site=eds-live&scope=site

Dibner, J. J., & Richards, J. D. (2005). Antibiotic Growth Promoters in Agriculture : History and Mode of Action Actual Usage of Antimicrobials in Denmark Voluntary and Legislated Bans. *Poultry Science*, *84*(July), 634–643.

Dibner, J. J., Richards, J. D., & Knight, C. D. (1963). Microbial imprinting in gut development and health. In *Lab. Invest* (pp. 355–364).

Ding, J., Dai, R., Yang, L., He, C., Xu, K., Liu, S., … Meng, H. (2017). Inheritance and establishment of gut microbiota in chickens. *Frontiers in Microbiology*, *8*(OCT), 1–11. https://doi.org/10.3389/fmicb.2017.01967

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., … Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

du Toit, J., & Casey, N. H. (2010). Effect of bromine and iodine in drinking water on production

parameters of broilers. *South African Journal of Animal Sciences*, *40*(4), 301–310. https://doi.org/10.4314/sajas.v40i4.65238

du Toit, J., & Casey, N. H. (2012). Iodine as an alleviator of bromine toxicity in thyroid, liver and kidney of broiler chickens. *Livestock Science*, *144*(3), 269–274. https://doi.org/10.1016/j.livsci.2011.12.011

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Emeash, H., Fayed, R., Essawy, G. (1994). Effect of iodine-treated water on the performance and some behavioural patterns of meat-strain chicks. *Vet Med J Giza*, *42(1),* 139–43.

Fernández-Rubio, C., Ordóñez, C., Abad-González, J., Garcia-Gallego, A., Honrubia, M. P., Mallo, J. J., & Balaña-Fouce, R. (2009). Butyric acid-based feed additives help protect broiler chickens from Salmonella enteritidis infection. *Poultry Science*, *88*(5), 943–948. https://doi.org/10.3382/ps.2008-00484

Forder, R. E. A., Howarth, G. S., Tivey, D. R., & Hughes, R. J. (2007). Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. *Poultry Science*, *86*(11), 2396–2403. https://doi.org/10.3382/ps.2007-00222

Fries, R., Akcan, M., Bandick, N., & Kobe, A. (2005). Microflora of two different types of poultry litter. *British Poultry Science*, *46*(6), 668–672. https://doi.org/10.1080/00071660500395483

Fukushima, K., Ogawa, H., Takahashi, K., Naito, H., Funayama, Y., Kitayama, T., … Sasaki, I. (2003). Non-pathogenic bacteria modulate colonic epithelial gene expression in germ-free mice. *Scandinavian Journal of Gastroenterology*, *38*(6), 626–634. https://doi.org/10.1080/00365510310000376

García-Hernández, Y., Pérez-Sánchez, T., Boucourt, R., Balcázar, J. L., Nicoli, J. R., Moreira-Silva, J., … Halaihel, N. (2016). Isolation, characterization and evaluation of probiotic lactic acid bacteria for potential use in animal production. *Research in Veterinary Science*, *108*, 125–132. https://doi.org/10.1016/j.rvsc.2016.08.009

Garcia, G. D., Carvalho, M. A. R., Diniz, C. G., Marques, J. L., Nicoli, J. R., & Farias, L. M. (2012). Isolation, identification and antimicrobial susceptibility of Bacteroides fragilis group strains recovered from broiler faeces. *British Poultry Science*, *53*(1), 71–76. https://doi.org/10.1080/00071668.2012.662272

Gaskins, H. R., Collier, C. T., & Anderson, D. B. (2002). Antibiotics as growth promotants: Mode of action. *Animal Biotechnology*, *13*(1), 29–42. https://doi.org/10.1081/ABIO-120005768

Good, K. D., & Vanbriesen, J. M. (2017). Power Plant Bromide Discharges and Downstream Drinking Water Systems in Pennsylvania. *Environmental Science and Technology*, *51*(20), 11829–11838. https://doi.org/10.1021/acs.est.7b03003

Gottardi, W. (1999). Iodine and disinfection: Theoretical study on mode of action, efficiency, stability, and analytical aspects in the aqueous system. *Archiv Der Pharmazie*, *332*(5), 151–157. https://doi.org/10.1002/(SICI)1521-4184(19995)332:5<151::AID-ARDP151>3.0.CO;2-E

Guo, A., Cai, J., Gong, W., Yan, H., Luo, X., Tian, G., … Cai, X. (2013). Transcriptome Analysis in Chicken Cecal Epithelia upon Infection by Eimeria tenella In Vivo. *PLoS ONE*, *8*(5), e64236. https://doi.org/10.1371/journal.pone.0064236

Hakkinen, M., & Schneitz, C. (1996). Efficacy of a commercial competitive exclusion product against a chicken pathogenic Escherichia coli and E coli O157:H7. *Vet Rec*, *139*(6), 139–141. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8863402

Hakkinen, M., & Schneitz, G. (1999). Efficacy of a commercial competitive exclusion product against Campylobacter jejuni. *British Poultry Science*, *40*(5), 619–621. https://doi.org/10.1080/00071669986990

Heyndrickx, M., Vandekerchove, D., Herman, L., Rollier, I., Grijspeerdt, K., & De zutter, L. (2002). Routes for salmonella contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. *Epidemiology and Infection*, *129*(02), 253–265. https://doi.org/10.1017/S0950268802007380

Higgins, S. E., Wolfenden, A. D., Tellez, G., Hargis, B. M., & Porter, T. E. (2011). Transcriptional profiling of cecal gene expression in probioticand Salmonella-challenged neonatal chicks. *Poultry Science*, *90*(4), 901–913. https://doi.org/10.3382/ps.2010-00907

Hitchon, B., Levinson, A. A., & Horn, M. K. (1977). Bromide, Iodide, and Boron in Alberta Formation Waters.

Hofacre, C. L., Beacorn, T., & Collett, S. (2003). Using Competitive Exclusion , Mannan-

Oligosaccharide and Other Necrotic Enteritis. *Biotechnology*, *12*(July), 60–64.

Hofacre, C. L., Johnson, A. C., Kelly, B. J., & Froyman, R. (2002). Effect of a commercial competitive exclusion culture on reduction of colonization of an antibiotic-resistant pathogenic Escherichia coli in day-old broiler chickens. *Avian Diseases*, *46*(1), 198–202. https://doi.org/10.1637/0005-2086(2002)046[0198:EOACCE]2.0.CO;2

Honjo, K., Hagiwara, T., Itoh, K., Takahashi, E., & Hirota, Y. (1993). Immunohistochemical Analysis of Tissue Distribution of B and T Cells in Germfree and Conventional Chickens. *The Journal of Veterinary Medical Science*, *55*(6), 1031–1034. https://doi.org/10.1292/jvms.55.1031

Hooper, L. V., Stappenbeck, T. S., Hong, C. V., & Gordon, J. I. (2003). Angiogenins: A new class of microbicidal proteins involved in innate immunity. *Nature Immunology*, *4*(3), 269–273. https://doi.org/10.1038/ni888

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., & Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science*, *291*(5505), 881–884. https://doi.org/10.1126/science.291.5505.881

Hu, Z., & Guo, Y. (2007). Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. *Animal Feed Science and Technology*, *132*(3–4), 240–249. https://doi.org/10.1016/j.anifeedsci.2006.03.017

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research*, *37*(1), 1–13. https://doi.org/10.1093/nar/gkn923

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*(1), 44–57. https://doi.org/10.1038/nprot.2008.211

Hugas, M., & Beloeil, P. A. (2014). Controlling *Salmonella* along the food chain in the European Union - progress over the last ten years. *Eurosurveillance*, *19*(19), 1–4. https://doi.org/10.2807/1560-7917.ES2014.19.19.20804

Hume, M. E., Kubena, L. F., Edrington, T. S., Donskey, C. J., Moore, R. W., Ricke, S. C., & Nisbet, D. J. (2003). Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. *Poultry Science*, *82*(7), 1100–1107. https://doi.org/10.1093/ps/82.7.1100

Ilina, L. A., Yildirim, E. A., Nikonov, I. N., Filippova, V. A., Laptev, G. Y., Novikova, N. I., … Fisinin, V. I. (2016). Metagenomic bacterial community profiles of chicken embryo gastrointestinal tract by using T-RFLP analysis. *Doklady Biochemistry and Biophysics*, *466*(1), 47–51. https://doi.org/10.1134/S1607672916010130

Impey, C. S., & Mead, G. C. (1989). Fate of salmonellas in the alimentary tract of chicks pre-treated with a mature caecal microflora to increase colonization resistance. *Journal of Applied Bacteriology*, *66*(6), 469–475. https://doi.org/10.1111/j.1365-2672.1989.tb04567.x

Jeffrey, J., Kirk, J., Atwill, E., & Cullor, J. (1998). Research notes: Prevalence of selected microbial pathogens in processed poultry waste used as dairy cattle feed. *Poultry Science*, *77*(6), 808–811. https://doi.org/10.1093/ps/77.6.808

Jiménez, E., Fernández, L., Marín, M. L., Martín, R., Odriozola, J. M., Nueno-Palop, C., … Rodríguez, J. M. (2005). Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Current Microbiology*, *51*(4), 270–274. https://doi.org/10.1007/s00284-005-0020-3

Jiménez, E., Marín, M. L., Martín, R., Odriozola, J. M., Olivares, M., Xaus, J., … Rodríguez, J. M. (2008). Is meconium from healthy newborns actually sterile? *Research in Microbiology*, *159*(3), 187–193. https://doi.org/10.1016/j.resmic.2007.12.007

Jones, F. S. (1913). An outbreak of an acute disease in adult fowls, due to bact. Pullorum. *The Journal of Medical Research*, *27*(4), 471–479.

Jones, F. T., & Hagler, W. M. (1983). Observations on New and Reused Litter for Growing Broilers. *Poultry Science*, *62*(1), 175–179. https://doi.org/10.3382/ps.0620175

Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J. I., & Krakoff, J. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *American Journal of Clinical Nutrition*, *94*(1), 58–65. https://doi.org/10.3945/ajcn.110.010132

Kaldhusdal, M., Schneitz, C., Hofshagen, M., & Skjerve, E. (2001). Reduced incidence of Clostridium perfringens-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. *Avian Diseases*, *45*(1), 149. https://doi.org/10.2307/1593022

Kennard, D. C., & Chamberlin, V. D. (1951). Growth and Mortality of Chickens as Affected by the Floor Litter. *Poultry Science*, *30*(1), 47–54.

Kizerwetter-Świda, M., & Binek, M. (2008). Bacterial microflora of the chicken embryos and newly hatched chicken. *Journal of Animal and Feed Sciences*, *17*(2), 224–232. https://doi.org/10.22358/jafs/66602/2008

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), 1–11. https://doi.org/10.1093/nar/gks808

Koch, B. J., Hungate, B. A., & Price, L. B. (2017). Food-animal production and the spread of antibiotic resistance: the role of ecology. *Frontiers in Ecology and the Environment*, *15*(6), 309–318. https://doi.org/10.1002/fee.1505

Lee, K. ., Lillehoj, H. . S., Lee, S. . H., Jang, S. . I., Ritter, G. D., Bautista, D. . A., … Lillehoj, E. . P. (2011). Impact of Fresh or Used Litter on the Posthatch Immune System of Commercial Broilers. *Avian Diseases Digest*, *6*(4), e54–e55. https://doi.org/10.1637/9916-969511-DIGEST.1

Lee, K. W., Kim, D. K., Lillehoj, H. S., Jang, S. I., & Lee, S. H. (2015). Immune modulation by Bacillus subtilis-based direct-fed microbials in commercial broiler chickens. *Animal Feed Science and Technology*, *200*(1), 76–85. https://doi.org/10.1016/j.anifeedsci.2014.12.006

Lee, K. W., Lee, S. H., Lillehoj, H. S., Li, G. X., Jang, S. I., Babu, U. S., … Siragusa, G. R. (2010). Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. *Poultry Science*, *89*(2), 203–216. https://doi.org/10.3382/ps.2009-00418

Lee, K. W., Lillehoj, H. S., Jang, S. I., Lee, S. H., Bautista, D. A., & Siragusa, G. R. (2013). Effect of Bacillus Subtilis-based direct-fed microbials on immune status in broiler chickens raised on fresh or used litter. *Asian-Australasian Journal of Animal Sciences*, *26*(11), 1592–1597. https://doi.org/10.5713/ajas.2013.13178

Li, J., Yang, K., Ju, T., Ho, T., McKay, C. A. C. A., Gao, Y., … Willing, B. P. B. P. (2017). Early life antibiotic exposure affects pancreatic islet development and metabolic regulation. *Scientific Reports*, *7*(February), 41778. https://doi.org/10.1038/srep41778

Li, X. Y., Swaggerty, C. L., Kogut, M. H., Chiang, H. I., Wang, Y., Genovese, K. J., … Zhou, H. J. (2011). Caecal transcriptome analysis of colonized and non-colonized chickens within two genetic lines that differ in caecal colonization by Campylobacter jejuni. *Animal*

Genetics, *42*(5), 491–500. https://doi.org/10.1111/j.1365-2052.2010.02168.x

Liao, Y., Smyth, G. K., & Shi, W. (2014). FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, *30*(7), 923–930. https://doi.org/10.1093/bioinformatics/btt656

Liljebjelke, K. A., Hofacre, C. L., Liu, T., White, D. G., Ayers, S., & Young, S. (2005). Vertical and Horizontal Transmission of Salmonella Within Integrated Broiler Production System, *2*(1), 90–102.

Lloyd, A. B., Cumming, R. B., & Kent, R. D. (1977). Prevention of Salmonella Typhimurium Infection in Poultry By Pretreatment of Chickens and Poults With Intestinal Extracts. *Australian Veterinary Journal*, *53*(2), 82–87. https://doi.org/10.1111/j.1751-0813.1977.tb14891.x

Lopetuso, L. R., Scaldaferri, F., Petito, V., & Gasbarrini, A. (2013). Commensal Clostridia: Leading players in the maintenance of gut homeostasis. *Gut Pathogens*. Gut Pathogens. https://doi.org/10.1186/1757-4749-5-23

Lovanh, N., Cook, K. L., Rothrock, M. J., Miles, D. M., & Sistani, K. (2007). Spatial Shifts in Microbial Population Structure Within Poultry Litter Associated with Physicochemical Properties. *Poultry Science*, *86*(9), 1840–1849. https://doi.org/10.1093/ps/86.9.1840

Lu, J., Sanchez, S., Hofacre, C., John, J., Harmon, B. G., Lee, M. D., & Maurer, J. J. (2003a). Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers. *Applied and Environmental Microbiology*, *69*(2), 901–908. https://doi.org/10.1128/AEM.69.2.901

Lu, J., Sanchez, S., Hofacre, C., John, J., Harmon, B. G., Lee, M. D., & Maurer, J. J. (2003b). Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene M. *Applied and Environmental Microbiology*, *69*(2), 901–908. https://doi.org/10.1128/AEM.69.2.901

Maiorka, A., Dahlke, F., & Morgulis, M. S. F. de A. (2006). Broiler adaptation to post-hatching period. *Ciência Rural*, *36*(2), 701–708. https://doi.org/10.1590/S0103-84782006000200057

Markovic, R., Sefer, D., Krstic, M., & Petrujkic, B. (2009). Effect of different growth promoters on broiler performance and gut morphology. *Arch Med Vet*, *41*, 163–169.

Martin, S. A., Mccann, M. A., & Waltman, W. D. (1998). Microbiological survey of Georgia poultry litter. *Journal of Applied Poultry Research*, *7*(1), 90–98. https://doi.org/10.1093/japr/7.1.90

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., & Neufeld, J. D. (2012). PANDAseq: Paired-end assembler for illumina sequences. *BMC Bioinformatics*, *13*(1), 1–7. https://doi.org/10.1186/1471-2105-13-31

Mccartney, M. G. (1971). Effect of Type of Housing and Litter on Production of Broilers. *Poultry Science*, *50*(4), 1200–1202.

Mead, G. C. (1989). Microbes of the avian cecum: Types present and substrates utilized. *Journal of Experimental Zoology*, *252*(3 S), 48–54. https://doi.org/10.1002/jez.1402520508

Medicine, I. of. (2006). *Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*. (J. J. Otten, J. P. Hellwig, & L. D. Meyers, Eds.). Washington, DC: The National Academies Press. https://doi.org/10.17226/11537

Moles, L., Gómez, M., Heilig, H., Bustos, G., Fuentes, S., de Vos, W., … Jiménez, E. (2013). Bacterial Diversity in Meconium of Preterm Neonates and Evolution of Their Fecal Microbiota during the First Month of Life. *PLoS ONE*, *8*(6). https://doi.org/10.1371/journal.pone.0066986

Molitoris, E., Krichevsky, M. I., Fagerberg, D. J., & Quarles, C. L. (1986). Effects of dietary chlortetracycline on the antimicrobial resistance of porcine faecal streptococcaceae. *Journal of Applied Bacteriology*, *60*(2), 111–120. https://doi.org/10.1111/j.1365-2672.1986.tb03367.x

Mooseker, M. S. (1985). Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annual Review of Cell Biology*, *1*(Mv), 209–41. https://doi.org/10.1146/annurev.cb.01.110185.001233

Morales-Erasto, V., Falconi-Agapito, F., Luna-Galaz, G. A., Saravia, L. E., Montalvan-Avalos, A., Soriano-Vargas E, E., & Fernandez-Diaz, M. (2016). Coinfection of Avibacterium paragallinarum and Ornithobacterium rhinotracheale in Chickens from Peru. *Avian Diseases*, *60*(1), 75–78. https://doi.org/10.1637/11265-082015-ResNote.1

Motulsky, H. J., & Brown, R. E. (2006). Detecting outliers when fitting data with nonlinear regression - A new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics*, *7*, 1–20. https://doi.org/10.1186/1471-2105-7-123

Murdoch, R., & Lagan, K. M. (2013). The role of povidone and cadexomer iodine in the management of acute and chronic wounds. *Physical Therapy Reviews*, *18*(3), 207–216. https://doi.org/10.1179/1743288X13Y.0000000082

Mutch, D. M., Simmering, R., Donnicola, D., Fotopoulos, G., Holzwarth, J. A., Williamson, G., & Corthésy-Theulaz, I. (2004). Impact of commensal microbiota on murine gastrointestinal tract gene ontologies. *Physiological Genomics*, *19*(1), 22–31. https://doi.org/10.1152/physiolgenomics.00105.2004

Mwangi, W. N., Beal, R. K., Powers, C., Wu, X., Humphrey, T., Watson, M., … Smith, A. L. (2010). Regional and global changes in TCRαβ T cell repertoires in the gut are dependent upon the complexity of the enteric microflora. *Developmental and Comparative Immunology*, *34*(4), 406–417. https://doi.org/10.1016/j.dci.2009.11.009

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., … Knight, R. (2013). *Advancing our understanding of the human microbiome using QIIME*. *Methods in Enzymology* (1st ed., Vol. 531). Elsevier Inc. https://doi.org/10.1016/B978-0-12-407863-5.00019-8

Nepelska, M., Cultrone, A., Béguet-Crespel, F., Le Roux, K., Doré, J., Arulampalam, V., & Blottière, H. M. (2012). Butyrate Produced by Commensal Bacteria Potentiates Phorbol Esters Induced AP-1 Response in Human Intestinal Epithelial Cells. *PLoS ONE*, *7*(12), 1–11. https://doi.org/10.1371/journal.pone.0052869

Newell, D. G., & Fearnley, C. (2003). Sources of Campylobacter Colonization in Broiler Chickens. *Applied and Environmental Microbiology*, *69*(8), 4343–4351. https://doi.org/10.1128/AEM.69.8.4343-4351.2003

Nodar, R., Acea, M. J., & Carballas, T. (1990). Microbial populations of poultry pine-sawdust litter. *Biological Wastes*, *33*(4), 295–306. https://doi.org/10.1016/0269-7483(90)90133-D

Nurmi, E., & Rantala, M. (1973). New Aspects of Salmonella Infection in Broiler Production. *Nature*, *241*(5386), 210–211. https://doi.org/10.1038/241210a0

Oakley, B. B., & Kogut, M. H. (2016). Spatial and Temporal Changes in the Broiler Chicken Cecal and Fecal Microbiomes and Correlations of Bacterial Taxa with Cytokine Gene Expression. *Frontiers in Veterinary Science*, *3*(February), 11. https://doi.org/10.3389/fvets.2016.00011

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., … Cox, N.

A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiology Letters*, *360*(2), 100–112. https://doi.org/10.1111/1574-6968.12608

Opalinski, S., Dolinska, B., Korczynski, M., Chojnacka, K., Dobrzanski, Z., & Ryszka, F. (2012). Effect of iodine-enriched yeast supplementation of diet on performance of laying hens, egg traits, and egg iodine content. *Poultry Science*, *91*(7), 1627–1632. https://doi.org/10.3382/ps.2011-02031

Oviedo-rondón, E. O. (2009). Molecular methods to evaluate effects of feed additives and nutrients in poultry gut microflora Edgar. *Revista Brasileira de Zootecnia*, *38*, 209–225.

Palumbo, S. a, & Alford, J. a. (1970). Inhibitory action of tetrathionate enrichment broth. *Applied Microbiology*, *20*(6), 970–976.

