Comparative isolation of bacteria from inflamed sites to identify inflammation-tailored bacteria

by

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Abstract

Microbial culture collections are fundamental to microbiology research. However, with the advent of next generation sequencing research, the generation and maintenance of comprehensive culture collections has become deprioritized. As such a need exists for the improved isolation of diverse bacterial communities enabling bacterial communities to be archived for future investigations. This need in particular is highlighted in animal agriculture, since modern production practises such as the ingestion of a consistent diet can lead to loss of autochthonous members of the microbiota. Culture collections can be improved through understanding how to better select isolation methods and how to better evaluate both previously-cultured and -uncultured bacteria, as well as evaluating mechanistic processes involved in bacteria colonization of enteric habitats such as the inflamed tissues of the pig gastro-intestinal tract. Therefore, the goals of my research were to: 1) provide a better understanding of distinct differences between the effectiveness of direct plating and enrichment strategies to isolate diverse communities of bacteria within a chicken model; 2) expand evaluation of these comparisons to include the Ichip and endospore selection methods to isolate diverse communities of bacteria in a pig model; and finally, 3) identify candidate bacteria that are able to selectively colonize inflamed tissues. In the first study, 899 bacteria were isolated from chickens, which included the isolation of 75 unique bacterial taxa using various direct plating and enrichment methods. Differences between isolation methods were observed, with direct plating onto De Man, Rogosa and Sharpe agar (MRS) medium representing the lowest diversity of medium evaluated. In contrast, no differences were observed when comparing the diversity of bacterial communities isolated between enteric locations. These traditional isolation methods were further compared against more novel methods including the Ichip, as well as the use of ethanol or heat (i.e. tyndallization) to select for endospore forming bacteria. With these methods, 1,523 bacteria were evaluated spanning 80 genera and 7 phyla. No differences were observed when comparing the diversity of specific isolation strategies; in addition, it was determined that the use of

heat to select for bacteria, generated collections that were less diverse than those obtained by enrichment broths, Ichips, or direct plating methods. The ability of these methods to isolate previously uncultured bacteria was also evaluated and demonstrated that enrichment and Ichip methods more frequently isolated previously uncultured bacteria than other isolation methods. In contrast, endospore selective methods were found to isolate novel bacteria less frequently than other methods; however, ethanol was more effective than heat at isolating novel bacteria. In the final study, these isolation methods were used to isolate bacteria from inflamed tissues of pigs. In this investigation 24 Landrace x Duroc piglets were inoculated with *S. enterica* var Typhimurium phage type DT104 or Columbia broth to generate inflammation (ST+; n = 12) or as a control (ST-; n = 12) respectively. The pigs challenged with *Salmonella* (ST+), had a reduction in feed consumption, and body weight, as well as increased body temperature, tissue inflammation with increased expression of mediators of inflammation and immune function. In this study it was also determined that the culturable diversity of bacteria differed from evaluations using culture-independent methods. This was most obvious as culture-dependent methods were predominantly populated by *Bacillus* spp., whereas culture-independent analyses were dominated by *Prevotella* spp. Culture-independent analyses determined that *Prevoteallaceae* and *Enterobacteriaceae* were more abundant within ST+ animals, whereas culturomics determined that *Bacteroides* spp. (*B. uniformis*, *B. fragilis*), *Streptococcus* spp. (*S. gallolyticus*), were associated with ST+ piglets. *Ruminococcacea* was associated with the microbiota of ST- piglets through both culture-dependant and -independent analyses. In conclusion, this research has evaluated various traditional and modern isolation strategies to provide researchers with a better understanding of the best methods to employ in future culturomics strategies, and identified members of the autochthonous microbiota that can be used as beneficial bacteria for host health or drug delivery strategies within the habitats of inflamed tissues.

Preface

This thesis is an original work by Paul E. Moote. All animal work conducted in this thesis received ethics approval from the Animal Care Committee at the Lethbridge Research and Development Centre (Agriculture and Agri-Food Canada) before research was conducted. Chapter 4 contains material that was jointly prepared by Danisa M. Bescucci and me and we share first co-authorship on the research, and accepted publications.