Park, J., Kotani, T., Konno, T., Setiawan, J., Kitamura, Y., Imada, S., … Matozaki, T. (2016). Promotion of Intestinal Epithelial Cell Turnover by Commensal Bacteria: Role of Short-Chain Fatty Acids. *Plos One*, *11*(5), e0156334. https://doi.org/10.1371/journal.pone.0156334

Patterson, E., Wall, R., Fitzgerald, G. F., Ross, R. P., & Stanton, C. (2012). Health Implications of High Dietary Omega-6 Polyunsaturated Fatty Acids. *Journal of Nutrition and Metabolism*, *2012*(11), 1–16. https://doi.org/10.1155/2012/539426

Pearson, A. D., Greenwood, M. H., Feltham, R. K. A., Healing, T. D., Donaldson, J., Jones, D. M., & Colwell, R. R. (1996). Microbial ecology of Campylobacter jejuni in a United Kingdom chicken supply chain: Intermittent common source, vertical transmission, and amplification by flock propagation. *Applied and Environmental Microbiology*, *62*(12), 4614–4620.

Perez-Muñoz, M. E., Arrieta, M. C., Ramer-Tait, A. E., & Walter, J. (2017). A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: Implications for research on the pioneer infant microbiome. *Microbiome*, *5*(1), 1–19. https://doi.org/10.1186/s40168-017-0268-4

Pourakbari, M., Seidavi, A., Asadpour, L., & Martínez, A. (2016). Probiotic level effects on growth performance, carcass traits, blood parameters, cecal microbiota, and immune response of broilers. *Anais Da Academia Brasileira de Ciencias*, *88*(2), 1011–1021. https://doi.org/10.1590/0001-3765201620150071

Rantala, M., & Nurmi, E. (1973). Prevention of the growth of salmonella infantis in chicks by

the flora of the alimentary tract of chickens. *British Poultry Science*, *14*(6), 627–630. https://doi.org/10.1080/00071667308416073

Rehman, H. U., Vahjen, W., Awad, W. A., & Zentek, J. (2007). Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. *Archives of Animal Nutrition*, *61*(5), 319–335. https://doi.org/10.1080/17450390701556817

Reif, J. S., Hatch, M. C., Bracken, M., Holmes, L. B., Schwetz, B. a, & Singer, P. C. (1996). Reproductive and Developmental Effects of Disinfection by-Products in Drinking Water. *Environmental Health Perspectives*, *104*(10), 1056. https://doi.org/10.2307/3433117

Rinttilä, T., & Apajalahti, J. (2013). Intestinal microbiota and metabolites — Implications for broiler chicken health and performance. *J. Appl. Poult Res.*, *22*(July), 647–658. https://doi.org/10.3382/japr.2013-00742

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*, e2584. https://doi.org/10.7717/peerj.2584

Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., & Thompson, F. (2014). *The prokaryotes: Actinobacteria*. *The Prokaryotes: Actinobacteria*. https://doi.org/10.1007/978-3-642-30138-4

Röttger, A. S., Halle, I., Wagner, H., Breves, G., Dänicke, S., & Flachowsky, G. (2012). The effects of iodine level and source on iodine carry-over in eggs and body tissues of laying hens. *Archives of Animal Nutrition*, *66*(5), 385–401. https://doi.org/10.1080/1745039X.2012.719795

Röttger, A. S., Halle, I., Wagner, H., Breves, G., & Flachowsky, G. (2011). The effect of various iodine supplementations and two different iodine sources on performance and iodine concentrations in different tissues of broilers. *British Poultry Science*, *52*(1), 115–123. https://doi.org/10.1080/00071668.2010.539591

Ruiz, V., Ruiz, D., Gernat, A. G., Grimes, J. L., Murillo, J. G., Wineland, M. J., … Maguire, R. O. (2008). The effect of quicklime (CaO) on litter condition and broiler performance. *Poultry Science*, *87*(5), 823–827. https://doi.org/10.3382/ps.2007-00101

Sasaki, T., Takasuga, S., Sasaki, J., Kofuji, S., Eguchi, S., Yamazaki, M., & Suzuki, A. (2009). Mammalian phosphoinositide kinases and phosphatases. *Progress in Lipid Research*, *48*(6), 307–343. https://doi.org/10.1016/j.plipres.2009.06.001

Schneitz, C. (2005). Competitive exclusion in poultry - 30 years of research. *Food Control*, *16*(8

SPEC. ISS.), 657–667. https://doi.org/10.1016/j.foodcont.2004.06.002

Segain, J.-P. (2000). Butyrate inhibits inflammatory responses through NFkappa B inhibition: implications for Crohn's disease. *Gut*, *47*(3), 397–403. https://doi.org/10.1136/gut.47.3.397

Seo, B. J., Rather, I. A., Kumar, V. J. R., Choi, U. H., Moon, M. R., Lim, J. H., & Park, Y. H. (2012). Evaluation of Leuconostoc mesenteroides YML003 as a probiotic against low-pathogenic avian influenza (H9N2) virus in chickens. *Journal of Applied Microbiology*, *113*(1), 163–171. https://doi.org/10.1111/j.1365-2672.2012.05326.x

Shakouri, M. D., Iji, P. A., Mikkelsen, L. L., & Cowieson, A. J. (2009). Intestinal function and gut microflora of broiler chickens as influenced by cereal grains and microbial enzyme supplementation. *Journal of Animal Physiology and Animal Nutrition*, *93*(5), 647–658. https://doi.org/10.1111/j.1439-0396.2008.00852.x

Shannon, M. A., Bohn, P. W., Elimelech, M., Georgiadis, J. G., Marinas, B. J., & Mayes, A. M. (2008). Science and technology for water purification in the coming decades. *Nature (London, U. K.)*, *452*(March), 301–310. https://doi.org/10.1038/nature06599

Sharma, R. B., Alegria, J. D., Talor, M. V, Rose, N. R., Caturegli, P., & Burek, C. L. (2005). Iodine and IFN-gamma synergistically enhance intercellular adhesion molecule 1 expression on NOD.H2h4 mouse thyrocytes. *Journal of Immunology (Baltimore, Md. : 1950)*, *174*(12), 7740–7745. https://doi.org/10.4049/jimmunol.174.12.7740

Shepherd, E. M., & Fairchild, B. D. (2010). Footpad dermatitis in poultry. *Poultry Science*, *89*(10), 2043–2051. https://doi.org/10.3382/ps.2010-00770

Shokryazdan, P., Sieo, C., & Kalavathy, R. (2017). Effects of Lactobacillus salivarius complex on performance, intestinal health status and serum lipids of chickens. *Downloads.Hindawi.Com*, 1–20. Retrieved from http://downloads.hindawi.com/journals/tswj/raa/187561.pdf

Slawinska, A., Plowiec, A., Siwek, M., Jaroszewski, M., & Bednarczyk, M. (2016). Long-term transcriptomic effects of prebiotics and synbiotics delivered in ovo in broiler chickens. *PLoS ONE*, *11*(12), 1–22. https://doi.org/10.1371/journal.pone.0168899

Słupczyńska, M., Jamroz, D., Orda, J., & Wiliczkiewicz, A. (2014). Effect of various sources and levels of iodine, as well as the kind of diet, on the performance of young laying hens, iodine accumulation in eggs, egg characteristics, and morphotic and biochemical indices in blood. *Poultry Science*, *93*(10), 2536–2547. https://doi.org/10.3382/ps.2014-03959

Smith, H., Cook, J., & Parsell, Z. (1985). The experimental infection of chickens with mixtures of infectious bronchitis virus and Escherichia coli. *Journal of General Virology*, *66*(1985), 777–786.

Smith, H. W., & Tucker, J. F. (1980). The virulence of salmonella strains for chickens: Their excretion by infected chickens. *Journal of Hygiene*, *84*(3), 479–488. https://doi.org/10.1017/S0022172400027017

Snoeyenbos, G. H., Weinack, O. M., & Smyser, C. F. (1978). Protecting Chicks and Poults from Salmonellae by Oral Administration of " Normal " Gut Microflora, *22*(2), 273–287.

Soerjadi-Liem, A. S., Snoeyenbos, G. H., & Weinack, O. M. (1984). Comparative studies on competitive exclusion of three isolates of Campylobacter fetus subsp. jejuni in chickens by native gut microflora. *Avian Dis*, *28*(1), 139–146. https://doi.org/10.2307/1590135

Soerjadi, A. S., Snoeyenbos, G. H., & Weinack, O. M. (1982). Intestinal Colonization and Competitive Exclusion of Campylobacter fetus subsp. jejuni in Young Chicks. *Avian Pathologists*, *26*(3), 520–524.

Soerjadi, A. S., Stehman, S. M., Snoeyenbos, G. H., Weinack, O. M., & Smyser, C. F. (1981). Some Measurements of Protection against Paratyphoid Salmonella and Escherichia coli by Competitive Exclusion in Chickens, *23*(3), 706–712.

Sommer, F., & Bäckhed, F. (2013). The gut microbiota-masters of host development and physiology. *Nature Reviews Microbiology*, *11*(4), 227–238. https://doi.org/10.1038/nrmicro2974

Stanley, D., Hughes, R. J., & Moore, R. J. (2014). Microbiota of the chicken gastrointestinal tract: Influence on health, productivity and disease. *Applied Microbiology and Biotechnology*, *98*(10), 4301–4310. https://doi.org/10.1007/s00253-014-5646-2

Stanley, V. G., Bailey, J. E., & Krueger, W. F. (1989). Research Note : Effect of Iodine-Treated Water on the Performance of Broiler Chickens Reared Under Various Stocking Densities It is well documented that iodine is an essential nutrient in poultry diets. *Poultry Science*, *68*(3), 435–437.

Stappenbeck, T. S., Hooper, L. V, & Gordon, J. I. (2002). Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(24), 15451–15455. https://doi.org/10.1073/pnas.202604299

Stavric, S., Gleeson, T. M., Blanchfield, B., & Pivnick, H. (1985). Competitive-Exclusion of Salmonella From Newly Hatched Chicks By Mixtures of Pure Bacterial Cultures Isolated From Fecal and Cecal Contents of Adult Birds. *Journal of Food Protection*, *48*(9), 778-. https://doi.org/10.4315/0362-028X-48.9.778

Stavric, S., Buchanan, B., & Gleeson, T. M. (1992). Competitive exclusion of Escherichia coli O157: H7 from chicks with anaerobic cultures of faecal microflora. *Letters in Applied Microbiology*, *14*(5), 191–193. https://doi.org/10.1111/j.1472-765X.1992.tb00682.x

Stern, N. (1994). Mucosal Competitive Exclusion to Diminish Colonization of Chickens by Campylobacter jejuni. *Poultry Science*, *73*(July), 402–407. https://doi.org/10.1007/s13398-014-0173-7.2

Stern, N. J., Clavero, M. R. S., Bailey, J. S., Cox, N. A., & Robach, M. C. (1995). Campylobacter spp. in broilers on the farm and after transport. *Poultry Science*, (74), 937–941.

Sunkara, L. T., Achanta, M., Schreiber, N. B., Bommineni, Y. R., Dai, G., Jiang, W., … Zhang, G. (2011). Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. *PLoS ONE*, *6*(11). https://doi.org/10.1371/journal.pone.0027225

Suzuki, S. (1994). Pathogenicity of Salmonella enteritidis in poultry. *International Journal of Food Microbiology*, *21*(1–2), 89–105. https://doi.org/10.1016/0168-1605(94)90203-8

Szabó, R., Wehmann, E., Makrai, L., Nemes, C., Gyuris, É., Thuma, Á., & Magyar, T. (2017). Characterization of Ornithobacterium rhinotracheale field isolates from Hungary. *Avian Pathology*, *46*(5), 506–514. https://doi.org/10.1080/03079457.2017.1321104

Taherparvar, G., Seidavi, A., Asadpour, L., Payan-Carreira, R., Laudadio, V., & Tufarelli, V. (2016). Effect of litter treatment on growth performance, intestinal development, and selected cecum microbiota in broiler chickens. *Revista Brasileira de Zootecnia*, *45*(5), 257–264. https://doi.org/10.1590/S1806-92902016000500008

Thiennimitr, P., Winter, S. E., Winter, M. G., Xavier, M. N., Tolstikov, V., Huseby, D. L., … Baumler, A. J. (2011). Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. *Proceedings of the National Academy of Sciences*, *108*(42), 17480–17485. https://doi.org/10.1073/pnas.1107857108

Thomke, S., & Elwinger, K. (1998). Growth promotants in feeding pigs and poultry. III.

Alternatives to antibiotic growth promotants. *Annales de Zootechnie*, *47*(4), 245–271. https://doi.org/10.1051/animres:19980402

Torok, V. A., Hughes, R. J., Mikkelsen, L. L., Perez-Maldonado, R., Balding, K., MacAlpine, R., … Ophel-Keller, K. (2011). Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Applied and Environmental Microbiology*, *77*(17), 5868–5878. https://doi.org/10.1128/AEM.00165-11

Tran, D. T., & Ten Hagen, K. G. (2013). Mucin-type o-glycosylation during development. *Journal of Biological Chemistry*, *288*(10), 6921–6929. https://doi.org/10.1074/jbc.R112.418558

Turk, D. E. (1982). Symposium: the avian gastrointestinal tract and digestion, *61*(7), 1225–1244.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, *444*(7122), 1027–1031. https://doi.org/10.1038/nature05414

Van der Wielen, P. W. J. J., Biesterveld, S., Hofstra, H., Urlings, B. a P., & Van, F. (2000). Role of Volatile Fatty Acids in Development of the Cecal Microflora in Broiler Chickens during Growth Role of Volatile Fatty Acids in Development of the Cecal Microflora in Broiler Chickens during Growth, *66*(6), 6–11. https://doi.org/10.1128/AEM.66.6.2536-2540.2000.Updated

Van Der Wielen, P. W. J. J., Keuzenkamp, D. A., Lipman, L. J. A., Van Knapen, F., & Biesterveld, S. (2002). Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbial Ecology*, *44*(3), 286–293. https://doi.org/10.1007/s00248-002-2015-y

Van Kruiningen, H. J., Ruiz, B., & Gumprecht, L. (1991). Experimental disease in young chickens induced by a Mycobacterium paratuberculosis isolate from a patient with Crohn's disease. *Canadian Journal of Veterinary Research = Revue Canadienne de Recherche Veterinaire*, *55*(2), 199–202.

Velický, J., Titlbach, M., Lojda, Z., Dušková, J., Vobecký, M., & Raška, I. (2004). The effect of bromide on the ultrastructure of rat thyrocytes. *Annals of Anatomy*, *186*(3), 209–216. https://doi.org/10.1016/S0940-9602(04)80004-9

Vieira, S. L., & Moran, E. T. (1999). Effects of Delayed Placement and Used Litter on Broiler

Yields. *Journal of Applied Poultry Research*, *8*(1), 75–81.

Volf, J., Polansky, O., Sekelova, Z., Velge, P., Schouler, C., Kaspers, B., & Rychlik, I. (2017). Gene expression in the chicken caecum is dependent on microbiota composition. *Veterinary Research*, *48*(1), 85. https://doi.org/10.1186/s13567-017-0493-7

Volf, J., Polansky, O., Varmuzova, K., Gerzova, L., Sekelova, Z., Faldynova, M., … Rychlik, I. (2016). Transient and prolonged response of chicken cecum mucosa to colonization with different gut microbiota. *PLoS ONE*, *11*(9), 1–19. https://doi.org/10.1371/journal.pone.0163932

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

Wealleans, A. L., Sirukhi, M., & Egorov, I. A. (2017). Performance, gut morphology and microbiology effects of a Bacillus probiotic, avilamycin and their combination in mixed grain broiler diets. *British Poultry Science*, *58*(5), 523–529. https://doi.org/10.1080/00071668.2017.1349298

Weinack, O. M., Snoeyenbos, G. H., Smyser, C. F., & Soerjadi, A. S. (1981). Competitive Exclusion of Intestinal Colonization of Escherichia coli in Chicks, *25*(3), 696–705.

Weinack, O. M., Snoeyenbos, G. H., Smyser, C. F., & Soerjadi, A. S. (1982). Reciprocal Competitive Exclusion of Salmonella and Escherichia coli by Native Intestinal Microflora of the Chicken and Turkey, *26*(3), 585–595.

Yamak, U. S., Sarica, M., Boz, M. A., & Ucar, A. (2015). Effect of reusing litter on broiler performance, foot pad dermatitis and litter quality in chickens with different growth rates. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, *22*(1), 85–91. https://doi.org/10.9775/kvfd.2015.13957

Yeoman, C. J., Chia, N., Jeraldo, P., Sipos, M., Goldenfeld, N. D., & White, B. A. (2012). The microbiome of the chicken gastrointestinal tract. *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases*, *13*(1), 89–99. https://doi.org/10.1017/S1466252312000138

Yu, H., Zhou, T., Gong, J., Young, C., Su, X., Li, X. Z., … Yang, R. (2010). Isolation of deoxynivalenol-transforming bacteria from the chicken intestines using the approach of PCR-DGGE guided microbial selection. *BMC Microbiology*, *10*.

https://doi.org/10.1186/1471-2180-10-182

Zhang, W. H., Jiang, Y., Zhu, Q. F., Gao, F., Dai, S. F., Chen, J., & Zhou, G. H. (2011). Sodium butyrate maintains growth performance by regulating the immune response in broiler chickens. *British Poultry Science*, *52*(3), 292–301. https://doi.org/10.1080/00071668.2011.578121

Ziemer, C. J., & Steadham, S. R. (2003). Evaluation of the specificity of Salmonella PCR primers using various intestinal bacterial species. *Letters in Applied Microbiology*, *37*(6), 463–469. https://doi.org/10.1046/j.1472-765X.2003.01430.x

Appendices

Appendix 1 Significantly differentially expressed up-regulated genes identified between used litter and clean litter treatment on H_2O.

Ensembl gene ID	Gene symbol	Entrez ID	Log_2FC	FDR
ENSGALG00000016964	EPSTI1	418837	2.55	5.12E-18
ENSGALG00000036915	SQLE	420335	1.66	5.11E-11
ENSGALG00000001558	MOV10	419872	1.89	2.53E-08
ENSGALG00000003879	MFSD2A	419679	1.30	5.86E-08
ENSGALG00000007701	B3GNT7	424930	3.21	1.40E-07
ENSGALG00000007311	CLDN2	422292	1.96	3.77E-07
ENSGALG00000004243	IFITM3	422993	1.67	5.11E-07
ENSGALG00000000516	FANCA	415854	1.30	5.90E-07
ENSGALG00000010560	EIF2AK2	395147	1.21	6.10E-07
ENSGALG00000028037	FOS	396512	2.86	7.74E-07
ENSGALG00000016400	RSAD2	428650	3.22	7.74E-07
ENSGALG00000041129	TMEM173	768990	1.45	7.74E-07
ENSGALG00000016759	MITD1	418698	1.12	8.43E-07
ENSGALG00000010798	DHCR24	424661	1.06	1.57E-06
ENSGALG00000028318	CDKN1A	378914	2.44	2.39E-06
ENSGALG00000016665	FDFT1	422038	1.14	5.45E-06
ENSGALG00000007651	STAT1	424044	1.59	5.70E-06
ENSGALG00000013723	OASL	395908	3.16	7.56E-06
ENSGALG00000041192	IFIH1	424185	1.57	1.64E-05
ENSGALG00000003144	TRIM25	417401	1.27	1.64E-05
ENSGALG00000013911	ZC3HAV1	426315	1.26	1.64E-05
ENSGALG00000037286	VIL1	396423	1.37	1.85E-05
ENSGALG00000009365	CYP51	420548	1.07	2.20E-05
ENSGALG00000031373	PADI3	395910	1.52	2.48E-05
ENSGALG00000012873	SERPINB5	420900	1.98	3.32E-05
ENSGALG00000017106	ATP8A2	418936	1.24	3.42E-05
ENSGALG00000004859	ZNFX1	419218	2.01	4.72E-05
ENSGALG00000006785	IRF1	396384	1.09	5.26E-05
ENSGALG00000010866	AREG	428752	4.30	1.10E-04
ENSGALG00000045511	PARP9	424269	1.26	1.13E-04
ENSGALG00000002708	LINGO1	415344	1.25	1.51E-04
ENSGALG00000019716	KRT20	420045	1.69	1.67E-04
ENSGALG00000043734	LIPG	426846	1.21	1.67E-04
ENSGALG00000001231	SAMHD1	419125	1.04	1.67E-04
ENSGALG00000039895	EPHA2	771550	1.48	1.82E-04
ENSGALG00000009560	MSMO1	422423	1.20	2.64E-04
ENSGALG00000002792	SPDEF	428271	1.61	2.94E-04
ENSGALG00000007817	EHF	425791	1.37	4.34E-04
ENSGALG00000010864	EREG	408036	1.62	5.68E-04
ENSGALG00000030941	KIAA1324	NA	1.02	5.68E-04
ENSGALG00000000901	CHST4	427567	1.27	6.63E-04
ENSGALG00000005852	SRMS	419246	1.85	6.91E-04
ENSGALG00000045936	FAM83F	770747	1.40	6.96E-04
ENSGALG00000004260	PKP3	422994	1.20	7.12E-04
ENSGALG00000016128	B3GALT5	427985	2.30	7.55E-04
ENSGALG00000019555	SERPINB1	420894	1.90	7.97E-04
ENSGALG00000020002	SLC22A3	421582	1.34	1.04E-03
ENSGALG00000028047	RHOV	428868	1.50	1.05E-03
ENSGALG00000029015	TM6SF2	425719	1.18	1.12E-03
ENSGALG00000003217	LITAF	374125	1.35	1.38E-03
ENSGALG00000014948	HMGCR	395145	1.13	1.43E-03
ENSGALG00000005763	VILL	420415	2.04	1.86E-03
ENSGALG00000010149	CMTR1	421434	1.12	1.87E-03
ENSGALG00000015691	SMC2	396156	1.27	1.87E-03
ENSGALG00000030002	KRT18	101749333	1.14	1.89E-03
ENSGALG00000032957	NOCT	404779	1.12	1.93E-03
ENSGALG00000008980	VWA2	423907	2.21	1.94E-03
ENSGALG00000001407	ADAMTS15	419733	1.11	1.98E-03
ENSGALG00000010579	STON2	423390	1.12	2.02E-03
ENSGALG00000010692	LRP8	396102	2.02	2.16E-03
ENSGALG00000001727	LZTS1	431331	1.20	2.16E-03
ENSGALG00000015422	NRG1	373906	2.14	2.18E-03
ENSGALG00000001442	OPCML	395422	1.49	2.51E-03
ENSGALG00000014684	ERAP1	427122	1.14	2.61E-03
ENSGALG00000033198	MYO5C	430027	1.08	2.66E-03
ENSGALG00000004285	HNF4A	419198	1.20	3.42E-03
ENSGALG00000008098	NAMPT	417707	1.05	3.42E-03
ENSGALG00000027786	SOCS3	395299	1.78	3.42E-03
ENSGALG00000017394	INSIG1	420442	1.14	3.45E-03
ENSGALG00000036645	KIF15	420708	1.12	3.48E-03
ENSGALG00000035845	JPH1	420190	1.22	3.75E-03
ENSGALG00000010312	PLCH1	425030	1.28	3.83E-03
ENSGALG00000004446	NPFFR1	378784	2.47	4.12E-03
ENSGALG00000005806	KIF24	427398	1.35	4.13E-03
ENSGALG00000006354	GAL3ST2	424854	6.00	4.22E-03
ENSGALG00000046187	PARP12	418100	1.12	4.90E-03
ENSGALG00000023347	PTCHD3	428417	1.55	4.90E-03
ENSGALG00000036456	TRIB1	428386	1.16	5.27E-03
ENSGALG00000000470	LMNB2	396222	1.14	5.43E-03
ENSGALG00000003922	TOP2A	395570	1.09	5.76E-03
ENSGALG00000015854	PGM3	421841	1.00	6.22E-03
ENSGALG00000011141	ITGB6	424191	1.37	6.35E-03
ENSGALG00000028308	TNS4	107055314	1.24	6.57E-03
ENSGALG00000005845	SLC7A5	415832	1.01	6.66E-03
ENSGALG00000036678	ETV4	395747	1.85	6.95E-03
ENSGALG00000002797	PGD	419450	1.15	7.24E-03
ENSGALG00000038311	COL18A1	NA	1.20	7.43E-03
ENSGALG00000007669	EGR1	373931	1.54	8.49E-03
ENSGALG00000002121	PLEKHN1	771179	1.15	8.49E-03
ENSGALG00000019795	NA	NA	1.91	9.18E-14
ENSGALG00000042001	NA	422513	1.95	6.97E-13
ENSGALG00000009479	NA	420559	4.37	3.42E-12
ENSGALG00000041621	NA	395550	3.35	4.28E-12

ENSGALG00000013575	NA	403120	4.17	8.76E-12
ENSGALG00000038140	NA	NA	4.64	1.22E-11
ENSGALG00000016142	NA	395313	3.96	4.14E-11
ENSGALG00000013057	NA	418167	3.41	2.02E-10
ENSGALG00000009639	NA	NA	5.12	3.63E-10
ENSGALG00000011190	NA	NA	3.00	4.36E-10
ENSGALG00000039585	NA	NA	4.83	1.85E-09
ENSGALG00000045085	NA	423790	3.18	2.37E-09
ENSGALG00000030318	NA	NA	3.16	3.81E-09
ENSGALG00000011796	NA	NA	2.86	1.67E-08
ENSGALG00000028982	NA	421921	2.77	4.22E-08
ENSGALG00000000720	NA	419563	1.38	3.40E-07
ENSGALG00000045534	NA	100857563	1.63	1.05E-06
ENSGALG00000026970	NA	770612	1.65	1.26E-06
ENSGALG00000037629	NA	NA	1.94	1.43E-06
ENSGALG00000030952	NA	418543	7.11	1.18E-05
ENSGALG00000044778	NA	NA	3.94	1.36E-05
ENSGALG00000039269	NA	NA	2.35	1.64E-05
ENSGALG00000046098	NA	NA	1.27	1.65E-05
ENSGALG00000045105	NA	NA	1.39	1.70E-05
ENSGALG00000012072	NA	101747378	1.65	1.76E-05
ENSGALG00000019063	NA	418981	2.19	2.55E-05
ENSGALG00000033300	NA	NA	1.19	2.75E-05
ENSGALG00000016761	NA	395708	3.61	2.91E-05
ENSGALG00000037416	NA	101747310	1.70	5.43E-05
ENSGALG00000032428	NA	100858381	1.68	7.97E-05
ENSGALG00000036747	NA	100858003	1.07	9.52E-05
ENSGALG00000045477	NA	NA	1.52	1.02E-04
ENSGALG00000026422	NA	424266	1.38	1.04E-04
ENSGALG00000038950	NA	423101	1.68	1.88E-04
ENSGALG00000041833	NA	NA	3.25	2.81E-04
ENSGALG00000045581	NA	420368	1.08	2.89E-04
ENSGALG00000023821	NA	100858653	1.71	3.80E-04
ENSGALG00000044985	NA	NA	1.31	3.96E-04
ENSGALG00000001320	NA	396051	4.15	4.10E-04
ENSGALG00000029521	NA	107054237	2.46	4.57E-04
ENSGALG00000000504	NA	419813	1.24	4.79E-04
ENSGALG00000001325	NA	396052	4.20	4.79E-04
ENSGALG00000046482	NA	417458	2.39	5.68E-04
ENSGALG00000034721	NA	NA	3.35	6.91E-04
ENSGALG00000014505	NA	422826	1.47	7.05E-04
ENSGALG00000031737	NA	NA	1.49	7.32E-04
ENSGALG00000005964	NA	422224	1.04	8.52E-04
ENSGALG00000039716	NA	NA	1.03	9.06E-04
ENSGALG00000012915	NA	426764	1.05	9.29E-04
ENSGALG00000019553	NA	395715	3.58	1.13E-03
ENSGALG00000039647	NA	NA	3.55	1.71E-03
ENSGALG00000045064	NA	NA	1.10	1.92E-03
ENSGALG00000045640	NA	776920	1.88	2.19E-03
ENSGALG00000034688	NA	NA	1.10	2.27E-03
ENSGALG00000033314	NA	417459	2.38	2.39E-03
ENSGALG00000044780	NA	NA	1.39	2.61E-03
ENSGALG00000041502	NA	395773	2.08	3.29E-03
ENSGALG00000023709	NA	417192	1.71	3.67E-03
ENSGALG00000033170	NA	NA	1.08	3.97E-03
ENSGALG00000035761	NA	420553	1.40	4.14E-03
ENSGALG00000021139	NA	416928	2.14	5.01E-03
ENSGALG00000006138	NA	NA	2.01	5.52E-03
ENSGALG00000034649	NA	415724	1.49	5.74E-03
ENSGALG00000005937	NA	NA	2.07	6.46E-03
ENSGALG00000004747	NA	NA	2.04	7.40E-03
ENSGALG00000032368	NA	NA	1.02	8.40E-03
ENSGALG00000015704	NA	396437	1.27	8.49E-03
ENSGALG00000038257	NA	NA	2.61	8.49E-03
ENSGALG00000032687	NA	423088	1.13	8.59E-03
ENSGALG00000032401	NA	NA	1.94	8.87E-03
ENSGALG00000042386	NA	NA	1.12	9.07E-03
ENSGALG00000044618	NA	415531	2.05	9.07E-03
ENSGALG00000011443	NA	423476	1.23	9.67E-03

Appendix 2 Significantly differentially expressed down-regulated genes identified between used litter and clean litter treatment on H_2O.

Ensembl gene ID	Gene symbol	Entrez ID	Log_2FC	FDR
ENSGALG00000002893	STC2	416208	-1.95	5.90E-07
ENSGALG00000011529	NAAA	422641	-1.30	9.57E-07
ENSGALG00000011921	DHRS7	423527	-1.68	1.74E-06
ENSGALG00000013414	PDLIM3	414873	-1.32	2.39E-06
ENSGALG00000033260	SIAT9	407775	-1.28	4.50E-06
ENSGALG00000036819	ARHGDIG	100858938	-1.17	2.31E-05
ENSGALG00000014975	DRD5	427552	-2.39	3.21E-05
ENSGALG00000015544	ALDOB	427308	-1.68	4.72E-05
ENSGALG00000002955	ADAMTSL2	417147	-1.04	1.13E-04
ENSGALG00000007320	FXYD2	419770	-2.16	1.41E-04
ENSGALG00000010027	CFL2	423320	-1.24	1.42E-04
ENSGALG00000013948	RASL11B	422756	-1.36	1.82E-04
ENSGALG00000026661	MSRB3	417833	-1.16	2.33E-04
ENSGALG00000010614	SLC25A4	422546	-1.06	2.36E-04
ENSGALG00000038043	HECTD2	427031	-1.20	2.53E-04
ENSGALG00000010769	HPGD	422567	-1.43	2.92E-04
ENSGALG00000028949	CORO6	100857679	-1.28	3.30E-04
ENSGALG00000002358	CDO1	427391	-2.99	3.33E-04
ENSGALG00000015970	COL9A1	771873	-2.35	3.52E-04
ENSGALG00000010663	TTC8	423401	-1.11	3.80E-04
ENSGALG00000019157	SMPX	771780	-1.35	4.57E-04
ENSGALG00000041365	SALL4	769286	-1.24	4.93E-04
ENSGALG00000009670	SGCE	420567	-1.10	4.97E-04
ENSGALG00000012997	DNAH5	NA	-2.44	6.63E-04
ENSGALG00000027255	NRXN3	423385	-1.53	6.78E-04
ENSGALG00000006106	TNFRSF6B	395096	-1.74	7.52E-04
ENSGALG00000008659	HACD1	420518	-1.04	8.52E-04
ENSGALG00000007048	SYNM	395599	-1.18	1.32E-03
ENSGALG00000033033	MEP1A	422060	-2.79	1.35E-03
ENSGALG00000022819	PCP4	771220	-1.12	1.81E-03
ENSGALG00000039376	DPT	768904	-1.09	1.92E-03
ENSGALG00000015252	ST3GAL6	395138	-1.01	2.16E-03
ENSGALG00000010741	MAP1LC3C	421504	-1.59	2.28E-03
ENSGALG00000008874	SLC13A1	770198	-4.11	2.33E-03
ENSGALG00000011742	ART4	427879	-2.91	2.56E-03
ENSGALG00000034741	ETNPPL	428743	-1.08	2.60E-03
ENSGALG00000011469	IGFBP2	396315	-1.04	3.02E-03
ENSGALG00000010068	KCNK17	421423	-1.85	3.29E-03
ENSGALG00000015253	COL8A1	418378	-1.28	3.62E-03
ENSGALG00000041575	PPP1R14C	421630	-1.18	3.75E-03
ENSGALG00000035104	HAND1	395812	-1.14	3.75E-03
ENSGALG00000045602	REM1	NA	-1.14	4.22E-03
ENSGALG00000007681	HTR2B	395581	-1.06	4.34E-03
ENSGALG00000000884	CXXC5	416138	-1.11	4.84E-03
ENSGALG00000001773	RFX2	420092	-1.23	5.50E-03
ENSGALG00000001857	C1QTNF12	419422	-1.17	5.67E-03
ENSGALG00000013297	BBOX1	426932	-1.01	5.80E-03
ENSGALG00000021158	SLC7A9	415768	-2.66	5.97E-03
ENSGALG00000029921	CSRP2	396128	-1.00	8.49E-03
ENSGALG00000030776	METTL7A	426871	-1.13	8.49E-03
ENSGALG00000015935	SMYD1	373960	-1.16	8.49E-03
ENSGALG00000023359	BTBD17	427813	-1.65	8.94E-03
ENSGALG00000003172	NA	423667	-2.23	5.07E-10
ENSGALG00000016287	NA	395285	-1.87	8.94E-07
ENSGALG00000020788	NA	770766	-1.24	2.39E-06
ENSGALG00000034919	NA	416826	-1.17	1.10E-04
ENSGALG00000045429	NA	NA	-2.34	1.55E-04
ENSGALG00000006025	NA	374266	-3.58	2.33E-04
ENSGALG00000038440	NA	NA	-1.08	2.70E-04
ENSGALG00000044347	NA	107052479	-4.68	2.80E-04
ENSGALG00000038679	NA	NA	-2.14	3.62E-04
ENSGALG00000010469	NA	424618	-2.57	3.81E-04
ENSGALG00000034813	NA	NA	-1.37	6.63E-04
ENSGALG00000012952	NA	373905	-1.20	7.15E-04
ENSGALG00000031527	NA	769134	-1.33	7.47E-04
ENSGALG00000010901	NA	420606	-2.08	7.85E-04
ENSGALG00000046293	NA	NA	-1.82	1.10E-03
ENSGALG00000032195	NA	NA	-1.50	1.29E-03
ENSGALG00000033139	NA	NA	-1.51	1.90E-03
ENSGALG00000019284	NA	418170	-1.01	1.91E-03
ENSGALG00000034846	NA	NA	-1.09	2.16E-03
ENSGALG00000005934	NA	420423	-4.15	2.19E-03
ENSGALG00000028283	NA	NA	-1.95	2.67E-03
ENSGALG00000037014	NA	420304	-1.16	3.48E-03
ENSGALG00000028560	NA	417943	-1.24	3.52E-03
ENSGALG00000041312	NA	NA	-2.04	3.70E-03
ENSGALG00000041375	NA	NA	-1.57	4.51E-03
ENSGALG00000029851	NA	NA	-1.49	4.76E-03
ENSGALG00000044832	NA	NA	-1.32	4.80E-03
ENSGALG00000013571	NA	NA	-2.16	4.84E-03
ENSGALG00000011356	NA	NA	-1.90	5.01E-03
ENSGALG00000005795	NA	414746	-1.88	5.80E-03
ENSGALG00000006018	NA	420422	-2.35	5.97E-03
ENSGALG00000039873	NA	NA	-1.94	6.92E-03
ENSGALG00000015410	NA	408032	-1.11	7.12E-03
ENSGALG00000030591	NA	107056096	-1.55	7.49E-03
ENSGALG00000011717	NA	417937	-1.26	8.49E-03
ENSGALG00000034639	NA	NA	-2.16	9.18E-03

Appendix 3 Detailed information on the corresponding GO terms of the up-regulated DE genes found in used vs clean litter on H_2O. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Accession No.	Ontology	Definition	No. of DEGs	P-value
GO:0009607	BP	response to biotic stimulus	15	7.35E-07
GO:0044764	BP	multi-organism cellular process	13	1.06E-06
GO:0016032	BP	viral process	13	1.06E-06
GO:0016126	BP	sterol biosynthetic process	6	1.65E-06
GO:0044403	BP	symbiosis, encompassing mutualism through parasitism	13	2.04E-06
GO:0044419	BP	interspecies interaction between organisms	13	2.04E-06
GO:0009615	BP	response to virus	10	3.78E-06
GO:0043207	BP	response to external biotic stimulus	13	1.39E-05
GO:0051707	BP	response to other organism	13	1.39E-05
GO:0048525	BP	negative regulation of viral process	6	1.51E-05
GO:0050792	BP	regulation of viral process	9	1.62E-05
GO:0006952	BP	defense response	16	2.41E-05
GO:1902653	BP	secondary alcohol biosynthetic process	5	2.48E-05
GO:0043903	BP	regulation of symbiosis, encompassing mutualism through parasitism	9	2.78E-05
GO:0051607	BP	defense response to virus	8	4.54E-05
GO:0016125	BP	sterol metabolic process	6	7.28E-05
GO:0045069	BP	regulation of viral genome replication	5	1.01E-04
GO:1903901	BP	negative regulation of viral life cycle	5	1.01E-04
GO:0043901	BP	negative regulation of multi-organism process	6	1.40E-04
GO:0043900	BP	regulation of multi-organism process	9	1.44E-04
GO:0051704	BP	multi-organism process	17	1.48E-04
GO:1903900	BP	regulation of viral life cycle	6	1.75E-04
GO:0019079	BP	viral genome replication	5	1.83E-04
GO:0098542	BP	defense response to other organism	9	1.90E-04
GO:0006694	BP	steroid biosynthetic process	6	2.01E-04
GO:0045071	BP	negative regulation of viral genome replication	4	2.09E-04
GO:0009605	BP	response to external stimulus	19	3.19E-04
GO:1901617	BP	organic hydroxy compound biosynthetic process	6	5.11E-04
GO:0006695	BP	cholesterol biosynthetic process	4	5.90E-04
GO:0002376	BP	immune system process	19	6.78E-04
GO:0046165	BP	alcohol biosynthetic process	5	7.10E-04
GO:1902652	BP	secondary alcohol metabolic process	5	7.65E-04
GO:0006950	BP	response to stress	25	1.03E-03
GO:0005154	MF	epidermal growth factor receptor binding	3	1.28E-03
GO:0045087	BP	innate immune response	8	1.73E-03
GO:0019058	BP	viral life cycle	6	1.82E-03
GO:0008202	BP	steroid metabolic process	6	1.97E-03
GO:0031347	BP	regulation of defense response	8	4.82E-03
GO:0008203	BP	cholesterol metabolic process	4	6.96E-03
GO:0030261	BP	chromosome condensation	3	7.05E-03
GO:0002252	BP	immune effector process	8	7.15E-03
GO:0005789	CC	endoplasmic reticulum membrane	8	7.50E-03
GO:0042175	CC	nuclear outer membrane-endoplasmic reticulum membrane network	8	8.45E-03
GO:0005783	CC	endoplasmic reticulum	12	1.12E-02
GO:0050896	BP	response to stimulus	42	1.20E-02
GO:0004497	MF	monooxygenase activity	4	1.25E-02
GO:0006066	BP	alcohol metabolic process	5	1.30E-02
GO:0044283	BP	small molecule biosynthetic process	7	1.37E-02
GO:1901615	BP	organic hydroxy compound metabolic process	6	1.38E-02
GO:0044432	CC	endoplasmic reticulum part	8	1.57E-02
GO:0071615	BP	oxidative deethylation	2	1.59E-02
GO:1901362	BP	organic cyclic compound biosynthetic process	24	1.92E-02
GO:0008610	BP	lipid biosynthetic process	7	2.14E-02
GO:1901360	BP	organic cyclic compound metabolic process	32	2.17E-02
GO:0070851	MF	growth factor receptor binding	3	2.36E-02
GO:0043385	BP	mycotoxin metabolic process	2	2.38E-02
GO:0045944	BP	positive regulation of transcription from RNA polymerase II promoter	9	2.77E-02
GO:0012505	CC	endomembrane system	21	2.99E-02
GO:0019221	BP	cytokine-mediated signaling pathway	5	3.12E-02
GO:0009404	BP	toxin metabolic process	2	3.16E-02
GO:0030097	BP	hemopoiesis	8	3.42E-02
GO:0070330	MF	aromatase activity	2	3.92E-02
GO:1901576	BP	organic substance biosynthetic process	31	3.93E-02
GO:0045935	BP	positive regulation of nucleobase-containing compound metabolic process	12	3.95E-02
GO:0006955	BP	immune response	9	4.16E-02
GO:0017076	MF	purine nucleotide binding	16	4.39E-02
GO:0008083	MF	growth factor activity	3	4.58E-02
GO:0048534	BP	hematopoietic or lymphoid organ development	8	4.65E-02
GO:0035458	BP	cellular response to interferon-beta	2	4.71E-02
GO:0006268	BP	DNA unwinding involved in DNA replication	2	4.71E-02
GO:0003824	MF	catalytic activity	37	4.74E-02
GO:0034097	BP	response to cytokine	6	4.74E-02
GO:0005737	CC	cytoplasm	48	4.79E-02
GO:0016705	MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	4	4.99E-02

Appendix 4 Detailed information on the corresponding GO terms of the down-regulated DE genes found in used vs clean litter on H_2O. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Accession No.	Ontology	Definition	No. of DEGs	P-value
GO:0008092	MF	Cytoskeletal protein binding	6	1.80E-03
GO:0016491	MF	Oxidoreductase activity	8	5.80E-03
GO:0044449	CC	Contractile fiber part	4	1.14E-02
GO:0005506	MF	Iron ion binding	4	1.23E-02
GO:0030016	CC	Myofibril	4	1.56E-02
GO:0043292	CC	Contractile fiber	4	1.78E-02
GO:0072359	BP	Circulatory system development	7	2.21E-02
GO:0072358	BP	Cardiovascular system development	7	2.21E-02
GO:0044699	BP	Single-organism process	35	2.81E-02
GO:0003012	BP	Muscle system process	4	2.82E-02
GO:0072348	BP	Sulfur compound transport	2	2.92E-02
GO:0009888	BP	Tissue development	9	3.43E-02
GO:0032501	BP	Multicellular organismal process	20	4.07E-02
GO:0043034	CC	Costamere	2	4.39E-02
GO:0007179	BP	Transforming growth factor beta receptor signaling pathway	3	4.57E-02
GO:0055085	BP	Transmembrane transport	6	4.59E-02
GO:0044763	BP	Single-organism cellular process	32	4.81E-02