The chapters contained in this thesis have either been accepted, or submitted for publication, or in the final stages of edits for submission in peer reviewed journals. Chapter 2 "Application of culturomics to characterize diverse anaerobic bacteria from the gastrointestinal tract of broiler chickens in relation to environmental reservoirs" was accepted and published in the Canadian Journal of Microbiology. Chapter 4 was submitted to Applied and Environmental Microbiology and was accepted with minor revisions on August 11, 2020. At the time of submission, chapter 3 is in the final stages of edits for submission to Applied and Environmental Microbiology for peer review.

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List of Abbreviations

Abbreviation	Name
AAC	Animal Care Committee
AAFC	Agriculture and Agri-Food Canada
ANCOM	Analysis of the Composition of Microbes
ASV	Amplicon Sequence Variant
BactBileEsc	Bacteroides Bile Esculin agar
BBE+Gent	Bacteroides Bile Esculin agar + 100mg/L Gentamicin
CA	Columbia Agar
CB	Columbia Broth
CBA	Columbia Blood Agar
CBAG	Columbia Blood Agar with 100mg/L Gentamycin
CO_2	Carbon Dioxide
CTAB	Cetyl trimethylammonium bromide
CTC	Chlortetracycline
DP	Direct Plating
Enrich	Enrichment
EtOH	Ethanol
FMT	Fecal Microbial Transplant
GALT	Gut Associated Lymphoid Tissue
Gent	Gentamycin
HDP	Host Defence Peptides
IFN-y	Interferon gamma
IL	Interleukin
LCU	Livestock Containment Unit
LeRDC	Lethbridge Research and Development Centre
MA	Mackonkeys Agar
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization
MMA	Minimal Media with Agarose
MRS	De Man, Rogosa and Sharpe agar
N_2	Nitrogen
RDP	Ribosomal Database Project
SCFA	Short Chain Fatty Acid
SNP	Single Nucleotide Polymorphism
ST	*Salmonella enterica* var Typhimurium
TGFb	Transforming growth factor beta
TNFa	Tumor necrosis factor (TNF)-alpha
Tyn	Tyndallization
VFA	Volatile Fatty Acid

Chapter 1 Literature Review

1.1 Introduction – The microbiome of humans, and livestock

The term 'microbiota' is defined as communities of microorganisms within a distinct environment. The term was first coined to describe bacteria that colonized the human body during periods of wellness and illness (Lederberg and McCray 2001). In contrast, the microbiome, is defined as the collection of microorganisms (bacteria, archaea, viruses, and fungi) within a defined habitat and also includes their genomes and surrounding abiotic environment (Marchesi and Ravel 2015). The interactions between members of the microbiota and their host are important as they provide an understanding of the ensuing relationships that exist between the microorganism and their host. These relationships can be beneficial, neutral, or detrimental to either the host or resident (autochthonous) bacteria, and therefore it is important to correctly define and characterize these interactions. As an example, interactions where one organism benefits and the other is unaffected is defined as commensalism. This is not to be mistaken with mutualism, where two organisms gain benefits from their relationship. Similarly, when two organisms benefit from their physical interaction, this relationship is considered a symbiotic relationship. Finally, interactions where one organism benefits and the other is adversely affected is defined as parasitism (Atlas and Bartha 1993). Relationships between the microbiota and the host are dynamic and can change over time. As such it is important to understand these changes since they can affect not only the microbial communities but the health of the host as well. As a result, gathering information from both culture-dependent and culture-independent methods are needed to fully understand and accurately evaluate the functional relationship between the microbiota and host. The information gathered will also assist in learning how these relationships affect gut health and in examining the role of these relationships in the induction of enteric disease.