Appendix 5 DAVID generated top 5 annotation clusters for up regulated DE genes found in used vs clean litter on H_2O. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Annotation Cluster 1	Enrichment Score: 3.823090259113751	
Category	Term	No. of DEGs
BP	GO:0009607~response to biotic stimulus	15
BP	GO:0016032~viral process	13
BP	GO:0044764~multi-organism cellular process	13
BP	GO:0044419~interspecies interaction between organisms	13
BP	GO:0044403~symbiosis, encompassing mutualism through parasitism	13
BP	GO:0009615~response to virus	10
BP	GO:0051707~response to other organism	13
BP	GO:0043207~response to external biotic stimulus	13
BP	GO:0048525~negative regulation of viral process	6
BP	GO:0050792~regulation of viral process	9
BP	GO:0006952~defense response	16
BP	GO:0043903~regulation of symbiosis, encompassing mutualism through parasitism	9
BP	GO:0051607~defense response to virus	8
BP	GO:1903901~negative regulation of viral life cycle	5
BP	GO:0045069~regulation of viral genome replication	5
BP	GO:0043901~negative regulation of multi-organism process	6
BP	GO:0043900~regulation of multi-organism process	9
BP	GO:0051704~multi-organism process	17
BP	GO:1903900~regulation of viral life cycle	6
BP	GO:0019079~viral genome replication	5
BP	GO:0098542~defense response to other organism	9
BP	GO:0045071~negative regulation of viral genome replication	4
BP	GO:0009605~response to external stimulus	19
BP	GO:0002376~immune system process	19
BP	GO:0006950~response to stress	25
BP	GO:0045087~innate immune response	8
BP	GO:0019058~viral life cycle	6
BP	GO:0002252~immune effector process	8
BP	GO:0006955~immune response	9
MF	GO:0003676~nucleic acid binding	20
MF	GO:0044822~poly(A) RNA binding	8
MF	GO:0003723~RNA binding	10
Annotation Cluster 2	Enrichment Score: 2.178774368723128	
Category	Term	No. of DEGs
BP	GO:0016126~sterol biosynthetic process	6
BP	GO:1902653~secondary alcohol biosynthetic process	5
BP	GO:0016125~sterol metabolic process	6
BP	GO:0006694~steroid biosynthetic process	6
BP	GO:1901617~organic hydroxy compound biosynthetic process	6
BP	GO:0006695~cholesterol biosynthetic process	4
BP	GO:0046165~alcohol biosynthetic process	5
BP	GO:1902652~secondary alcohol metabolic process	5
BP	GO:0008202~steroid metabolic process	6
BP	GO:0008203~cholesterol metabolic process	4
CC	GO:0005789~endoplasmic reticulum membrane	8
CC	GO:0042175~nuclear outer membrane-endoplasmic reticulum membrane network	8
CC	GO:0005783~endoplasmic reticulum	12
BP	GO:0006066~alcohol metabolic process	5
BP	GO:0044283~small molecule biosynthetic process	7
BP	GO:1901615~organic hydroxy compound metabolic process	6
CC	GO:0044432~endoplasmic reticulum part	8
BP	GO:0008610~lipid biosynthetic process	7
BP	GO:0044711~single-organism biosynthetic process	10
MF	GO:0016491~oxidoreductase activity	8
BP	GO:0006629~lipid metabolic process	9
BP	GO:0044281~small molecule metabolic process	12
MF	GO:0016614~oxidoreductase activity, acting on CH-OH group of donors	3
BP	GO:0044710~single-organism metabolic process	20
CC	GO:0031090~organelle membrane	10
BP	GO:0044255~cellular lipid metabolic process	5
Annotation Cluster 3	Enrichment Score: 1.698863282992058	
Category	Term	No. of DEGs
MF	GO:0005154~epidermal growth factor receptor binding	3
MF	GO:0070851~growth factor receptor binding	3
MF	GO:0005102~receptor binding	5
Annotation Cluster 4	Enrichment Score: 1.2415987331449243	
Category	Term	No. of DEGs
MF	GO:0004497~monooxygenase activity	4
MF	GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	4
MF	GO:0005506~iron ion binding	4
MF	GO:0016491~oxidoreductase activity	8
MF	GO:0020037~heme binding	3
MF	GO:0046906~tetrapyrrole binding	3
BP	GO:0055114~oxidation-reduction process	4
Annotation Cluster 5	Enrichment Score: 1.23498671620941	
Category	Term	No. of DEGs
BP	GO:0048525~negative regulation of viral process	6
BP	GO:1903901~negative regulation of viral life cycle	5
BP	GO:0043901~negative regulation of multi-organism process	6
BP	GO:0009966~regulation of signal transduction	12
BP	GO:1902531~regulation of intracellular signal transduction	7
BP	GO:1902533~positive regulation of intracellular signal transduction	4
BP	GO:0048584~positive regulation of response to stimulus	7
BP	GO:0035556~intracellular signal transduction	10
BP	GO:0044093~positive regulation of molecular function	5
BP	GO:0009967~positive regulation of signal transduction	5
BP	GO:0010647~positive regulation of cell communication	5
BP	GO:0023056~positive regulation of signaling	5

Appendix 6 DAVID generated top 5 annotation clusters for down-regulated DE genes found in used vs clean litter on H_2O. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Annotation Cluster 1	Enrichment Score: 1.2361293239486049	
Category	Term	No. of DEGs
MF	GO:0008092~cytoskeletal protein binding	6
CC	GO:0005856~cytoskeleton	7
CC	GO:0015629~actin cytoskeleton	3
Annotation Cluster 2	Enrichment Score: 1.0834873665447624	
Category	Term	No. of DEGs
BP	GO:0003012~muscle system process	4
BP	GO:0006936~muscle contraction	3
BP	GO:0003008~system process	6
Annotation Cluster 3	Enrichment Score: 0.950133720845698	
Category	Term	No. of DEGs
MF	GO:0016491~oxidoreductase activity	8
MF	GO:0005506~iron ion binding	4
MF	GO:0046914~transition metal ion binding	8
MF	GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	3
MF	GO:0046872~metal ion binding	11
MF	GO:0043169~cation binding	11
MF	GO:0008270~zinc ion binding	5
MF	GO:0043167~ion binding	11
Annotation Cluster 4	Enrichment Score: 0.931630172868112	
Category	Term	No. of DEGs
CC	GO:0044463~cell projection part	5
CC	GO:0031253~cell projection membrane	3
CC	GO:0042995~cell projection	7
CC	GO:0044459~plasma membrane part	9
CC	GO:0098590~plasma membrane region	4
Annotation Cluster 5	Enrichment Score: 0.8821801423458001	
Category	Term	No. of DEGs
MF	GO:0008092~cytoskeletal protein binding	6
MF	GO:0003779~actin binding	3
BP	GO:0030036~actin cytoskeleton organization	4
BP	GO:0030029~actin filament-based process	4
BP	GO:1902589~single-organism organelle organization	6
BP	GO:0007010~cytoskeleton organization	4
BP	GO:0006996~organelle organization	8

Appendix 7 Detailed information on the up-regulated DE genes found in 1 ppm iodinated water on clean litter.

Ensembl gene ID	Gene symbol	Entrez ID	Log_2FC	FDR
ENSGALG00000014537	BMF	769140	1.75	8.13E-08
ENSGALG00000014321	SMIM14	422793	1.53	8.13E-08
ENSGALG00000033954	TMEM248	417549	1.45	8.13E-08
ENSGALG00000003144	TRIM25	417401	1.60	8.13E-08
ENSGALG00000030157	MCTP2	426324	1.74	7.93E-07
ENSGALG00000010579	STON2	423390	1.68	9.61E-07
ENSGALG00000008933	CASP7	423901	1.52	1.61E-06
ENSGALG00000008744	MCF2L2	424959	1.13	1.61E-06
ENSGALG00000010202	ENTPD5	423343	1.40	2.08E-06
ENSGALG00000003336	DAPK2	100857587	2.12	7.53E-06
ENSGALG00000009413	BROX	NA	1.48	1.05E-05
ENSGALG00000007808	HID1	422113	1.54	1.05E-05
ENSGALG00000010347	CCNG2	422512	1.05	1.06E-05
ENSGALG00000013468	TLR3	NA	1.41	1.54E-05
ENSGALG00000037375	MAN1B1	417296	1.19	1.74E-05
ENSGALG00000016707	CLIC5	422054	1.94	1.78E-05
ENSGALG00000008632	ACSS1	416714	1.44	1.99E-05
ENSGALG00000045936	FAM83F	770747	1.73	1.99E-05
ENSGALG00000046187	PARP12	418100	1.60	1.99E-05
ENSGALG00000040000	ERO1B	421509	1.31	2.55E-05
ENSGALG00000004710	TMOD3	415421	1.11	3.74E-05
ENSGALG00000019228	SFT2D2	418373	1.77	3.76E-05
ENSGALG00000007077	CPT1A	423118	1.34	5.39E-05
ENSGALG00000027397	DUSP22	NA	1.66	5.60E-05
ENSGALG00000009674	CLCN3	422432	1.81	5.80E-05
ENSGALG00000015828	BACH1	418488	1.18	5.94E-05
ENSGALG00000002242	GALNT9	416796	1.70	6.17E-05
ENSGALG00000000901	CHST4	427567	1.44	8.37E-05
ENSGALG00000034316	CNOT6	416293	1.18	8.37E-05
ENSGALG00000011934	DESI1	770448	1.05	8.37E-05
ENSGALG00000011664	RASSF6	422653	1.30	8.37E-05
ENSGALG00000005966	SEC61A1	416023	1.31	8.37E-05
ENSGALG00000010581	SEL1L	423391	1.23	8.37E-05
ENSGALG00000016205	PDXK	418549	1.30	8.40E-05
ENSGALG00000028994	SAP30L	416259	1.22	8.83E-05
ENSGALG00000006689	ABHD2	415493	2.20	1.01E-04
ENSGALG00000011635	TFCP2L1	424238	1.23	1.03E-04
ENSGALG00000005245	SGPP2	424813	1.14	1.20E-04
ENSGALG00000008663	DDAH1	378898	1.51	1.32E-04
ENSGALG00000017196	ARHGAP42	418990	1.17	1.32E-04
ENSGALG00000031843	ARHGAP18	421706	1.02	1.38E-04
ENSGALG00000041470	PPARA	374120	1.22	1.48E-04
ENSGALG00000012978	CDYL2	425886	1.71	1.49E-04
ENSGALG00000039139	TNS3	420792	1.10	1.49E-04
ENSGALG00000010320	PAWR	417870	1.09	1.52E-04
ENSGALG00000014337	NWD2	428789	1.73	1.52E-04
ENSGALG00000010593	GALC	423394	1.84	1.54E-04
ENSGALG00000007994	PARD6B	419352	1.57	1.58E-04
ENSGALG00000004228	USP40	100859211	1.70	1.58E-04
ENSGALG00000031637	LICOS	395968	1.17	1.68E-04
ENSGALG00000045642	TNFRSF11A	769909	1.32	1.68E-04
ENSGALG00000006785	IRF1	396384	1.03	1.71E-04
ENSGALG00000031380	DENND3	420309	1.08	1.72E-04
ENSGALG00000041362	SECISBP2L	415435	1.25	2.01E-04
ENSGALG00000014979	FRK	421747	1.07	2.05E-04
ENSGALG00000034077	KCNK5	NA	1.93	2.16E-04
ENSGALG00000014759	STYK1	NA	1.22	2.18E-04
ENSGALG00000001777	MFSD11	417352	1.12	2.23E-04
ENSGALG00000005408	BCO1	395346	1.11	2.27E-04
ENSGALG00000013911	ZC3HAV1	426315	1.11	2.33E-04
ENSGALG00000034971	SLC45A4	420308	1.41	2.49E-04
ENSGALG00000006425	SLC6A6	416041	1.77	2.50E-04
ENSGALG00000007075	ATP13A3	424898	1.65	2.57E-04
ENSGALG00000001603	NARF	417341	1.26	2.57E-04
ENSGALG00000007052	PXK	416065	1.01	2.57E-04
ENSGALG00000034948	UAP1L1	417295	1.04	2.57E-04
ENSGALG00000005259	VIPR1	395329	1.24	2.57E-04
ENSGALG00000005251	CMTM4	415794	1.71	2.62E-04
ENSGALG00000001662	ATP10B	416159	1.32	2.68E-04
ENSGALG00000004435	RAB27A	415410	1.14	2.69E-04
ENSGALG00000001030	SCAMP4	420081	1.14	2.78E-04
ENSGALG00000004814	RHPN2	415771	1.13	3.04E-04
ENSGALG00000017106	ATP8A2	418936	1.10	3.11E-04
ENSGALG00000012874	KIAA1147	427917	1.15	3.11E-04
ENSGALG00000014551	TTC38	426193	1.50	3.13E-04
ENSGALG00000003743	VPS37B	416835	1.22	3.13E-04
ENSGALG00000029679	PLEKHA7	423069	1.33	3.27E-04
ENSGALG00000008956	CCDC186	423905	1.28	3.30E-04
ENSGALG00000000893	TAT	415884	1.85	3.36E-04
ENSGALG00000002710	IPMK	101751177	1.31	3.40E-04
ENSGALG00000030802	SHROOM2	418651	1.28	3.42E-04
ENSGALG00000045511	PARP9	424269	1.17	3.51E-04
ENSGALG00000004162	SLC16A6	417435	1.59	3.69E-04
ENSGALG00000008702	OVOL2	416720	1.22	3.82E-04
ENSGALG00000013197	GALNT12	420978	1.26	3.83E-04
ENSGALG00000010988	MPP6	420622	1.33	3.88E-04
ENSGALG00000031130	GGH	421144	1.25	3.93E-04
ENSGALG00000017125	MICU2	418946	1.08	4.06E-04
ENSGALG00000004033	GALNT10	416260	1.40	4.13E-04
ENSGALG00000015854	PGM3	421841	1.20	4.35E-04
ENSGALG00000011227	CDS1	422611	1.23	4.45E-04
ENSGALG00000041644	ATP11A	418749	1.41	4.49E-04
ENSGALG00000009807	FLVCR1	421365	1.36	4.57E-04
ENSGALG00000016375	PHEX	395777	1.60	4.69E-04
ENSGALG00000009170	NCEH1	NA	1.22	4.74E-04
ENSGALG00000010840	SNX13	420598	1.08	4.80E-04
ENSGALG00000017037	NHLRC3	NA	1.19	4.97E-04
ENSGALG00000028807	AP1AR	422693	1.11	5.08E-04
ENSGALG00000012513	STAM2	424317	1.04	5.17E-04
ENSGALG00000015669	PTBP3	427333	1.15	5.54E-04
ENSGALG00000007132	ACOX2	416068	1.04	6.27E-04
ENSGALG00000034548	PLEKHA1	423940	1.05	6.27E-04
ENSGALG00000035478	FAM91A1	770621	1.04	6.29E-04
ENSGALG00000036920	SLC22A5	416328	1.50	6.37E-04
ENSGALG00000027908	CYP2U1	422528	1.30	6.38E-04

ENSGALG00000006623	ATP11C	422254	1.19	6.57E-04
ENSGALG00000038678	EPB41L3	421055	1.13	6.63E-04
ENSGALG00000000438	ERMP1	426644	1.44	6.71E-04
ENSGALG00000041884	MFSD6	423978	1.07	6.87E-04
ENSGALG00000014509	BST1	422828	1.30	6.89E-04
ENSGALG00000007234	CLCN5	422285	1.04	6.94E-04
ENSGALG00000004974	PPARG	373928	1.17	6.94E-04
ENSGALG00000015142	DSG2	428529	1.60	7.03E-04
ENSGALG00000004397	MLXIP	416857	1.14	7.03E-04
ENSGALG00000028250	ANKRD22	771419	1.01	7.07E-04
ENSGALG00000036028	GNA13	417434	1.28	7.25E-04
ENSGALG00000001895	PANK3	416170	1.47	7.25E-04
ENSGALG00000005519	TLL2	107053676	1.37	7.25E-04
ENSGALG00000008347	ATG13	423192	1.19	7.46E-04
ENSGALG00000043573	CHMP4C	420201	1.02	7.46E-04
ENSGALG00000011185	PLEKHA8	420637	1.33	7.71E-04
ENSGALG00000009207	TRIM2	100857562	1.43	7.94E-04
ENSGALG00000011141	ITGB6	424191	1.58	7.96E-04
ENSGALG00000015051	CDK19	421756	1.21	8.16E-04
ENSGALG00000015141	GDA	427253	1.48	8.57E-04
ENSGALG00000012119	MARCO	395488	1.17	8.57E-04
ENSGALG00000005004	SAMD8	428946	1.18	8.57E-04
ENSGALG00000012988	B4GALNT3	418150	1.65	8.61E-04
ENSGALG00000010540	TTC39A	424632	1.39	8.61E-04
ENSGALG00000037410	HEPH	422168	1.40	8.68E-04
ENSGALG00000034007	ITGA6	396226	1.24	9.23E-04
ENSGALG00000011531	APAF1	417926	1.22	9.94E-04
ENSGALG00000003765	A1CF	423680	1.49	1.00E-03
ENSGALG00000013205	ETNK1	418196	1.29	1.05E-03
ENSGALG00000011296	FRMD1	421562	1.25	1.07E-03
ENSGALG00000016964	EPSTI1	418837	1.09	1.07E-03
ENSGALG00000009320	PSEN1	373977	1.29	1.08E-03
ENSGALG00000011447	GADL1	100857134	1.93	1.16E-03
ENSGALG00000010875	ITGB8	395470	1.06	1.16E-03
ENSGALG00000003479	LMTK2	769200	1.50	1.16E-03
ENSGALG00000001564	ATP2A3	395707	1.50	1.16E-03
ENSGALG00000010885	BMP2K	422578	1.00	1.22E-03
ENSGALG00000012913	PKP2	418130	1.15	1.24E-03
ENSGALG00000010889	HOOK1	424675	1.17	1.24E-03
ENSGALG00000014201	PARVB	418235	1.18	1.26E-03
ENSGALG00000016055	KCNJ15	427984	1.10	1.31E-03
ENSGALG00000040478	CAPN5	419086	1.36	1.36E-03
ENSGALG00000011213	DEGS2	423454	1.25	1.38E-03
ENSGALG00000008081	PIK3CG	417706	1.13	1.38E-03
ENSGALG00000043724	NFX1	420796	1.05	1.42E-03
ENSGALG00000005021	RAB43	415970	1.02	1.45E-03
ENSGALG00000016361	FAM110C	100859913	1.46	1.45E-03
ENSGALG00000002141	UGGT1	424757	1.03	1.46E-03
ENSGALG00000042621	HNF4G	428364	1.54	1.48E-03
ENSGALG00000033720	CA7	415791	1.46	1.51E-03
ENSGALG00000015546	TBC1D14	422862	1.05	1.54E-03
ENSGALG00000008469	SPINT1	423206	1.39	1.59E-03
ENSGALG00000016144	TMPRSS2	418528	1.17	1.59E-03
ENSGALG00000016675	PAQR8	422042	1.08	1.68E-03
ENSGALG00000014094	STK38L	418217	1.54	1.78E-03
ENSGALG00000007620	GFRA3	416353	1.91	1.81E-03
ENSGALG00000043025	EPS8L2	770081	1.43	1.82E-03
ENSGALG00000009859	TBC1D30	417830	1.30	1.82E-03
ENSGALG00000002172	FAM168B	424759	1.07	1.83E-03
ENSGALG00000033338	GPT2	415746	1.12	1.95E-03
ENSGALG00000016870	TMTC4	418772	1.32	1.97E-03
ENSGALG00000042766	TRAPPC9	420312	1.30	2.09E-03
ENSGALG00000013377	LMBRD2	429640	1.32	2.13E-03
ENSGALG00000007550	TPCN2	423141	1.17	2.21E-03
ENSGALG00000005074	FAM102A	417228	1.05	2.24E-03
ENSGALG00000028685	SEMA5A	420931	1.26	2.30E-03
ENSGALG00000039673	GALNS	NA	1.13	2.37E-03
ENSGALG00000023670	NAALADL2	429156	1.66	2.44E-03
ENSGALG00000040447	CDCP1	420703	1.19	2.46E-03
ENSGALG00000007651	STAT1	424044	1.11	2.56E-03
ENSGALG00000001936	MYO7B	NA	1.70	2.59E-03
ENSGALG00000032868	AHCYL2	NA	1.15	2.61E-03
ENSGALG00000012601	GOLM1	427462	1.77	2.64E-03
ENSGALG00000010707	GPR68	428904	1.30	2.71E-03
ENSGALG00000012185	PLA2G12A	NA	1.03	2.72E-03
ENSGALG00000011005	KCNK1	421519	1.23	2.74E-03
ENSGALG00000002655	ITGAV	396420	1.02	2.79E-03
ENSGALG00000008701	XDH	396025	1.62	2.85E-03
ENSGALG00000011490	CMTM6	NA	1.22	2.92E-03
ENSGALG00000004332	PLA2G12B	423705	1.45	2.93E-03
ENSGALG00000006076	RASGEF1C	416296	1.17	2.94E-03
ENSGALG00000013043	FAM173B	428500	1.31	2.95E-03
ENSGALG00000030276	SHTN1	423919	1.22	3.05E-03
ENSGALG00000028047	RHOV	428868	1.33	3.10E-03
ENSGALG00000001239	SLC26A2	427611	1.43	3.10E-03
ENSGALG00000001546	ZZEF1	NA	1.34	3.14E-03
ENSGALG00000012791	TBXAS1	418101	1.17	3.19E-03
ENSGALG00000040424	SCNN1A	396050	1.26	3.21E-03
ENSGALG00000026816	PITPNC1	NA	1.21	3.30E-03
ENSGALG00000014267	RBM47	770355	1.39	3.31E-03
ENSGALG00000015016	SLC22A15	418336	1.37	3.36E-03
ENSGALG00000009805	PPM1H	771760	1.25	3.38E-03
ENSGALG00000010868	MACC1	420604	1.26	3.49E-03
ENSGALG00000006627	UPB1	416949	1.46	3.50E-03
ENSGALG00000042357	ARHGAP1	428865	1.08	3.55E-03
ENSGALG00000005103	GDPD1	417631	1.12	3.59E-03
ENSGALG00000010793	SLC24A4	772279	1.36	3.60E-03
ENSGALG00000014730	ELOVL7	431579	1.10	3.66E-03
ENSGALG00000009880	INPP4B	422454	1.05	3.69E-03
ENSGALG00000003849	TRPM1	427494	1.70	3.69E-03
ENSGALG00000005360	CA4	417647	1.15	3.73E-03
ENSGALG00000013026	HDHD5	418159	1.09	3.83E-03
ENSGALG00000004285	HNF4A	419198	1.13	3.92E-03
ENSGALG00000015433	ABCA1	373945	1.08	3.92E-03
ENSGALG00000007817	EHF	425791	1.12	3.92E-03
ENSGALG00000008603	ITPKA	395694	1.03	4.02E-03
ENSGALG00000016406	RPS6KA3	418605	1.01	4.07E-03
ENSGALG00000001042	MTMR4	417472	1.15	4.10E-03
ENSGALG00000020002	SLC22A3	421582	1.16	4.10E-03
ENSGALG00000010583	VIT	421471	1.15	4.10E-03
ENSGALG00000033630	LRP12	420267	1.19	4.12E-03
ENSGALG00000019231	CD200R1A	768673	1.04	4.22E-03
ENSGALG00000006382	PTPRJ	395330	1.35	4.30E-03
ENSGALG00000035345	TXNRD1	418082	1.12	4.44E-03
ENSGALG00000007701	B3GNT7	424930	1.79	4.51E-03
ENSGALG00000000255	MAN2A1	415611	1.11	5.03E-03

ENSGALG00000016755	KIAA1211L	100858050	1.00	5.30E-03
ENSGALG00000004246	SLC6A4	404747	1.17	5.30E-03
ENSGALG00000026482	uc_338	NA	1.28	5.30E-03
ENSGALG00000016047	HLCS	418516	1.02	5.45E-03
ENSGALG00000015352	GAK	427291	1.39	5.55E-03
ENSGALG00000015877	SH3BGRL2	NA	1.27	5.73E-03
ENSGALG00000034500	CIDEA	768659	1.09	6.23E-03
ENSGALG00000030550	ATP2B1	374244	1.05	6.40E-03
ENSGALG00000004255	SNX8	416468	1.06	6.40E-03
ENSGALG00000042851	PPARGC1A	422815	1.25	6.58E-03
ENSGALG00000010036	FOSL2	421416	1.32	6.86E-03
ENSGALG00000042679	RETREG1	100859798	1.23	6.96E-03
ENSGALG00000021378	UBAP1	431652	1.13	7.04E-03
ENSGALG00000004663	ARFGEF2	419211	1.02	7.17E-03
ENSGALG00000008076	TMEM164	422344	1.07	7.18E-03
ENSGALG00000041787	PLA2G15	NA	1.04	7.22E-03
ENSGALG00000041275	SLC25A38	420717	1.01	7.35E-03
ENSGALG00000016128	B3GALT5	427985	1.80	7.38E-03
ENSGALG00000017227	SLC36A4	419007	1.10	7.39E-03
ENSGALG00000005763	VILL	420415	1.69	7.80E-03
ENSGALG00000015492	PDZK1	418460	1.39	8.01E-03
ENSGALG00000015439	SLC44A1	427301	1.32	8.02E-03
ENSGALG00000009948	HHIP	422460	1.07	8.04E-03
ENSGALG00000000820	HTR1D	769107	3.20	8.07E-03
ENSGALG00000040663	RSC1A1	425541	1.02	8.19E-03
ENSGALG00000005303	CCDC13	420392	1.19	8.51E-03
ENSGALG00000041233	FAM84B	NA	1.55	8.67E-03
ENSGALG00000016412	MBOAT2	421925	1.21	9.02E-03
ENSGALG00000000783	PTAFR	428209	1.30	9.09E-03
ENSGALG00000032363	GPR141	420733	1.57	9.31E-03
ENSGALG00000006672	LARP4B	420457	1.11	9.31E-03
ENSGALG00000026287	IRS2	NA	1.29	9.35E-03
ENSGALG00000013723	OASL	395908	1.86	9.42E-03
ENSGALG00000004859	ZNFX1	419218	1.31	9.42E-03
ENSGALG00000016132	NA	NA	1.84	6.37E-06
ENSGALG00000028982	NA	421921	2.34	1.48E-05
ENSGALG00000045085	NA	423790	2.47	1.54E-05
ENSGALG00000016285	NA	418589	1.74	2.42E-05
ENSGALG00000030719	NA	NA	1.86	2.42E-05
ENSGALG00000011544	NA	419096	1.28	3.56E-05
ENSGALG00000043150	NA	NA	1.44	5.82E-05
ENSGALG00000042042	NA	NA	2.74	6.93E-05
ENSGALG00000009479	NA	420559	2.72	7.02E-05
ENSGALG00000039037	NA	NA	1.07	8.37E-05
ENSGALG00000044330	NA	NA	1.27	8.59E-05
ENSGALG00000001762	NA	NA	2.07	8.59E-05
ENSGALG00000006613	NA	396090	1.30	1.03E-04
ENSGALG00000023934	NA	NA	2.70	1.03E-04
ENSGALG00000003022	NA	100857724	1.37	1.21E-04
ENSGALG00000013033	NA	NA	1.51	1.21E-04
ENSGALG00000005350	NA	417646	1.19	1.42E-04
ENSGALG00000004769	NA	NA	1.35	1.44E-04
ENSGALG00000005393	NA	420398	1.09	1.45E-04
ENSGALG00000030350	NA	NA	1.18	1.49E-04
ENSGALG00000013091	NA	NA	1.76	1.49E-04
ENSGALG00000012187	NA	424286	1.57	1.52E-04
ENSGALG00000011443	NA	423476	1.65	1.68E-04
ENSGALG00000030316	NA	NA	1.78	1.68E-04
ENSGALG00000003129	NA	NA	1.50	2.03E-04
ENSGALG00000013057	NA	418167	2.10	2.27E-04
ENSGALG00000022720	NA	428083	1.59	2.28E-04
ENSGALG00000019795	NA	NA	1.03	2.90E-04
ENSGALG00000009639	NA	NA	2.92	3.88E-04
ENSGALG00000009728	NA	428427	1.21	3.95E-04
ENSGALG00000016142	NA	395313	2.19	4.74E-04
ENSGALG00000011285	NA	417897	1.04	5.90E-04
ENSGALG00000045127	NA	NA	1.26	7.94E-04
ENSGALG00000045319	NA	NA	3.18	8.35E-04
ENSGALG00000019553	NA	395715	3.57	8.35E-04
ENSGALG00000012072	NA	101747378	1.32	8.37E-04
ENSGALG00000013575	NA	403120	2.12	8.39E-04
ENSGALG00000041564	NA	NA	1.12	8.51E-04
ENSGALG00000044780	NA	NA	1.47	8.57E-04
ENSGALG00000022909	NA	420963	1.28	8.62E-04
ENSGALG00000024272	NA	426356	3.27	1.03E-03
ENSGALG00000014505	NA	422826	1.39	1.08E-03
ENSGALG00000002021	NA	NA	1.02	1.09E-03
ENSGALG00000016196	NA	418545	2.54	1.12E-03
ENSGALG00000000311	NA	419527	1.79	1.16E-03
ENSGALG00000014071	NA	418212	1.24	1.26E-03
ENSGALG00000036172	NA	107056878	1.18	1.31E-03
ENSGALG00000035561	NA	NA	1.02	1.38E-03
ENSGALG00000011455	NA	422633	3.48	1.39E-03
ENSGALG00000016761	NA	395708	2.75	1.43E-03
ENSGALG00000036828	NA	NA	3.06	1.49E-03
ENSGALG00000036437	NA	NA	1.46	1.55E-03
ENSGALG00000038574	NA	NA	1.48	1.56E-03
ENSGALG00000019570	NA	770433	1.00	1.61E-03
ENSGALG00000002783	NA	NA	1.22	1.76E-03
ENSGALG00000039168	NA	NA	1.00	1.76E-03
ENSGALG00000045477	NA	NA	1.23	1.93E-03
ENSGALG00000010315	NA	421449	1.00	1.94E-03
ENSGALG00000011805	NA	422660	1.37	2.03E-03
ENSGALG00000002492	NA	NA	1.72	2.20E-03
ENSGALG00000044716	NA	NA	1.41	2.21E-03
ENSGALG00000011320	NA	417905	1.15	2.28E-03
ENSGALG00000010072	NA	NA	1.33	2.53E-03
ENSGALG00000041009	NA	NA	1.59	2.71E-03
ENSGALG00000006565	NA	416945	1.52	2.99E-03
ENSGALG00000034822	NA	770055	1.43	3.38E-03
ENSGALG00000038402	NA	NA	1.31	3.44E-03
ENSGALG00000015388	NA	NA	1.15	3.45E-03
ENSGALG00000038140	NA	NA	2.05	3.45E-03
ENSGALG00000008056	NA	107054826	1.67	3.66E-03
ENSGALG00000015138	NA	427250	1.67	3.73E-03
ENSGALG00000027704	NA	NA	1.33	3.79E-03
ENSGALG00000040800	NA	107055804	1.62	3.79E-03
ENSGALG00000036951	NA	NA	1.42	3.88E-03
ENSGALG00000028304	NA	771888	1.27	4.09E-03
ENSGALG00000008491	NA	395280	1.14	4.10E-03
ENSGALG00000037629	NA	NA	1.22	4.10E-03
ENSGALG00000011796	NA	NA	1.54	4.21E-03
ENSGALG00000028551	NA	100859645	1.31	4.29E-03
ENSGALG00000045106	NA	NA	1.08	4.44E-03
ENSGALG00000015263	NA	418380	1.17	4.44E-03
ENSGALG00000028570	NA	NA	1.13	4.51E-03

ENSGALG00000016328	NA	414895	1.10	4.53E-03
ENSGALG00000035108	NA	NA	1.22	4.92E-03
ENSGALG00000042471	NA	395294	1.71	4.93E-03
ENSGALG00000015345	NA	NA	1.84	5.20E-03
ENSGALG00000024469	NA	423205	1.24	5.50E-03
ENSGALG00000025738	NA	NA	1.13	5.74E-03
ENSGALG00000038136	NA	415616	1.05	5.87E-03
ENSGALG00000045484	NA	NA	1.24	5.87E-03
ENSGALG00000034674	NA	418940	1.06	6.32E-03
ENSGALG00000034100	NA	415569	1.57	6.58E-03
ENSGALG00000011733	NA	420696	1.24	6.64E-03
ENSGALG00000040099	NA	NA	1.35	6.94E-03
ENSGALG00000041621	NA	395550	1.40	6.97E-03
ENSGALG00000043601	NA	NA	1.37	7.00E-03
ENSGALG00000043529	NA	NA	1.86	7.62E-03
ENSGALG00000023761	NA	101749599	1.18	8.33E-03
ENSGALG00000010032	NA	NA	1.89	8.60E-03
ENSGALG00000033644	NA	NA	1.02	8.75E-03
ENSGALG00000010934	NA	NA	1.41	8.79E-03
ENSGALG00000045901	NA	101749800	1.93	9.15E-03
ENSGALG00000039585	NA	NA	2.33	9.23E-03
ENSGALG00000046382	NA	NA	1.84	9.42E-03
ENSGALG00000042168	NA	100859636	1.56	9.43E-03
ENSGALG00000044074	NA	NA	2.28	9.77E-03

Appendix 8 Detailed information on the down regulated DE genes found in 1 ppm iodinated water on clean litter.

Ensembl gene ID	Gene symbol	Entrez ID	Log_2FC	FDR
ENSGALG00000011468	IGFBP5	424220	-1.15	1.53E-06
ENSGALG00000005554	ADIPOQ	404536	-1.38	1.81E-05
ENSGALG00000002955	ADAMTSL2	417147	-1.10	5.28E-05
ENSGALG00000027255	NRXN3	423385	-1.75	8.59E-05
ENSGALG00000034453	SAMD11	419434	-1.14	9.09E-05
ENSGALG00000010924	cNFI-A	396210	-1.12	1.11E-04
ENSGALG00000003529	SYNC	NA	-1.27	1.38E-04
ENSGALG00000010027	CFL2	423320	-1.22	2.00E-04
ENSGALG00000039634	KRT80	431302	-1.45	2.16E-04
ENSGALG00000040165	5_8S_rRNA	NA	-4.06	2.57E-04
ENSGALG00000009690	CENPF	395357	-1.30	2.57E-04
ENSGALG00000028949	CORO6	100857679	-1.29	2.62E-04
ENSGALG00000045602	REM1	NA	-1.37	3.04E-04
ENSGALG00000020151	NMU	422748	-1.52	3.27E-04
ENSGALG00000005483	CDH13	414849	-1.06	4.35E-04
ENSGALG00000037257	HLX	107052956	-1.02	4.45E-04
ENSGALG00000011469	IGFBP2	396315	-1.13	6.71E-04
ENSGALG00000033471	CALD1	373965	-1.27	7.03E-04
ENSGALG00000033304	MYBL1	396158	-1.08	7.78E-04
ENSGALG00000042458	ACTN1	373918	-1.10	8.35E-04
ENSGALG00000039118	Meis2a.1	395588	-1.02	8.57E-04
ENSGALG00000040648	RGS11	395621	-1.34	8.57E-04
ENSGALG00000038789	CLIC3	417293	-1.06	8.61E-04
ENSGALG00000021442	CARMIL2	415666	-1.03	1.05E-03
ENSGALG00000009670	SGCE	420567	-1.02	1.07E-03
ENSGALG00000030027	MCAM	448832	-1.31	1.24E-03
ENSGALG00000002960	HMGCS2	424380	-2.61	1.24E-03
ENSGALG00000001926	HSPB1	396227	-1.21	1.26E-03
ENSGALG00000009844	ACTC1	423298	-1.41	1.35E-03
ENSGALG00000016431	Pax5B	387330	-2.54	1.38E-03
ENSGALG00000002678	CSPG4	425524	-1.43	1.41E-03
ENSGALG00000015895	HTR1B	421858	-1.49	1.54E-03
ENSGALG00000029439	HOXC9	425723	-1.93	1.84E-03
ENSGALG00000014988	POPDC2	404229	-1.06	1.97E-03
ENSGALG00000007623	CACNA1G	769385	-1.46	2.05E-03
ENSGALG00000045312	SEMA6C	100859892	-1.25	2.23E-03
ENSGALG00000007048	SYNM	395599	-1.09	2.26E-03
ENSGALG00000013297	BBOX1	426932	-1.05	2.31E-03
ENSGALG00000041266	CNN1	396522	-1.35	2.32E-03
ENSGALG00000019157	SMPX	771780	-1.16	2.37E-03
ENSGALG00000004820	FAM57A	427836	-1.13	2.56E-03
ENSGALG00000016473	OSR1	100316920	-1.13	2.96E-03
ENSGALG00000032984	MEX3B	107054196	-1.13	3.26E-03
ENSGALG00000000558	SLC1A6	420057	-1.43	3.83E-03
ENSGALG00000035419	CDON	419710	-1.10	4.29E-03
ENSGALG00000027415	GRIN2C	431090	-1.31	5.02E-03
ENSGALG00000011708	MYLK	396445	-1.08	5.20E-03
ENSGALG00000017308	CHRDL2	NA	-1.47	5.40E-03
ENSGALG00000011902	TAGLN	396490	-1.15	5.87E-03
ENSGALG00000026146	CRLF1	NA	-1.35	5.90E-03
ENSGALG00000026983	CHRD	395828	-1.00	6.18E-03
ENSGALG00000041634	ACTG2	396084	-1.24	6.74E-03
ENSGALG00000043915	PYY	107049570	-2.24	6.96E-03
ENSGALG00000029917	SGCA	100859263	-1.79	7.44E-03
ENSGALG00000021592	TLCD2	427825	-1.16	7.98E-03
ENSGALG00000008367	MDK	423196	-1.08	8.21E-03
ENSGALG00000023772	HSPB7	430280	-1.11	8.59E-03
ENSGALG00000028376	FGF19	395394	-1.54	8.75E-03
ENSGALG00000032903	RTN4RL2	NA	-1.04	8.75E-03
ENSGALG00000036175	CAPG	NA	-1.01	9.03E-03
ENSGALG00000040045	DBH	395549	-1.71	9.42E-03
ENSGALG00000005797	COL20A1	419243	-1.09	9.43E-03
ENSGALG00000032344	NA	100858068	-1.76	3.69E-06
ENSGALG00000040226	NA	NA	-1.65	2.72E-05
ENSGALG00000043377	NA	NA	-1.83	3.69E-05
ENSGALG00000026605	NA	421880	-1.13	5.14E-05
ENSGALG00000032369	NA	NA	-1.19	5.26E-05
ENSGALG00000032220	NA	NA	-1.21	1.01E-04
ENSGALG00000034919	NA	416826	-1.18	1.20E-04
ENSGALG00000005843	NA	419244	-1.21	2.28E-04
ENSGALG00000044619	NA	NA	-1.37	3.11E-04
ENSGALG00000034913	NA	NA	-1.11	4.35E-04
ENSGALG00000036073	NA	NA	-1.58	5.67E-04
ENSGALG00000007178	NA	423122	-1.12	6.37E-04
ENSGALG00000038293	NA	NA	-1.25	6.94E-04
ENSGALG00000031638	NA	NA	-1.12	7.94E-04
ENSGALG00000000791	NA	419841	-1.14	8.44E-04
ENSGALG00000039864	NA	NA	-1.41	1.16E-03
ENSGALG00000042030	NA	419057	-1.12	1.26E-03
ENSGALG00000003921	NA	419180	-1.05	1.42E-03
ENSGALG00000025937	NA	770406	-1.13	1.57E-03
ENSGALG00000031398	NA	NA	-1.48	1.80E-03
ENSGALG00000014999	NA	NA	-1.54	1.92E-03
ENSGALG00000046483	NA	NA	-1.50	1.95E-03
ENSGALG00000045548	NA	NA	-1.57	2.21E-03
ENSGALG00000011687	NA	NA	-1.61	2.26E-03
ENSGALG00000034387	NA	NA	-1.15	2.32E-03
ENSGALG00000035757	NA	NA	-1.07	2.42E-03
ENSGALG00000031341	NA	425545	-1.23	2.71E-03
ENSGALG00000040901	NA	100859134	-4.75	2.75E-03
ENSGALG00000007507	NA	420487	-1.12	2.82E-03
ENSGALG00000038264	NA	107049501	-1.17	3.45E-03
ENSGALG00000042686	NA	NA	-1.05	3.46E-03
ENSGALG00000036752	NA	NA	-1.20	4.15E-03
ENSGALG00000038851	NA	NA	-1.10	4.15E-03
ENSGALG00000043381	NA	420716	-1.55	4.30E-03
ENSGALG00000045289	NA	NA	-1.07	4.44E-03
ENSGALG00000036270	NA	NA	-1.09	4.62E-03
ENSGALG00000035616	NA	NA	-1.52	4.96E-03
ENSGALG00000034893	NA	NA	-1.24	5.20E-03
ENSGALG00000036498	NA	107049991	-1.08	5.52E-03
ENSGALG00000034956	NA	NA	-1.47	5.78E-03
ENSGALG00000027490	NA	100858412	-1.53	5.84E-03
ENSGALG00000032326	NA	NA	-1.53	7.78E-03
ENSGALG00000041375	NA	NA	-1.40	7.94E-03
ENSGALG00000029697	NA	NA	-1.41	8.19E-03
ENSGALG00000039156	NA	101747651	-1.00	8.29E-03
ENSGALG00000032183	NA	NA	-1.01	8.41E-03
ENSGALG00000031737	NA	NA	-1.15	9.09E-03
ENSGALG00000001206	NA	NA	-1.19	9.74E-03
ENSGALG00000034498	NA	NA	-1.14	9.91E-03