The microbiota of humans and other animals varies greatly in the number, diversity, and composition of organisms. In fact, changes to the diversity of bacteria can be found within similar animals of the same species on the same diets, and consequently, finding a link between the microbiota and the factors that cause variations in community structure can be complex. For example, in beef cattle, inter-animal variations of the intestinal microbial communities have been identified between similar animals. This observation highlights how these differences in bacterial populations exist within animals that are the same breed, age, fed an identical diet, and raised in the same geographical location (Durso et al. 2010). In addition to cattle, the genetic backgrounds of different poultry breeds have been evaluated, and substantive variations in the intestinal microbiota of different breeds have similarly been observed. For example, in one study evaluating the microbiota in chicken ceca, marked differences in

enteric bacterial populations were detected in different poultry breeds. Specifically, high numbers of *Bacteroides* spp., were present in Tibetan and free-range birds. In contrast, the ceca of Ross 308 broiler birds had fewer numbers of *Bacteroides* spp., with higher numbers of *Lachnospiraceae* spp (Zou et al. 2018).

In addition to these studies, investigations have focused on identifying a 'core microbiota' present within the intestine of animals. In swine, meta-analyses of fecal samples were evaluated from different research projects from various locations around the world. Through this work, these sequenced samples were evaluated, and a core intestinal microbiota was observed within sequenced material. The core microbiota of pigs contains species of *Alloprevotella*, *Clostridium*, *Prevotella*, *Ruminococcus* and the RC9 gut group bacteria. Notably, these bacterial genera were found in 99% of all fecal samples evaluated through this meta-analysis, suggesting that there may indeed be a global core intestinal microbiota in swine (Holman et al. 2017).

The specific and often unique relationship that individual bacterial species have with hosts is critical for both the intestinal health of the host and viability of the microbiota within the host. In humans and animals, complex carbohydrates represent an abundant energy source, obtained by fermentation and metabolism of indigestible complex carbohydrates (Livesey 1991). Within humans and animals complex carbohydrate hydrolysis is almost exclusively accomplished by the enteric microbiota of the host. Indeed, these enteric bacteria have developed numerous metabolic mechanisms directed at carbohydrate metabolism (Salyers 1979). To illustrate this point, the human genome contains genetic sequences for 80 glycoside hydrolase (GH) proteins; however, only 17 of these glycoside hydrolase proteins are associated with the metabolism of complex carbohydrates contained in nutritional foods. These GHs are involved with the breakdown of carbohydrates through the addition of water groups into glycoside linkages between the monosaccharides that comprise the complex carbohydrates (Lehninger 1985). In contrast, the bacteria within the human enteric microbiota contain over 9,120 glycoside hydrolase genes directed at the hydrolysis of complex polysaccharides, with members of the *Bacteroides* spp., containing 3,976 of these genes. Moreover, without the presence of the intestinal microbiota, humans would be limited to the metabolism of a select number of carbohydrates, namely: sucrose, lactose and starch. This inability to digest complex carbohydrates would impact the production of fermentation by-products, such as volatile short chain fatty acids (SCFAs; to be discussed later); and thereby, impacting host intestinal health (Kaoutari et al. 2013).

Although many metabolic pathways accessed by the microbiota are important for metabolism of carbohydrates, the interactions between members of the microbiota and the host are not always

beneficial. For instance, *Lactobacillus* spp., can have both positive and negative affects on bacteria within the microbiota contained in the ceca of chickens and as such this relationship can vary between mutualism, commensalism and rarely parasitism. Specifically, the presence of *Lactobacillus* spp. in samples was positively correlated with the presence of bacteria from the *Bacteroides* genera and *Christensenellaceae* family, whereas the presence of *Lactobacillus* spp. was inversely correlated with the presence of *Ruminococcaceae* and *Lachnospiraceae* taxa. These interactions can play an important role in host health as members of *Ruminococcaceae* and *Lachnospiraceae* families are commonly associated with the production of SCFAs required for host health (Zou et al. 2018). The interactions between the host and its autochthonous organisms are more commonly associated with mutualism. A good example of mutualism observed between the host and its microbiota can be seen between the host and various bacteria of the *Bacteroidetes* spp. These commensal bacteria metabolize indigestible complex carbohydrates, such as homogalacturonan, as an energy source and in turn produce fermentation by-products, such as SCFAs (ex. butyrate), that can be used by the host (Chen et al. 2017; Luis et al. 2018). Additionally, these bacteria can compete with pathogens for environmental and localized anatomical habitats, such as the colonic crypts; potentially preventing the occurrence of disease within the host (Lee et al. 2013). In contrast, opportunistic pathogenic microorganisms such as several strains of *Salmonella enterica*, are capable of inducing marked interstitial inflammation and injury, a process that may ultimately lead to host death. Diseases associated with gastro-intestinal tract (GIT) infections, such as those induced by *S. enterica* are important causes of illness in livestock and can substantively diminish livestock performance (Moon et al. 1999). Therefore, it is important to accurately identify bacterial populations within the microbiota of host species that are associated with colonizing specific tissues as well as determining the best methods for isolating these bacteria. In this way, specific bacteria that have important roles in microbiota of chickens, and pigs can be identified.

1.2 Intestine physiology, an overview

The gastrointestinal tract (GIT) of animals acts as a barrier between the host and its environment. The GIT also has the important function in the absorption of nutrients required for growth and maintenance of the host. The absorption of nutrients and excretion of waste is accomplished through the ingestion, digestion, absorption, and egestion of nutritive components within diets (Jennings and Premanandan 2017). Although the same general processes occur in all animals, the anatomic structure of the alimentary tract between animal groups (avian vs mammal species) varies; possibly due to adaptations needed for mechanical digestion, absorption, fermentation, and excretion of foodstuffs. There are many anatomical similarities in the GIT of mammals such as mice, pigs and humans, whereas

the GIT of chickens has several structural differences as compared to mammals (Figure 1.1). Similarities that exist within these animals include the presence of an oral cavity and digestive organs, the presence of a cecum (ceca in chickens) as well as the presence of a small and large intestinal tract.

1.2.1 Oral cavity and stomach

Mammals, such as mice, pigs and humans, have an oral cavity that is important for the mastication of food. The oral cavity is connected to the stomach by the esophagus, a tubular structure that is lined by protective non-keratinized squamous epithelium. This epithelial layer is modestly resistant to abrasion caused by the movement of ingesta within the esophagus. The stomach is composed of a glandular and non-glandular region. The glandular area contains parietal cells that are involved in the secretion of hydrochloric acid (pH <2) and chief cells that secrete pepsinogen into the lumen of the stomach. The acidity of the stomach is important in both the hydrolysis of food stuffs and as an important chemical barrier to reduce pathogenic infections within the gut. Although the low pH of this region provides a barrier to pathogens, some microorganisms can survive within the stomach of mammals such as pigs. For instance, bacteria such as *Lactobacillus* spp., *Mitsuokella* spp., *Selenomonas ruminatinum*, and *Megasphera elsdenii* are common colonizers of the stomach in pigs and can on occasion induce injury (Mikkelsen et al. 2007).

In contrast, chickens have evolved a slightly different mechanism for digestion of feed within the diet. The crop, which is a dilation of the esophagus and located at the opening of the body cavity, functions as a storage compartment for foods and helps in the pre-digestion of poorly digestible feed in birds (Jennings and Premandandan 2019). The stomach in chickens is separated into two parts, the proventriculus and ventriculus. The proventriculus, which is distal to the crop, functions to soften food. The proventriculus contains numerous proventricular glands and these glands contain secretory cells, which produce pepsinogen and secrete hydrochloric acid and goblet cells that produce mucin. The ventriculus, also known as the gizzard, is the muscular part of the stomach and is important in mechanical breakdown of food. The mucosa is covered by koilin, a solid keratin like protein matrix that protects the stomach mucosa from ulceration and further aids in mechanical disruption of foodstuffs. In chickens, the pH of the gizzard is variable, however it is commonly more basic than the proventriculus ranging between 4-5 pH. Although the presence of these three organs (crop, proventriculus, and gizzard) differ from mammals they provide the same desired functions; the digestion and mastication of ingested feeds. A variety of microorganisms colonize the oral cavity and stomach of birds. In particular, organisms such as *L. salivarius* are commonly observed within this area and represent the dominant organisms

colonizing the crop. In contrast, *L. aviaries* can be found in all areas of the oral cavity and stomach of birds, but have not been identified within the crop (Gong et al. 2007).