Appendix 9 GO enrichment analysis of the up regulated DE genes found in 1 ppm iodinated water on clean litter. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Accession No.	Ontology	Definition	No. of DEGs	P-value
GO:0031224	CC	Intrinsic component of membrane	86	9.01E-06
GO:0016021	CC	Integral component of membrane	85	1.01E-05
GO:0044425	CC	Membrane part	95	3.22E-05
GO:0007033	BP	Vacuole organization	10	1.96E-04
GO:0005794	CC	Golgi apparatus	26	2.32E-04
GO:0022892	MF	Substrate-specific transporter activity	28	2.48E-04
GO:0005768	CC	Endosome	18	4.37E-04
GO:0022891	MF	Substrate-specific transmembrane transporter activity	24	5.31E-04
GO:0005215	MF	Transporter activity	30	6.54E-04
GO:0012505	CC	Endomembrane system	51	7.03E-04
GO:0022857	MF	Transmembrane transporter activity	25	8.76E-04
GO:0015075	MF	Ion transmembrane transporter activity	21	1.12E-03
GO:0016032	BP	Viral process	14	1.52E-03
GO:0044764	BP	Multi-organism cellular process	14	1.52E-03
GO:0044403	BP	Symbiosis, encompassing mutualism through parasitism	14	2.63E-03
GO:0044419	BP	Interspecies interaction between organisms	14	2.63E-03
GO:0006665	BP	Sphinlipid metabolic process	7	2.67E-03
GO:0031226	CC	Intrinsic component of plasma membrane	25	3.41E-03
GO:0005887	CC	Integral component of plasma membrane	24	4.54E-03
GO:0005773	CC	Vacuole	20	4.73E-03
GO:0043413~	BP	Macromolecule glycosylation	9	4.78E-03
GO:0006486	BP	Protein glycosylation	9	4.78E-03
GO:0098588	CC	Bounding membrane of organelle	22	4.97E-03
GO:0015085	MF	Calcium ion transmembrane transporter activity	7	5.12E-03
GO:0070085	BP	Glycosylation	9	5.20E-03
GO:0001881	BP	Receptor recycling	4	5.97E-03
GO:0043901	BP	Negative regulation of multi-organism process	6	6.61E-03
GO:0019751	BP	Polyol metabolic process	5	6.63E-03
GO:0006952	BP	Defense response	20	8.04E-03
GO:0045087	BP	Innate immune response	11	8.31E-03
GO:0043903	BP	Regulation of symbiosis, encompassing mutualism through parasitism	9	8.41E-03
GO:0051704	BP	Multi-organism process	24	9.19E-03
GO:0060333	BP	Interferon-gamma-mediated signaling pathway	3	9.23E-03
GO:0000139	CC	Golgi membrane	10	1.02E-02
GO:0043900	BP	Regulation of multi-organism process	10	1.09E-02
GO:0006643	BP	Membrane lipid metabolic process	7	1.10E-02
GO:0044255	BP	Cellular lipid metabolic process	17	1.17E-02
GO:0005262	MF	Calcium channel activity	6	1.18E-02
GO:0044710	BP	Single-organism metabolic process	49	1.19E-02
GO:0003824	MF	Catalytic activity	83	1.23E-02
GO:0006936	BP	Muscle contraction	8	1.25E-02
GO:0034220	BP	Ion transmembrane transport	14	1.31E-02
GO:0005216	MF	Ion channel activity	12	1.36E-02
GO:0007032	BP	Endosome organization	4	1.39E-02
GO:0016020	CC	Membrane	106	1.39E-02
GO:0006811	BP	Ion transport	20	1.43E-02
GO:0055085	BP	Transmembrane transport	17	1.58E-02
GO:1901135	BP	Carbohydrate derivative metabolic process	20	1.69E-02
GO:0022838	MF	Substrate-specific channel activity	12	1.73E-02
GO:0019058	BP	Viral life cycle	7	1.78E-02
GO:0050792	BP	Regulation of viral process	8	1.80E-02
GO:0008509	MF	Anion transmembrane transporter activity	7	1.93E-02
GO:0072509	MF	Divalent inorganic cation transmembrane transporter activity	7	1.93E-02
GO:0006629	BP	Lipid metabolic process	20	1.98E-02
GO:0098656	BP	Anion transmembrane transport	5	1.99E-02
GO:0043492	MF	Atpase activity, coupled to movement of substances	6	2.05E-02
GO:0098900	BP	Regulation of action potential	3	2.07E-02
GO:0006820	BP	Anion transport	9	2.08E-02
GO:0015267	MF	Channel activity	12	2.08E-02
GO:0022803	MF	Passive transmembrane transporter activity	12	2.08E-02
GO:0006066	BP	Alcohol metabolic process	7	2.08E-02
GO:0022832	MF	Voltage-gated channel activity	7	2.09E-02
GO:0005244	MF	Voltage-gated ion channel activity	7	2.09E-02
GO:0048037	MF	Cofactor binding	9	2.10E-02
GO:0009101	BP	Glycoprotein biosynthetic process	9	2.14E-02
GO:0005548	MF	Phospholipid transporter activity	4	2.16E-02
GO:0098660	BP	Inorganic ion transmembrane transport	11	2.17E-02
GO:0043168	MF	Anion binding	8	2.19E-02
GO:0046873	MF	Metal ion transmembrane transporter activity	11	2.21E-02
GO:0006487	BP	Protein N-linked glycosylation	5	2.24E-02
GO:0006810	BP	Transport	52	2.24E-02
GO:1902476	BP	Chloride transmembrane transport	4	2.39E-02
GO:0043112	BP	Receptor metabolic process	6	2.42E-02
GO:0009897	CC	External side of plasma membrane	6	2.50E-02
GO:0098661	BP	Inorganic anion transmembrane transport	4	2.59E-02
GO:0051607	BP	Defense response to virus	7	2.60E-02
GO:0008324	MF	Cation transmembrane transporter activity	14	2.61E-02
GO:0043207	BP	Response to external biotic stimulus	13	2.75E-02
GO:0051707	BP	Response to other organism	13	2.75E-02
GO:0009615	BP	Response to virus	8	2.84E-02
GO:0098552	CC	Side of membrane	9	2.88E-02
GO:0044431	CC	Golgi apparatus part	12	2.92E-02
GO:0003012	BP	Muscle system process	8	2.92E-02
GO:0051234	BP	Establishment of localization	53	3.02E-02
GO:0004012	MF	Phospholipid-translocating atpase activity	3	3.13E-02
GO:0005319	MF	Lipid transporter activity	5	3.17E-02
GO:0030003	BP	Cellular cation homeostasis	11	3.27E-02
GO:0002376	BP	Immune system process	28	3.45E-02
GO:0071702	BP	Organic substance transport	31	3.49E-02
GO:0000287	MF	Magnesium ion binding	7	3.53E-02
GO:0043028	MF	Cysteine-type endopeptidase regulator activity involved in apoptotic process	3	3.53E-02
GO:1903900	BP	Regulation of viral life cycle	5	3.59E-02
GO:0005975	BP	Carbohydrate metabolic process	14	3.64E-02
GO:0034686	CC	Integrin alphav-beta complex	2	3.65E-02
GO:0016554	BP	Cytidine to uridine editing	2	3.74E-02
GO:0060075	BP	Regulation of resting membrane potential	2	3.74E-02
GO:0010008	CC	Endosome membrane	7	3.83E-02

GO:0048525	BP	Negative regulation of viral process	4	3.93E-02
GO:0009607	BP	Response to biotic stimulus	13	3.94E-02
GO:0016787	MF	Hydrolase activity	38	3.95E-02
GO:0006873	BP	Cellular ion homeostasis	11	3.95E-02
GO:0031988	CC	Membrane-bounded vesicle	43	4.09E-02
GO:0030170	MF	Pyridoxal phosphate binding	4	4.10E-02
GO:0009100	BP	Glycoprotein metabolic process	9	4.12E-02
GO:0044459	CC	Plasma membrane part	31	4.21E-02
GO:0031090	CC	Organelle membrane	27	4.22E-02
GO:0008305	CC	Integrin complex	3	4.25E-02
GO:0004190	MF	Aspartic-type endopeptidase activity	3	4.39E-02
GO:0005737	CC	Cytoplasm	110	4.44E-02
GO:0098636	CC	Protein complex involved in cell adhesion	3	4.69E-02
GO:1901565	BP	Organonitrogen compound catabolic process	7	4.80E-02
GO:0006874	BP	Cellular calcium ion homeostasis	8	4.80E-02
GO:0070001	MF	Aspartic-type peptidase activity	3	4.85E-02
GO:0034113	BP	Heterotypic cell-cell adhesion	3	4.92E-02
GO:0044763	BP	Single-organism cellular process	127	4.95E-02

Appendix 10 GO enrichment analysis of the down regulated DE genes found in 1 ppm iodinated water on clean litter. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Accession No.	Ontology	Definition	No. of DEGs	P-value
GO:0032501	BP	Multicellular organismal process	24	2.31E-05
GO:0060537	BP	Muscle tissue development	7	8.01E-05
GO:0003008	BP	System process	11	1.34E-04
GO:0009888	BP	Tissue development	12	1.94E-04
GO:0003012	BP	Muscle system process	6	2.09E-04
GO:0048731	BP	System development	18	3.03E-04
GO:0044707	BP	Single-multicellular organism process	21	3.68E-04
GO:0044449	CC	Contractile fiber part	5	6.30E-04
GO:0007167	BP	Enzyme linked receptor protein signaling pathway	8	9.77E-04
GO:0030016	CC	Myofibril	5	9.77E-04
GO:0007275	BP	Multicellular organism development	18	1.05E-03
GO:0043292	CC	Contractile fiber	5	1.18E-03
GO:0048856	BP	Anatomical structure development	19	1.71E-03
GO:0032502	BP	Developmental process	19	2.60E-03
GO:0005515	MF	Protein binding	11	2.90E-03
GO:0007517	BP	Muscle organ development	5	3.06E-03
GO:0006928	BP	Movement of cell or subcellular component	10	3.30E-03
GO:0005576	CC	Extracellular region	17	3.60E-03
GO:0007169	BP	Transmembrane receptor protein tyrosine kinase signaling pathway	6	3.84E-03
GO:0061061	BP	Muscle structure development	6	4.12E-03
GO:0048519	BP	Negative regulation of biological process	16	4.34E-03
GO:0005912	CC	Adherens junction	6	4.90E-03
GO:0048659	BP	Smooth muscle cell proliferation	3	5.35E-03
GO:0044767	BP	Single-organism developmental process	18	5.41E-03
GO:0031327	BP	Negative regulation of cellular biosynthetic process	8	5.52E-03
GO:0070161	CC	Anchoring junction	6	5.56E-03
GO:0016477	BP	Cell migration	8	5.61E-03
GO:0031995	MF	Insulin-like growth factor II binding	2	5.85E-03
GO:0030017	CC	Sarcomere	4	5.91E-03
GO:0009890	BP	Negative regulation of biosynthetic process	8	6.01E-03
GO:0048523	BP	Negative regulation of cellular process	15	6.20E-03
GO:0048660	BP	Regulation of smooth muscle cell proliferation	3	6.41E-03
GO:0031324	BP	Negative regulation of cellular metabolic process	10	6.71E-03
GO:0048522	BP	Positive regulation of cellular process	16	8.15E-03
GO:0009986	CC	Cell surface	6	8.50E-03
GO:0031994	MF	Insulin-like growth factor I binding	2	8.77E-03
GO:0005615	CC	Extracellular space	8	8.93E-03
GO:0030334	BP	Regulation of cell migration	6	9.13E-03
GO:0048870	BP	Cell motility	8	9.53E-03
GO:0051674	BP	Localization of cell	8	9.61E-03
GO:0072358	BP	Cardiovascular system development	7	9.75E-03
GO:0072359	BP	Circulatory system development	7	9.75E-03
GO:0071495	BP	Cellular response to endogenous stimulus	7	9.84E-03
GO:0009892	BP	Negative regulation of metabolic process	10	1.07E-02
GO:0071310	BP	Cellular response to organic substance	9	1.07E-02
GO:2000145	BP	Regulation of cell motility	6	1.07E-02
GO:0006936	BP	Muscle contraction	4	1.12E-02
GO:0040012	BP	Regulation of locomotion	6	1.32E-02
GO:0048513	BP	Animal organ development	12	1.45E-02
GO:0051270	BP	Regulation of cellular component movement	6	1.47E-02
GO:0005924	CC	Cell-substrate adherens junction	5	1.52E-02
GO:0030055	CC	Cell-substrate junction	5	1.56E-02
GO:0015629	CC	Actin cytoskeleton	5	1.72E-02
GO:0014912	BP	Negative regulation of smooth muscle cell migration	2	1.77E-02
GO:0040011	BP	Locomotion	8	1.88E-02
GO:0010605	BP	Negative regulation of macromolecule metabolic process	9	1.89E-02
GO:0030054	CC	Cell junction	7	1.89E-02
GO:0005520	MF	Insulin-like growth factor binding	2	2.03E-02
GO:0032879	BP	Regulation of localization	10	2.07E-02
GO:0048518	BP	Positive regulation of biological process	16	2.15E-02
GO:0033002	BP	Muscle cell proliferation	3	2.16E-02
GO:0009653	BP	Anatomical structure morphogenesis	11	2.19E-02
GO:0051172	BP	Negative regulation of nitrogen compound metabolic process	7	2.21E-02
GO:0010629	BP	Negative regulation of gene expression	7	2.25E-02
GO:0009719	BP	Response to endogenous stimulus	7	2.34E-02
GO:0031674	CC	I band	3	2.42E-02
GO:0072202	BP	Cell differentiation involved in metanephros development	2	2.46E-02
GO:0001568	BP	Blood vessel development	5	2.48E-02
GO:0044421	CC	Extracellular region part	14	2.58E-02
GO:2000696	BP	Regulation of epithelial cell differentiation involved in kidney development	2	2.81E-02
GO:0048646	BP	Anatomical structure formation involved in morphogenesis	7	2.95E-02
GO:0001944	BP	Vasculature development	5	2.99E-02
GO:0044420	CC	Extracellular matrix component	3	3.25E-02
GO:0051239	BP	Regulation of multicellular organismal process	10	3.35E-02
GO:0043567	BP	Regulation of insulin-like growth factor receptor signaling pathway	2	3.50E-02
GO:0008015	BP	Blood circulation	4	3.56E-02
GO:0044057	BP	Regulation of system process	4	3.56E-02
GO:0070887	BP	Cellular response to chemical stimulus	9	3.57E-02
GO:0003013	BP	Circulatory system process	4	3.65E-02
GO:0043034	CC	Costamere	2	3.72E-02
GO:0043205	CC	Fibril	2	3.72E-02
GO:0031326	BP	Regulation of cellular biosynthetic process	12	3.75E-02
GO:0009966	BP	Regulation of signal transduction	10	3.78E-02
GO:0005578	CC	Proteinaceous extracellular matrix	4	3.88E-02
GO:0005856	CC	Cytoskeleton	9	4.02E-02
GO:0009889	BP	Regulation of biosynthetic process	12	4.11E-02
GO:0072224	BP	Metanephric glomerulus development	2	4.19E-02
GO:0090185	BP	Negative regulation of kidney development	2	4.19E-02
GO:0009968	BP	Negative regulation of signal transduction	6	4.44E-02
GO:0010033	BP	Response to organic substance	9	4.50E-02
GO:0072243	BP	Metanephric nephron epithelium development	2	4.53E-02
GO:0060074	BP	Synapse maturation	2	4.53E-02
GO:0048662	BP	Negative regulation of smooth muscle cell proliferation	2	4.53E-02
GO:0045934	BP	Negative regulation of nucleobase-containing compound metabolic process	6	4.71E-02
GO:0080090	BP	Regulation of primary metabolic process	15	4.83E-02
GO:2000113	BP	Negative regulation of cellular macromolecule biosynthetic process	6	4.86E-02
GO:0030154	BP	Cell differentiation	12	4.91E-02
GO:0097458	CC	Neuron part	6	4.96E-02

Appendix 11 DAVID generated top 5 annotation clusters for up regulated DE genes found in 1 ppm iodinated water on clean litter. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Annotation Cluster 1	Enrichment Score: 4.097190277775898	
Category	Term	No. of DEGs
CC	GO:0031224~intrinsic component of membrane	86
CC	GO:0016021~integral component of membrane	85
CC	GO:0044425~membrane part	95
CC	GO:0016020~membrane	106
Annotation Cluster 2	Enrichment Score: 2.3865015276216663	
Category	Term	No. of DEGs
CC	GO:0005794~Golgi apparatus	26
CC	GO:0000139~Golgi membrane	10
CC	GO:0044431~Golgi apparatus part	12
Annotation Cluster 3	Enrichment Score: 2.0058669819338375	
Category	Term	No. of DEGs
BP	GO:0007033~vacuole organization	10
BP	GO:0007032~endosome organization	4
BP	GO:0010256~endomembrane system organization	8
Annotation Cluster 4	Enrichment Score: 1.6502561367041615	
Category	Term	No. of DEGs
BP	GO:0044764~multi-organism cellular process	14
BP	GO:0016032~viral process	14
BP	GO:0044419~interspecies interaction between organisms	14
BP	GO:0044403~symbiosis, encompassing mutualism through parasitism	14
BP	GO:0043901~negative regulation of multi-organism process	6
BP	GO:0006952~defense response	20
BP	GO:0043903~regulation of symbiosis, encompassing mutualism through parasitism	9
BP	GO:0051704~multi-organism process	24
BP	GO:0043900~regulation of multi-organism process	10
BP	GO:0019058~viral life cycle	7
BP	GO:0050792~regulation of viral process	8
BP	GO:0051607~defense response to virus	7
BP	GO:0043207~response to external biotic stimulus	13
BP	GO:0051707~response to other organism	13
BP	GO:0009615~response to virus	8
BP	GO:1903900~regulation of viral life cycle	5
BP	GO:0048525~negative regulation of viral process	4
BP	GO:0009607~response to biotic stimulus	13
BP	GO:0098542~defense response to other organism	8
BP	GO:0009605~response to external stimulus	24
BP	GO:1903901~negative regulation of viral life cycle	3
BP	GO:0002252~immune effector process	9
BP	GO:0031347~regulation of defense response	8
BP	GO:0002697~regulation of immune effector process	3
Annotation Cluster 5	Enrichment Score: 1.5924264742926117	
Category	Term	No. of DEGs
BP	GO:0019751~polyol metabolic process	5
BP	GO:0006066~alcohol metabolic process	7
BP	GO:1901615~organic hydroxy compound metabolic process	7

Appendix 12 DAVID generated top 5 annotation clusters for down regulated DE genes found in 1 ppm iodinated water on clean litter. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Annotation Cluster 1	Enrichment Score: 1.707218737090698	
Category	Term	No. of DEGs
BP	GO:0060537~muscle tissue development	7
BP	GO:0007517~muscle organ development	5
BP	GO:0061061~muscle structure development	6
BP	GO:0051146~striated muscle cell differentiation	3
BP	GO:0014706~striated muscle tissue development	3
BP	GO:0042692~muscle cell differentiation	3
BP	GO:0006996~organelle organization	8
Annotation Cluster 2	Enrichment Score: 1.3340353503830193	
Category	Term	No. of DEGs
CC	GO:0044420~extracellular matrix component	3
CC	GO:0005578~proteinaceous extracellular matrix	4
CC	GO:0031012~extracellular matrix	4
Annotation Cluster 3	Enrichment Score: 1.015861968406669	
Category	Term	No. of DEGs
BP	GO:0032501~multicellular organismal process	24
BP	GO:0009888~tissue development	12
BP	GO:0048731~system development	18
BP	GO:0044707~single-multicellular organism process	21
BP	GO:0007275~multicellular organism development	18
BP	GO:0048856~anatomical structure development	19
BP	GO:0032502~developmental process	19
MF	GO:0005515~protein binding	11
BP	GO:0006928~movement of cell or subcellular component	10
BP	GO:0048519~negative regulation of biological process	16
BP	GO:0044767~single-organism developmental process	18
BP	GO:0031327~negative regulation of cellular biosynthetic process	8
BP	GO:0016477~cell migration	8
BP	GO:0009890~negative regulation of biosynthetic process	8
BP	GO:0048523~negative regulation of cellular process	15
BP	GO:0031324~negative regulation of cellular metabolic process	10
BP	GO:0048522~positive regulation of cellular process	16
BP	GO:0048870~cell motility	8
BP	GO:0051674~localization of cell	8
BP	GO:0009892~negative regulation of metabolic process	10
BP	GO:0071310~cellular response to organic substance	9
BP	GO:0048513~animal organ development	12
BP	GO:0040011~locomotion	8
BP	GO:0010605~negative regulation of macromolecule metabolic process	9
BP	GO:0048518~positive regulation of biological process	16
BP	GO:0009653~anatomical structure morphogenesis	11
BP	GO:0051172~negative regulation of nitrogen compound metabolic process	7
BP	GO:0010629~negative regulation of gene expression	7
BP	GO:0070887~cellular response to chemical stimulus	9
BP	GO:0031326~regulation of cellular biosynthetic process	12
BP	GO:0009889~regulation of biosynthetic process	12
BP	GO:0010033~response to organic substance	9
BP	GO:0045934~negative regulation of nucleobase-containing compound metabolic process	6
BP	GO:0080090~regulation of primary metabolic process	15
BP	GO:2000113~negative regulation of cellular macromolecule biosynthetic process	6
BP	GO:0030154~cell differentiation	12
BP	GO:0031323~regulation of cellular metabolic process	15
BP	GO:0007369~gastrulation	3
BP	GO:0010558~negative regulation of macromolecule biosynthetic process	6
BP	GO:0030278~regulation of ossification	3
BP	GO:0008283~cell proliferation	7
BP	GO:0050789~regulation of biological process	24
BP	GO:0050793~regulation of developmental process	8
BP	GO:0050794~regulation of cellular process	23
BP	GO:0045892~negative regulation of transcription, DNA-templated	5
BP	GO:1903507~negative regulation of nucleic acid-templated transcription	5
BP	GO:0019222~regulation of metabolic process	15
BP	GO:0007399~nervous system development	8
BP	GO:1902679~negative regulation of RNA biosynthetic process	5
BP	GO:0048869~cellular developmental process	12
BP	GO:0031325~positive regulation of cellular metabolic process	9
BP	GO:0051253~negative regulation of RNA metabolic process	5
BP	GO:0042127~regulation of cell proliferation	6
BP	GO:0001822~kidney development	3
BP	GO:0045596~negative regulation of cell differentiation	4
BP	GO:0072001~renal system development	3
BP	GO:0007420~brain development	4
BP	GO:0016043~cellular component organization	16
MF	GO:0005488~binding	21
BP	GO:0048598~embryonic morphogenesis	4
BP	GO:0010556~regulation of macromolecule biosynthetic process	10
BP	GO:0009893~positive regulation of metabolic process	9
BP	GO:0060322~head development	4
BP	GO:0001655~urogenital system development	3
BP	GO:0044763~single-organism cellular process	26
BP	GO:0044249~cellular biosynthetic process	14
BP	GO:0071840~cellular component organization or biogenesis	16
BP	GO:0051093~negative regulation of developmental process	4
BP	GO:0001503~ossification	3
BP	GO:0030900~forebrain development	3
BP	GO:0065007~biological regulation	24
BP	GO:1901576~organic substance biosynthetic process	14
BP	GO:0044699~single-organism process	28
BP	GO:0051171~regulation of nitrogen compound metabolic process	10
BP	GO:0060255~regulation of macromolecule metabolic process	13
BP	GO:0010468~regulation of gene expression	10
BP	GO:0009058~biosynthetic process	14
BP	GO:0007417~central nervous system development	4
BP	GO:0010604~positive regulation of macromolecule metabolic process	8
BP	GO:1901362~organic cyclic compound biosynthetic process	10
BP	GO:0006351~transcription, DNA-templated	8