1.2.2 The small intestine

The small intestine of mammals is a long structure that is partitioned into three anatomical segments: duodenum, jejunum and ileum. The small intestine is the principle site for food digestion and absorption and the small intestine is lined by villi covered with cuboidal and columnar epithelium; cells that readily absorb nutrients. The epithelia layer is also covered by the brush border. The mucosa, in addition to enterocytes, is also populated by specialized cells that have various function, namely: mucin production (goblet cells), secretion of proteins and enzymes required in nutrient digestion (enterocytes, crypt cells), regulation of the enteroendocrine system (enteroendocrine cells) and immunosurveillance and antigen uptake (M-cells, antigen presenting cells). Underneath the epithelium is the lamina propria. The lamina propria area is filled with capillaries and lacteals and importantly populated with cells, such as lymphocytes, macrophages, dendritic cells, and granulocytes involved in innate and adaptive immune function. The lymphocytes form discrete, well circumscribed aggregates called Peyer's patches. The lamina propria overlays the submucosa, and within the duodenum the submucosa contains Brunner's glands, which are specialized structures with zymogen and mucin secreting cells. Finally, the small intestine is surrounded by circular and longitudinal muscle of the muscularis externa and this smooth muscle propels digesta distally towards the large intestine. The intestinal microbiota of mammals is also present within the small intestine, but the numbers and diversity of bacteria are markedly reduced as compared to the large intestine. Most certainly there are populations of *Clostridia* spp., *Bacilli* spp., and *Proteobacteria* spp., bacteria within the duodenum, jejunum, and ileum, however the numbers of *Clostridia* spp., markedly increases distally towards the ileum and cecum (De Rodas et al. 2018). To assist with the digestion and absorption of fats within the intestinal tract bile salts and acids are produced in the liver and stored in the gall bladder. These primary bile acids and salts are eventually released into the lumen of the small intestine from the gall bladder. The bile salts can affect the community structure of microbiota (to be discussed later).

The small intestine of chickens is similar to mammals with one exception. The jejunum and ileum of chickens are separated by the Meckel's diverticulum, an appendage formed *in ovo.* Notably, this separation between the jejunum and ileum has no distinctive morphological features between the segment of small intestine-which is similar in mammals (Jacob 2012a; König et al. 2016). There are variations in microbiota of bacterial population within the small intestine in chickens. For example, members of the *Lactobacillus* spp., which may be important for maintaining intestinal physiological and

immunological homeostasis, dominate the upper small intestine while decreasing in numbers distally towards the ceca. In addition, members of the *Arthromitus* spp. also dominate the jejunum and ileum of birds (Gong et al. 2007).

1.2.3 The cecum

The cecum is a 'blind pouch' juxtaposed to the ileocecal value. The mucosa of the cecum has elongated glands that are lined by simple columnar epithelium and goblet cells and the numbers of goblet cells are greater within the cecum than within the small intestine. Similarly, the lamina propria has more Peyer's patches than the upper small intestine. The teniae coli are three thick segments of the muscularis extrema that surround the cecum and are important in moving contents towards the colon. There are greater numbers of bacteria, specifically members of the *Clostridia* spp., within the cecum as compared to the small intestine where it is hypothesized that the cecum 'seeds' the large intestine with bacteria important for the fermentation of indigestible carbohydrates (Brown et al. 2017; De Rodas et al. 2018). Microbial fermentation produces SCFAs, such as butyrate, which are essential to host health and are utilized by the host enterocytes for energy (Jiminez et al. 2017). Previous research using cecectomized mice demonstrated that that SCFA production was significantly reduced in these mice as compared to controls. It was suggested that in cecectomized mice there was a reduction of *Ruminococcacea* and *Lachnospiraceae* families of bacteria and these bacteria can ferment different complex carbohydrates to produce SCFAs (Brown et al. 2017).