BP	GO:2000112~regulation of cellular macromolecule biosynthetic process	9
BP	GO:0009790~embryo development	4
BP	GO:0044271~cellular nitrogen compound biosynthetic process	11
BP	GO:0019219~regulation of nucleobase-containing compound metabolic process	9
BP	GO:0006355~regulation of transcription, DNA-templated	8
BP	GO:1903506~regulation of nucleic acid-templated transcription	8
BP	GO:2001141~regulation of RNA biosynthetic process	8
BP	GO:0051241~negative regulation of multicellular organismal process	4
BP	GO:0010557~positive regulation of macromolecule biosynthetic process	5
BP	GO:0001501~skeletal system development	3
BP	GO:0000122~negative regulation of transcription from RNA polymerase II promoter	3
BP	GO:0006366~transcription from RNA polymerase II promoter	5
BP	GO:0051179~localization	13
BP	GO:0051252~regulation of RNA metabolic process	8
BP	GO:0034654~nucleobase-containing compound biosynthetic process	9
BP	GO:0031328~positive regulation of cellular biosynthetic process	5
BP	GO:0097659~nucleic acid-templated transcription	8
BP	GO:0045595~regulation of cell differentiation	5
BP	GO:0032774~RNA biosynthetic process	8
BP	GO:0009891~positive regulation of biosynthetic process	5
BP	GO:0018130~heterocycle biosynthetic process	9
BP	GO:0019438~aromatic compound biosynthetic process	9
BP	GO:0006357~regulation of transcription from RNA polymerase II promoter	5
BP	GO:1901701~cellular response to oxygen-containing compound	3
BP	GO:1903508~positive regulation of nucleic acid-templated transcription	4
BP	GO:0045893~positive regulation of transcription, DNA-templated	4
BP	GO:0050896~response to stimulus	16
BP	GO:1902680~positive regulation of RNA biosynthetic process	4
BP	GO:0051254~positive regulation of RNA metabolic process	4
BP	GO:0009059~macromolecule biosynthetic process	10
BP	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	3
BP	GO:0051716~cellular response to stimulus	13
BP	GO:0045935~positive regulation of nucleobase-containing compound metabolic process	4
BP	GO:0010467~gene expression	10
BP	GO:0010628~positive regulation of gene expression	4
BP	GO:0051173~positive regulation of nitrogen compound metabolic process	4
BP	GO:0034645~cellular macromolecule biosynthetic process	9
BP	GO:0006996~organelle organization	8
BP	GO:1901700~response to oxygen-containing compound	3
BP	GO:0016070~RNA metabolic process	8
BP	GO:0044260~cellular macromolecule metabolic process	15
BP	GO:0044238~primary metabolic process	18
BP	GO:0071704~organic substance metabolic process	19
BP	GO:0034641~cellular nitrogen compound metabolic process	11
BP	GO:0044237~cellular metabolic process	18
BP	GO:1901360~organic cyclic compound metabolic process	10
BP	GO:0043170~macromolecule metabolic process	15
BP	GO:0008152~metabolic process	19
BP	GO:0090304~nucleic acid metabolic process	8
BP	GO:0006139~nucleobase-containing compound metabolic process	9
BP	GO:0006807~nitrogen compound metabolic process	11
BP	GO:0071702~organic substance transport	4
BP	GO:0046483~heterocycle metabolic process	9
BP	GO:0006725~cellular aromatic compound metabolic process	9
MF	GO:0003676~nucleic acid binding	5
BP	GO:0009987~cellular process	28
Annotation Cluster 4	Enrichment Score: 0.9980456336800122	
Category	Term	No. of DEGs
BP	GO:0048731~system development	18
CC	GO:0044449~contractile fiber part	5
BP	GO:0007167~enzyme linked receptor protein signaling pathway	8
CC	GO:0030016~myofibril	5
BP	GO:0007275~multicellular organism development	18
CC	GO:0043292~contractile fiber	5
BP	GO:0006928~movement of cell or subcellular component	10
CC	GO:0005576~extracellular region	17
BP	GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	6
BP	GO:0048519~negative regulation of biological process	16
CC	GO:0005912~adherens junction	6
BP	GO:0048659~smooth muscle cell proliferation	3
BP	GO:0044767~single-organism developmental process	18
CC	GO:0070161~anchoring junction	6
BP	GO:0016477~cell migration	8
CC	GO:0030017~sarcomere	4
BP	GO:0048523~negative regulation of cellular process	15
BP	GO:0048660~regulation of smooth muscle cell proliferation	3
BP	GO:0031324~negative regulation of cellular metabolic process	10
BP	GO:0048522~positive regulation of cellular process	16
CC	GO:0009986~cell surface	6
CC	GO:0005615~extracellular space	8
BP	GO:0030334~regulation of cell migration	6
BP	GO:0048870~cell motility	8
BP	GO:0051674~localization of cell	8
BP	GO:0072359~circulatory system development	7
BP	GO:0072358~cardiovascular system development	7
BP	GO:0071495~cellular response to endogenous stimulus	7
BP	GO:0009892~negative regulation of metabolic process	10
BP	GO:0071310~cellular response to organic substance	9
BP	GO:2000145~regulation of cell motility	6
BP	GO:0040012~regulation of locomotion	6
BP	GO:0051270~regulation of cellular component movement	6
CC	GO:0005924~cell-substrate adherens junction	5
CC	GO:0030055~cell-substrate junction	5
CC	GO:0015629~actin cytoskeleton	5
BP	GO:0040011~locomotion	8
BP	GO:0010605~negative regulation of macromolecule metabolic process	9
CC	GO:0030054~cell junction	7
BP	GO:0032879~regulation of localization	10
BP	GO:0048518~positive regulation of biological process	16
BP	GO:0033002~muscle cell proliferation	3
BP	GO:0009653~anatomical structure morphogenesis	11
BP	GO:0009719~response to endogenous stimulus	7
CC	GO:0031674~I band	3
BP	GO:0001568~blood vessel development	5
CC	GO:0044421~extracellular region part	14
BP	GO:0048646~anatomical structure formation involved in morphogenesis	7
BP	GO:0001944~vasculature development	5
BP	GO:0051239~regulation of multicellular organismal process	10
BP	GO:0070887~cellular response to chemical stimulus	9

BP	GO:0031326~regulation of cellular biosynthetic process	12
BP	GO:0009966~regulation of signal transduction	10
CC	GO:0005856~cytoskeleton	9
BP	GO:0009889~regulation of biosynthetic process	12
BP	GO:0009968~negative regulation of signal transduction	6
BP	GO:0010033~response to organic substance	9
BP	GO:0080090~regulation of primary metabolic process	15
BP	GO:0031323~regulation of cellular metabolic process	15
BP	GO:0010648~negative regulation of cell communication	6
BP	GO:0023057~negative regulation of signaling	6
BP	GO:0030336~negative regulation of cell migration	3
BP	GO:0051128~regulation of cellular component organization	9
BP	GO:0032269~negative regulation of cellular protein metabolic process	5
BP	GO:2000146~negative regulation of cell motility	3
BP	GO:0008283~cell proliferation	7
BP	GO:0010646~regulation of cell communication	10
CC	GO:0005925~focal adhesion	4
BP	GO:0019220~regulation of phosphate metabolic process	7
BP	GO:0051174~regulation of phosphorus metabolic process	7
BP	GO:0048583~regulation of response to stimulus	11
BP	GO:0048514~blood vessel morphogenesis	4
BP	GO:0023051~regulation of signaling	10
BP	GO:0031400~negative regulation of protein modification process	4
BP	GO:0050789~regulation of biological process	24
BP	GO:1902533~positive regulation of intracellular signal transduction	5
BP	GO:0051248~negative regulation of protein metabolic process	5
BP	GO:0010563~negative regulation of phosphorus metabolic process	4
BP	GO:0045936~negative regulation of phosphate metabolic process	4
BP	GO:0090066~regulation of anatomical structure size	4
BP	GO:0050793~regulation of developmental process	8
BP	GO:2000026~regulation of multicellular organismal development	7
BP	GO:0050794~regulation of cellular process	23
BP	GO:0051271~negative regulation of cellular component movement	3
BP	GO:0019222~regulation of metabolic process	15
BP	GO:0040013~negative regulation of locomotion	3
BP	GO:0071363~cellular response to growth factor stimulus	4
BP	GO:0019216~regulation of lipid metabolic process	3
BP	GO:0048585~negative regulation of response to stimulus	6
BP	GO:0048584~positive regulation of response to stimulus	7
BP	GO:0070848~response to growth factor	4
BP	GO:0031325~positive regulation of cellular metabolic process	9
BP	GO:0042221~response to chemical	10
BP	GO:0042127~regulation of cell proliferation	6
BP	GO:0007166~cell surface receptor signaling pathway	8
BP	GO:0030036~actin cytoskeleton organization	4
BP	GO:0045596~negative regulation of cell differentiation	4
MF	GO:0008092~cytoskeletal protein binding	3
BP	GO:0050673~epithelial cell proliferation	3
BP	GO:0051094~positive regulation of developmental process	5
BP	GO:0071417~cellular response to organonitrogen compound	3
BP	GO:0016310~phosphorylation	7
BP	GO:0010556~regulation of macromolecule biosynthetic process	10
BP	GO:0009893~positive regulation of metabolic process	9
BP	GO:0008284~positive regulation of cell proliferation	4
BP	GO:0030029~actin filament-based process	4
BP	GO:0043549~regulation of kinase activity	4
BP	GO:0032101~regulation of response to external stimulus	4
BP	GO:0007015~actin filament organization	3
BP	GO:0051093~negative regulation of developmental process	4
BP	GO:0001667~ameboidal-type cell migration	3
BP	GO:1902531~regulation of intracellular signal transduction	6
BP	GO:0006629~lipid metabolic process	5
BP	GO:0065007~biological regulation	24
BP	GO:0042592~homeostatic process	6
BP	GO:0022411~cellular component disassembly	3
BP	GO:1901699~cellular response to nitrogen compound	3
BP	GO:0031399~regulation of protein modification process	6
BP	GO:0030335~positive regulation of cell migration	3
BP	GO:0032535~regulation of cellular component size	3
BP	GO:0006468~protein phosphorylation	6
BP	GO:0071900~regulation of protein serine/threonine kinase activity	3
BP	GO:2000147~positive regulation of cell motility	3
BP	GO:0001525~angiogenesis	3
BP	GO:0051272~positive regulation of cellular component movement	3
BP	GO:0040017~positive regulation of locomotion	3
BP	GO:0060255~regulation of macromolecule metabolic process	13
BP	GO:0010468~regulation of gene expression	10
BP	GO:0051338~regulation of transferase activity	4
BP	GO:0033674~positive regulation of kinase activity	3
BP	GO:0007010~cytoskeleton organization	5
BP	GO:0010604~positive regulation of macromolecule metabolic process	8
BP	GO:0032268~regulation of cellular protein metabolic process	7
BP	GO:1901362~organic cyclic compound biosynthetic process	10
BP	GO:0009967~positive regulation of signal transduction	5
BP	GO:0042327~positive regulation of phosphorylation	4
BP	GO:0007155~cell adhesion	5
BP	GO:0051240~positive regulation of multicellular organismal process	5
BP	GO:0022610~biological adhesion	5
BP	GO:0006796~phosphate-containing compound metabolic process	8
BP	GO:0042325~regulation of phosphorylation	5
BP	GO:0006793~phosphorus metabolic process	8
BP	GO:0051347~positive regulation of transferase activity	3
BP	GO:0010243~response to organonitrogen compound	3
BP	GO:1902589~single-organism organelle organization	6
CC	GO:0043232~intracellular non-membrane-bounded organelle	11
CC	GO:0043228~non-membrane-bounded organelle	11
BP	GO:0051241~negative regulation of multicellular organismal process	4
BP	GO:0044700~single organism signaling	13
BP	GO:0043086~negative regulation of catalytic activity	3
BP	GO:0008285~negative regulation of cell proliferation	3
BP	GO:0051246~regulation of protein metabolic process	7
BP	GO:0023052~signaling	13
BP	GO:0048878~chemical homeostasis	4
BP	GO:0010647~positive regulation of cell communication	5
BP	GO:0023056~positive regulation of signaling	5
BP	GO:0007154~cell communication	13
BP	GO:0010562~positive regulation of phosphorus metabolic process	4
BP	GO:0045937~positive regulation of phosphate metabolic process	4
BP	GO:0030155~regulation of cell adhesion	3
BP	GO:0007507~heart development	3
BP	GO:0051179~localization	13
CC	GO:0070062~extracellular exosome	8
CC	GO:1903561~extracellular vesicle	8
CC	GO:0043230~extracellular organelle	8
BP	GO:0022607~cellular component assembly	7
BP	GO:0045595~regulation of cell differentiation	5

BP	GO:1901698~response to nitrogen compound	3
BP	GO:0035556~intracellular signal transduction	7
BP	GO:0080134~regulation of response to stress	4
BP	GO:0050790~regulation of catalytic activity	5
BP	GO:0051129~negative regulation of cellular component organization	3
BP	GO:0045859~regulation of protein kinase activity	3
BP	GO:0000165~MAPK cascade	3
BP	GO:0043408~regulation of MAPK cascade	3
BP	GO:0023014~signal transduction by protein phosphorylation	3
BP	GO:0050896~response to stimulus	16
BP	GO:0044711~single-organism biosynthetic process	4
BP	GO:0044085~cellular component biogenesis	7
BP	GO:0044092~negative regulation of molecular function	3
BP	GO:0001932~regulation of protein phosphorylation	4
BP	GO:0007165~signal transduction	11
BP	GO:0065008~regulation of biological quality	8
BP	GO:0009059~macromolecule biosynthetic process	10
BP	GO:0051960~regulation of nervous system development	3
BP	GO:0009605~response to external stimulus	5
BP	GO:0098609~cell-cell adhesion	3
BP	GO:0051247~positive regulation of protein metabolic process	4
BP	GO:0001934~positive regulation of protein phosphorylation	3
BP	GO:0051716~cellular response to stimulus	13
BP	GO:0009628~response to abiotic stimulus	3
CC	GO:0031988~membrane-bounded vesicle	8
BP	GO:0044710~single-organism metabolic process	8
BP	GO:0006950~response to stress	7
BP	GO:0010467~gene expression	10
BP	GO:0043085~positive regulation of catalytic activity	3
BP	GO:0065009~regulation of molecular function	5
CC	GO:0031982~vesicle	8
MF	GO:0043167~ion binding	7
BP	GO:0006996~organelle organization	8
BP	GO:0018193~peptidyl-amino acid modification	3
BP	GO:0031401~positive regulation of protein modification process	3
BP	GO:0044260~cellular macromolecule metabolic process	15
BP	GO:0006464~cellular protein modification process	7
BP	GO:0036211~protein modification process	7
BP	GO:0033554~cellular response to stress	4
BP	GO:0044238~primary metabolic process	18
BP	GO:0044281~small molecule metabolic process	4
BP	GO:0044093~positive regulation of molecular function	3
BP	GO:0043412~macromolecule modification	7
MF	GO:0046872~metal ion binding	6
MF	GO:0043169~cation binding	6
BP	GO:0044237~cellular metabolic process	18
BP	GO:1901360~organic cyclic compound metabolic process	10
BP	GO:0032270~positive regulation of cellular protein metabolic process	3
BP	GO:0043170~macromolecule metabolic process	15
BP	GO:0071822~protein complex subunit organization	3
BP	GO:0044267~cellular protein metabolic process	8
BP	GO:0032989~cellular component morphogenesis	3
BP	GO:0043933~macromolecular complex subunit organization	4
BP	GO:0019538~protein metabolic process	8
CC	GO:0044422~organelle part	12
CC	GO:0044446~intracellular organelle part	10
Annotation Cluster 5	Enrichment Score: 0.8594043375859217	
Category	Term	No. of DEGs
BP	GO:0042981~regulation of apoptotic process	6
BP	GO:0043067~regulation of programmed cell death	6
BP	GO:0031325~positive regulation of cellular metabolic process	9
BP	GO:0010941~regulation of cell death	6
BP	GO:0006915~apoptotic process	6
BP	GO:0009893~positive regulation of metabolic process	9
BP	GO:0043549~regulation of kinase activity	4
BP	GO:0012501~programmed cell death	6
BP	GO:0043066~negative regulation of apoptotic process	4
BP	GO:0043069~negative regulation of programmed cell death	4
BP	GO:0008219~cell death	6
BP	GO:0051338~regulation of transferase activity	4
BP	GO:0060548~negative regulation of cell death	4
BP	GO:0010604~positive regulation of macromolecule metabolic process	8

Appendix 13 Detailed information on the up regulated DE genes found in used vs. clean litter on 1 ppm.

Ensembl gene ID	Gene symbol	Entrez ID	Log_2FC	FDR
ENSGALG00000033157	ADAMTS10	NA	1.02	7.69E-04
ENSGALG00000001407	ADAMTS15	419733	1.48	1.65E-05
ENSGALG00000030038	C3	396370	1.65	7.52E-03
ENSGALG00000017308	CHRDL2	NA	1.93	4.00E-04
ENSGALG00000037675	COL14A1	396276	1.27	3.53E-03
ENSGALG00000038311	COL18A1	NA	1.35	3.20E-03
ENSGALG00000037437	EGR3	NA	1.42	8.73E-04
ENSGALG00000016964	EPSTI1	418837	1.67	8.89E-08
ENSGALG00000002108	EVPL	427805	3.04	5.66E-03
ENSGALG00000026677	F10	395876	1.64	7.76E-03
ENSGALG00000041192	IFIH1	424185	1.20	3.41E-03
ENSGALG00000040651	IGF1R	395889	1.10	6.27E-04
ENSGALG00000011468	IGFBP5	424220	1.19	2.91E-07
ENSGALG00000002708	LINGO1	415344	1.13	1.42E-03
ENSGALG00000001558	MOV10	419872	1.07	8.29E-03
ENSGALG00000009107	NRXN1	395398	1.14	1.42E-03
ENSGALG00000001442	OPCML	395422	1.63	1.34E-03
ENSGALG00000013929	PDGFRA	395509	1.12	5.94E-03
ENSGALG00000004888	RAI1	427664	1.20	3.20E-03
ENSGALG00000008911	RNF208	417279	1.51	3.90E-03
ENSGALG00000016400	RSAD2	428650	2.12	3.69E-03
ENSGALG00000037274	SNPH	100858058	1.61	6.64E-03
ENSGALG00000026465	TMEM100	417398	1.08	1.42E-03
ENSGALG00000033966	NA	101748831	1.18	1.20E-03
ENSGALG00000041621	NA	395550	1.69	2.38E-03
ENSGALG00000013575	NA	403120	1.99	4.07E-03
ENSGALG00000039585	NA	NA	2.47	2.97E-03
ENSGALG00000009479	NA	420559	2.61	2.65E-05
ENSGALG00000016556	NA	428660	1.98	4.99E-03
ENSGALG00000042001	NA	422513	1.30	1.43E-05
ENSGALG00000038140	NA	NA	2.64	3.55E-04
ENSGALG00000009639	NA	NA	2.71	2.11E-03
ENSGALG00000011190	NA	NA	3.78	1.37E-14
ENSGALG00000026970	NA	770612	1.14	3.82E-03
ENSGALG00000045534	NA	100857563	1.03	9.24E-03
ENSGALG00000032428	NA	100858381	1.57	5.57E-04
ENSGALG00000026152	NA	395366	2.02	1.31E-06
ENSGALG00000023709	NA	417192	1.72	5.29E-03
ENSGALG00000000720	NA	419563	1.01	8.73E-04
ENSGALG00000000478	NA	NA	1.21	7.13E-03
ENSGALG00000023819	NA	772158	3.14	3.88E-03
ENSGALG00000044619	NA	NA	1.47	2.27E-04
ENSGALG00000045640	NA	776920	1.92	1.96E-03
ENSGALG00000044326	NA	426820	1.08	7.25E-03

Appendix 14 Detailed information on the down regulated DE genes found in used vs. clean litter on 1 ppm.

Ensembl gene ID	Gene symbol	Entrez ID	Log_2FC	FDR
ENSGALG00000008744	MCF2L2	424959	-1.54	1.34E-12
ENSGALG00000011921	DHRS7	423527	-2.08	3.03E-09
ENSGALG00000015492	PDZK1	418460	-2.95	5.40E-09
ENSGALG00000029102	PXYLP1	424815	-1.08	3.38E-08
ENSGALG00000033695	CYP26A1	408183	-2.89	4.86E-07
ENSGALG00000008608	FETUB	395404	-1.42	1.96E-06
ENSGALG00000015595	GPR78	428798	-4.85	1.96E-06
ENSGALG00000015544	ALDOB	427308	-1.96	2.36E-06
ENSGALG00000007234	CLCN5	422285	-1.36	8.52E-06
ENSGALG00000010202	ENTPD5	423343	-1.31	1.14E-05
ENSGALG00000002849	DER	374066	-1.17	1.31E-05
ENSGALG00000004669	ASPA	417609	-1.60	3.09E-05
ENSGALG00000010001	EGLN3	423316	-1.55	1.19E-04
ENSGALG00000002132	KCNIP1	416173	-2.92	2.05E-04
ENSGALG00000006425	SLC6A6	416041	-1.88	2.07E-04
ENSGALG00000010769	HPGD	422567	-1.51	2.27E-04
ENSGALG00000012997	DNAH5	NA	-2.62	3.15E-04
ENSGALG00000016828	GRTP1	418742	-1.08	3.41E-04
ENSGALG00000016300	TINAG	421888	-1.22	3.51E-04
ENSGALG00000036819	ARHGDIG	100858938	-1.05	4.77E-04
ENSGALG00000039326	HSBP1L1	420812	-1.15	5.15E-04
ENSGALG00000002893	STC2	416208	-1.48	7.29E-04
ENSGALG00000001252	CREB3L3	428333	-2.17	7.37E-04
ENSGALG00000014201	PARVB	418235	-1.30	7.46E-04
ENSGALG00000004162	SLC16A6	417435	-1.60	7.53E-04
ENSGALG00000012166	SLC35F5	424281	-1.08	8.47E-04
ENSGALG00000010427	TM4SF4	771806	-1.36	8.50E-04
ENSGALG00000008914	NRAP	423899	-2.54	1.03E-03
ENSGALG00000004332	PLA2G12B	423705	-1.68	1.20E-03
ENSGALG00000002169	PLEKHB2	424758	-1.10	1.20E-03
ENSGALG00000006627	UPB1	416949	-1.69	1.58E-03
ENSGALG00000023738	IRX6	NA	-1.91	1.60E-03
ENSGALG00000002066	NCOA4	423615	-1.04	1.60E-03
ENSGALG00000016858	ASBT	428018	-1.72	1.66E-03
ENSGALG00000034960	MLXIPL	101751014	-1.61	2.53E-03
ENSGALG00000039536	C8orf22	425711	-2.32	2.58E-03
ENSGALG00000010935	PTPRQ	772163	-1.99	2.70E-03
ENSGALG00000000667	EDN2	419559	-1.25	3.07E-03
ENSGALG00000015147	ALDH1A1	395264	-1.12	4.19E-03
ENSGALG00000013265	GYS2	418201	-1.50	5.08E-03
ENSGALG00000015937	FABP1	374015	-1.20	5.14E-03
ENSGALG00000006534	PEX11A	NA	-1.25	5.99E-03
ENSGALG00000031590	B3GAT2	428638	-1.17	6.25E-03
ENSGALG00000013097	SLC15A5	418177	-2.43	6.52E-03
ENSGALG00000033720	CA7	415791	-1.37	7.24E-03
ENSGALG00000003456	CA12	415370	-2.11	7.25E-03
ENSGALG00000006217	S100B	424038	-1.18	7.28E-03
ENSGALG00000014938	ABHD3	421068	-1.15	8.87E-03
ENSGALG00000003172	NA	423667	-2.20	3.50E-09
ENSGALG00000007382	NA	771849	-1.29	2.69E-06
ENSGALG00000010901	NA	420606	-2.82	3.90E-06
ENSGALG00000016027	NA	418512	-1.16	8.83E-06
ENSGALG00000009830	NA	422448	-4.84	8.83E-06
ENSGALG00000034813	NA	NA	-1.75	1.05E-05
ENSGALG00000016287	NA	395285	-1.70	3.08E-05
ENSGALG00000033139	NA	NA	-1.97	3.08E-05
ENSGALG00000046639	NA	107049058	-3.16	1.03E-04
ENSGALG00000009538	NA	423274	-1.26	1.03E-04
ENSGALG00000030614	NA	771920	-1.26	1.15E-04
ENSGALG00000021314	NA	416425	-2.03	3.15E-04
ENSGALG00000015416	NA	421130	-1.20	3.41E-04
ENSGALG00000045738	NA	NA	-1.03	3.51E-04
ENSGALG00000004637	NA	417607	-1.14	4.77E-04
ENSGALG00000008599	NA	NA	-3.78	7.37E-04
ENSGALG00000009291	NA	421321	-1.04	1.03E-03
ENSGALG00000028451	NA	396212	-1.57	1.14E-03
ENSGALG00000003022	NA	100857724	-1.23	1.20E-03
ENSGALG00000019284	NA	418170	-1.07	1.42E-03
ENSGALG00000011356	NA	NA	-2.19	1.42E-03
ENSGALG00000002595	NA	NA	-1.22	1.82E-03
ENSGALG00000016690	NA	422046	-5.42	1.93E-03
ENSGALG00000010736	NA	395940	-1.34	1.93E-03
ENSGALG00000010554	NA	421465	-1.12	2.33E-03
ENSGALG00000006018	NA	420422	-2.54	3.17E-03
ENSGALG00000043529	NA	NA	-2.14	5.05E-03
ENSGALG00000013571	NA	NA	-2.12	5.05E-03
ENSGALG00000028551	NA	100859645	-1.40	5.66E-03
ENSGALG00000038582	NA	770250	-1.49	5.99E-03
ENSGALG00000012869	NA	396058	-2.19	6.57E-03
ENSGALG00000008185	NA	424071	-1.16	6.97E-03
ENSGALG00000043484	NA	101747789	-1.06	7.24E-03
ENSGALG00000016483	NA	421965	-2.27	7.52E-03
ENSGALG00000030700	NA	NA	-1.97	7.76E-03
ENSGALG00000016132	NA	NA	-1.20	7.91E-03
ENSGALG00000023070	NA	423434	-1.91	7.91E-03

Appendix 15 GO enrichment analysis of the up regulated DE genes found in used vs. clean litter on 1 ppm. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Accession No.	Ontology	Definition	No. of DEGs	P-value
GO:0097367	MF	Carbohydrate derivative binding	8	4.97E-03
GO:0043491	BP	Protein kinase B signaling	3	6.63E-03
GO:0035639	MF	Purine ribonucleoside triphosphate binding	7	1.05E-02
GO:0032550	MF	Purine ribonucleoside binding	7	1.07E-02
GO:0001883	MF	Purine nucleoside binding	7	1.07E-02
GO:0032549	MF	Ribonucleoside binding	7	1.08E-02
GO:0001882	MF	Nucleoside binding	7	1.10E-02
GO:0032555	MF	Purine ribonucleotide binding	7	1.15E-02
GO:0017076	MF	Purine nucleotide binding	7	1.16E-02
GO:0032553	MF	Ribonucleotide binding	7	1.20E-02
GO:0005488	MF	Binding	15	2.10E-02
GO:0048731	BP	System development	8	2.91E-02
GO:0000166	MF	Nucleotide binding	7	3.46E-02
GO:1901265	MF	Nucleoside phosphate binding	7	3.46E-02
GO:0001525	BP	Angiogenesis	3	4.47E-02
GO:0044707	BP	Single-multicellular organism process	9	4.56E-02
GO:0036094	MF	Small molecule binding	7	4.63E-02
GO:0007275	BP	Multicellular organism development	8	4.84E-02

Appendix 16 GO enrichment analysis of the down regulated DE genes found in used vs. clean litter on 1 ppm. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Accession No.	Ontology	Definition	No. of DEGs	P-value
GO:0015718	BP	monocarboxylic acid transport	4	2.40E-03
GO:0006690	BP	icosanoid metabolic process	3	8.10E-03
GO:0006820	BP	anion transport	5	8.80E-03
GO:0033559	BP	unsaturated fatty acid metabolic process	3	1.10E-02
GO:0046942	BP	carboxylic acid transport	4	1.23E-02
GO:0044710	BP	single-organism metabolic process	16	1.42E-02
GO:0006811	BP	ion transport	8	1.54E-02
GO:0044281	BP	small molecule metabolic process	10	2.05E-02
GO:0015711	BP	organic anion transport	4	2.11E-02
GO:0032787	BP	monocarboxylic acid metabolic process	5	2.47E-02
GO:0002478	BP	antigen processing and presentation of exogenous peptide antigen	2	3.59E-02
GO:0055114	BP	oxidation-reduction process	5	3.99E-02
GO:0002474	BP	antigen processing and presentation of peptide antigen via MHC class I	2	4.03E-02
GO:0042445	BP	hormone metabolic process	3	4.67E-02
GO:0044723	BP	single-organism carbohydrate metabolic process	5	4.96E-02
GO:0005576	CC	extracellular region	18	1.00E-02
GO:0044421	CC	extracellular region part	16	2.21E-02
GO:0016491	MF	oxidoreductase activity	12	0.00E+00
GO:0004623	MF	phospholipase A2 activity	3	3.50E-03
GO:0003824	MF	catalytic activity	27	2.50E-02
GO:0004620	MF	phospholipase activity	3	2.63E-02
GO:0016298	MF	lipase activity	3	3.85E-02

Appendix 17 DAVID generated top 5 annotation clusters for up regulated DE genes found in used vs. clean litter on 1 ppm. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Annotation Cluster 1	Enrichment Score: 1.1994903987792247	
Category	Term	No. of DEGs
MF	GO:0097367~carbohydrate derivative binding	8
MF	GO:0035639~purine ribonucleoside triphosphate binding	7
MF	GO:0032550~purine ribonucleoside binding	7
MF	GO:0001883~purine nucleoside binding	7
MF	GO:0032549~ribonucleoside binding	7
MF	GO:0001882~nucleoside binding	7
MF	GO:0032555~purine ribonucleotide binding	7
MF	GO:0017076~purine nucleotide binding	7
MF	GO:0032553~ribonucleotide binding	7
MF	GO:0000166~nucleotide binding	7
MF	GO:1901265~nucleoside phosphate binding	7
MF	GO:0036094~small molecule binding	7
MF	GO:0005525~GTP binding	3
MF	GO:0032561~guanyl ribonucleotide binding	3
MF	GO:0019001~guanyl nucleotide binding	3
MF	GO:0017111~nucleoside-triphosphatase activity	3
MF	GO:0016462~pyrophosphatase activity	3
MF	GO:0016818~hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	3
MF	GO:0016817~hydrolase activity, acting on acid anhydrides	3
MF	GO:0005524~ATP binding	4
MF	GO:0032559~adenyl ribonucleotide binding	4
MF	GO:0030554~adenyl nucleotide binding	4
MF	GO:0016787~hydrolase activity	5
MF	GO:1901363~heterocyclic compound binding	8
MF	GO:0097159~organic cyclic compound binding	8
MF	GO:0003824~catalytic activity	8
Annotation Cluster 2	Enrichment Score: 0.6610966537664664	
Category	Term	No. of DEGs
BP	GO:0043491~protein kinase B signaling	3
BP	GO:0048731~system development	8
BP	GO:0001525~angiogenesis	3
BP	GO:0044707~single-multicellular organism process	9
BP	GO:0007275~multicellular organism development	8
BP	GO:0048514~blood vessel morphogenesis	3
BP	GO:0032501~multicellular organismal process	9
BP	GO:0001568~blood vessel development	3
BP	GO:0009967~positive regulation of signal transduction	4
BP	GO:0048856~anatomical structure development	8
BP	GO:0001944~vasculature development	3
BP	GO:0044767~single-organism developmental process	8
CC	GO:0005783~endoplasmic reticulum	4
BP	GO:0010647~positive regulation of cell communication	4
BP	GO:0023056~positive regulation of signaling	4
BP	GO:0032502~developmental process	8
BP	GO:0051239~regulation of multicellular organismal process	5
BP	GO:0009966~regulation of signal transduction	5
BP	GO:0030154~cell differentiation	6
BP	GO:0048584~positive regulation of response to stimulus	4
BP	GO:0007167~enzyme linked receptor protein signaling pathway	3
BP	GO:0009888~tissue development	4
BP	GO:0007165~signal transduction	7
BP	GO:0010646~regulation of cell communication	5
BP	GO:0023051~regulation of signaling	5
BP	GO:0048869~cellular developmental process	6
BP	GO:0072359~circulatory system development	3
BP	GO:0072358~cardiovascular system development	3
BP	GO:0071495~cellular response to endogenous stimulus	3
BP	GO:0048513~animal organ development	5
BP	GO:0050896~response to stimulus	9
BP	GO:0050793~regulation of developmental process	4
BP	GO:0044700~single organism signaling	7
BP	GO:0023052~signaling	7
BP	GO:0007154~cell communication	7
BP	GO:0009719~response to endogenous stimulus	3
BP	GO:0007166~cell surface receptor signaling pathway	4
BP	GO:0048583~regulation of response to stimulus	5
CC	GO:0005886~plasma membrane	6
BP	GO:0048522~positive regulation of cellular process	6
BP	GO:0048646~anatomical structure formation involved in morphogenesis	3
BP	GO:0051240~positive regulation of multicellular organismal process	3
CC	GO:0071944~cell periphery	6
BP	GO:0003008~system process	3
BP	GO:0010468~regulation of gene expression	5
BP	GO:0032989~cellular component morphogenesis	3
BP	GO:1902531~regulation of intracellular signal transduction	3
BP	GO:0048518~positive regulation of biological process	6
BP	GO:0051716~cellular response to stimulus	7
BP	GO:0009653~anatomical structure morphogenesis	4
BP	GO:0071310~cellular response to organic substance	3
CC	GO:0044425~membrane part	8
CC	GO:0031224~intrinsic component of membrane	7
BP	GO:0048519~negative regulation of biological process	5
BP	GO:0048468~cell development	3
BP	GO:0070887~cellular response to chemical stimulus	3
BP	GO:0065008~regulation of biological quality	4
BP	GO:0050794~regulation of cellular process	9
BP	GO:0010033~response to organic substance	3
BP	GO:0010467~gene expression	5
BP	GO:0032879~regulation of localization	3
BP	GO:0016043~cellular component organization	6
CC	GO:0012505~endomembrane system	4
BP	GO:0060255~regulation of macromolecule metabolic process	5
CC	GO:0016021~integral component of membrane	6
BP	GO:0071840~cellular component organization or biogenesis	6
BP	GO:0035556~intracellular signal transduction	3
BP	GO:0050789~regulation of biological process	9
CC	GO:0016020~membrane	9
BP	GO:0044260~cellular macromolecule metabolic process	7
BP	GO:0019222~regulation of metabolic process	5
BP	GO:0042221~response to chemical	3

BP	GO:0065007~biological regulation	9
CC	GO:0044444~cytoplasmic part	6
BP	GO:0009987~cellular process	13
CC	GO:0031982~vesicle	3
BP	GO:0006996~organelle organization	3
BP	GO:0051179~localization	4
BP	GO:0044763~single-organism cellular process	9
BP	GO:0044237~cellular metabolic process	7
BP	GO:0044699~single-organism process	10
CC	GO:0043229~intracellular organelle	9
Annotation Cluster 3	Enrichment Score: 0.5234163839091858	
Category	Term	No. of DEGs
BP	GO:0009605~response to external stimulus	4
BP	GO:0050896~response to stimulus	9
BP	GO:0006950~response to stress	4
BP	GO:0002376~immune system process	3
Annotation Cluster 4	Enrichment Score: 0.4707859109968255	
Category	Term	No. of DEGs
CC	GO:0005615~extracellular space	3
CC	GO:0005576~extracellular region	6
CC	GO:0044421~extracellular region part	5
Annotation Cluster 5	Enrichment Score: 0.2192400210579103	
Category	Term	No. of DEGs
BP	GO:0051239~regulation of multicellular organismal process	5
BP	GO:0009888~tissue development	4
BP	GO:0050793~regulation of developmental process	4
BP	GO:0007166~cell surface receptor signaling pathway	4
BP	GO:0048522~positive regulation of cellular process	6
BP	GO:0010468~regulation of gene expression	5
BP	GO:0019538~protein metabolic process	6
BP	GO:0048518~positive regulation of biological process	6
BP	GO:0048519~negative regulation of biological process	5
BP	GO:2000112~regulation of cellular macromolecule biosynthetic process	4
BP	GO:0010556~regulation of macromolecule biosynthetic process	4
BP	GO:0044267~cellular protein metabolic process	5
BP	GO:0043170~macromolecule metabolic process	8
BP	GO:0031326~regulation of cellular biosynthetic process	4
BP	GO:0050794~regulation of cellular process	9
BP	GO:0009889~regulation of biosynthetic process	4
BP	GO:0010467~gene expression	5
BP	GO:0051171~regulation of nitrogen compound metabolic process	4
BP	GO:0060255~regulation of macromolecule metabolic process	5
BP	GO:0050789~regulation of biological process	9
BP	GO:0044260~cellular macromolecule metabolic process	7
BP	GO:0019222~regulation of metabolic process	5
BP	GO:0044271~cellular nitrogen compound biosynthetic process	4
BP	GO:0034645~cellular macromolecule biosynthetic process	4
BP	GO:0009059~macromolecule biosynthetic process	4
BP	GO:0044238~primary metabolic process	8
BP	GO:0065007~biological regulation	9
BP	GO:0006464~cellular protein modification process	3
BP	GO:0036211~protein modification process	3
CC	GO:0044444~cytoplasmic part	6
BP	GO:0080090~regulation of primary metabolic process	4
BP	GO:0071704~organic substance metabolic process	8
BP	GO:0031323~regulation of cellular metabolic process	4
BP	GO:0043412~macromolecule modification	3
BP	GO:0044249~cellular biosynthetic process	4
BP	GO:0008152~metabolic process	8
BP	GO:0016070~RNA metabolic process	3
BP	GO:1901576~organic substance biosynthetic process	4
BP	GO:0009058~biosynthetic process	4
BP	GO:0044237~cellular metabolic process	7
BP	GO:0034641~cellular nitrogen compound metabolic process	4
BP	GO:0090304~nucleic acid metabolic process	3
BP	GO:0006807~nitrogen compound metabolic process	4
BP	GO:0006139~nucleobase-containing compound metabolic process	3
BP	GO:0046483~heterocycle metabolic process	3
BP	GO:0006725~cellular aromatic compound metabolic process	3
BP	GO:1901360~organic cyclic compound metabolic process	3

Appendix 18 DAVID generated top 5 annotation clusters for down regulated DE genes found in used vs. clean litter on 1 ppm. GO = gene ontology; BP = biological process; MF = molecular function; CC = cellular components; DEG = differentially expressed gene.

Annotation Cluster 1	Enrichment Score: 1.4648015890374169	
Category	Term	No. of DEGs
MF	GO:0004623~phospholipase A2 activity	3
MF	GO:0004620~phospholipase activity	3
MF	GO:0016298~lipase activity	3
MF	GO:0052689~carboxylic ester hydrolase activity	3
MF	GO:0016788~hydrolase activity, acting on ester bonds	5
Annotation Cluster 2	Enrichment Score: 1.157328766572954	
Category	Term	No. of DEGs
BP	GO:0006690~eicosanoid metabolic process	3
BP	GO:0033559~unsaturated fatty acid metabolic process	3
BP	GO:0044281~small molecule metabolic process	10
BP	GO:0032787~monocarboxylic acid metabolic process	5
BP	GO:0006082~organic acid metabolic process	6
BP	GO:0044255~cellular lipid metabolic process	6
BP	GO:0006629~lipid metabolic process	7
BP	GO:0019752~carboxylic acid metabolic process	5
BP	GO:0043436~oxoacid metabolic process	5
BP	GO:0006631~fatty acid metabolic process	3
BP	GO:0007275~multicellular organism development	5
BP	GO:0048731~system development	4
Annotation Cluster 3	Enrichment Score: 1.0999765106567414	
Category	Term	No. of DEGs
BP	GO:0015718~monocarboxylic acid transport	4
BP	GO:0006820~anion transport	5
BP	GO:0046942~carboxylic acid transport	4
BP	GO:0006811~ion transport	8
BP	GO:0015711~organic anion transport	4
BP	GO:0055085~transmembrane transport	6
BP	GO:0044765~single-organism transport	10
BP	GO:1902578~single-organism localization	10
BP	GO:0006810~transport	12
BP	GO:0051234~establishment of localization	12
BP	GO:0071702~organic substance transport	7
BP	GO:0034220~ion transmembrane transport	3
BP	GO:0051179~localization	14
Annotation Cluster 4	Enrichment Score: 0.8423609508861539	
Category	Term	No. of DEGs
CC	GO:0005576~extracellular region	18
CC	GO:0044421~extracellular region part	16
CC	GO:0070062~extracellular exosome	11
CC	GO:1903561~extracellular vesicle	11
CC	GO:0043230~extracellular organelle	11
CC	GO:0031988~membrane-bounded vesicle	12
CC	GO:0031982~vesicle	12
CC	GO:0005829~cytosol	5
CC	GO:0043227~membrane-bounded organelle	23
CC	GO:0043226~organelle	24

Annotation Cluster 5	Enrichment Score: 0.8215774660129734	
Category	Term	No. of DEGs
MF	GO:0004866~endopeptidase inhibitor activity	3
MF	GO:0030414~peptidase inhibitor activity	3
MF	GO:0061135~endopeptidase regulator activity	3
MF	GO:0061134~peptidase regulator activity	3
MF	GO:0004857~enzyme inhibitor activity	3
MF	GO:0030234~enzyme regulator activity	4
MF	GO:0098772~molecular function regulator	4

Appendix 19 Detailed information on the up-regulated DE genes found in 1 ppm iodinated water on used litter.

Ensembl gene ID	Gene symbol	Entrez ID	Chr	Log2 FC	P-value	FDR
ENSGALG00000012119	MARCO	395488	7	1.59	4.37E-09	6.44E-05
ENSGALG00000002431	NA	429057	8	1.33	1.63E-08	1.20E-04
ENSGALG00000028304	NA	771888	2	1.88	2.89E-08	1.42E-04
ENSGALG00000043921	NA	NA		1.40	9.31E-07	2.91E-03
ENSGALG00000044799	NA	NA	4	1.22	1.91E-06	4.69E-03

Appendix 20 Detailed information on the down-regulated DE genes found in 1 ppm iodinated water on used litter.

Ensembl gene ID	Gene symbol	Entrez ID	Chr	Log2 FC	P-value	FDR
ENSGALG00000025958	U3	NA	19	-1.78	3.25E-06	6.84E-03
ENSGALG00000031737	NA	NA	5	-1.63	9.86E-07	2.91E-03