In contrast to mammals, chickens have two ceca, that communicate directly with the rectum at the ostium caeci. It has been suggested that this difference in location of their ceca may reduce the ability of chickens to absorb nutrients (Jacob 2012a). The histological structures and functions of the chicken ceca are similar to the mammals, and this includes fermentation of polysaccharides. The bacteria that colonize the ceca in chickens vary by breed and diet. Notably, members of the *Bacteroidetes* spp., are present in most chicken breeds, with Tibetan and free-range birds having these organisms represent between 40 and 20% of their cecal microbiota, respectively. Interestingly, some breeds such as the ROSS 308 have relatively low numbers of *Bacteroidetes* spp., within the cecal microbiota, while in contrast *Bacteroidetes* spp. can represent 5% of the bacterial populations of different ROSS birds such as the ROSS 708 breed (Bortoluzzi et al. 2017; Zou et al. 2018).

1.2.4 The large intestine

The large intestines of mammals have three primary functions: resorption of water and electrolytes; formation, storage, and movement of waste; and the production of fermentation by-products such as vitamins and SCFAs. The anatomic segments and therefore the nomenclature of the

large intestine varies between species. As examples, the large intestine in people is comprised of the ascending, traverse, descending and sigmoid colon (Ross and Pawlina 2011). In contrast, the colon of pigs is partitioned into the ascending, transverse (spiral colon), and the descending colon (Sisson et al. 1975). The structure of the large intestines of mammals is similar to the cecum. In particular, the mucosa contains columnar epithelium, a lamina propria with numerous populations of lymphoid cells and *teniae coli*. The colonic microbiota of mammals has the greatest number and the highest diversity of bacteria within the GIT. For example in swine; the numbers of *Clostridia* spp. and *Bacteroidetes* spp. are greatest in numbers within the colon and the bacterial load increases distally along the colonic tract. (De Rodas et al. 2018). The large intestine of the chickens contains the ceca and rectum, with the rectum connecting with the cloaca. The large intestine of birds has many structures similar to the large intestine of mammals (König et al. 2016).

1.2.5 Short-chain fatty acids (SCFAs) within the GIT

SCFAs are a diverse class of organic molecules including acetic acid, butyric acid, propionic acid, valeric acid and other variations of these small molecules (Cummings and Branch 1986). Within the lower GIT of animals, the fermentation of complex carbohydrates results in the generation in SCFAs (Henningsson et al. 2001). The concentration of SCFAs produced by the fermentation of dietary fibre varies but approaches approximately 100 mM within the cecum and proximal colon, while decreasing in concentration moving towards the distal colon (Hamer et al. 2008). Of these organic molecules, acetate is produced at the highest concentration following microbial fermentation and acetate may account for nearly of 80% of the total SCFAs (Wong et al. 2006). Other SCFAs produced during microbial fermentation include propionate and butyrate, which can represent 20-30% and 10-20% of the total SCFAs produced within the small and large colons of humans respectively (Bergman 1990; Knudsen 2015).

A primary function of SCFAs is providing energy for enterocytes, particularly colonocytes. Similarities exists within the microbiota of many animals where colonocytes have adapted to utilizing their metabolic by-products as energy sources (Nagpal et al. 2018). A secondary function of SCFAs is signal transduction within the GIT of animals. For instance, the production of the antibacterial peptide LL-37 in mice is modulated by the utilization of SCFAs in enterocytes (Schauber et al. 2003). Collectively, these observations describe a beneficial interaction between the host and the production of SCFAs by the microbiota. Indeed, the ability of the microbiota to ferment non-digestible dietary ingredients plays a critical role in the intestinal health of animals.

1.3 *Salmonella* and interactions within the GIT

Host illnesses, such as chronic inflammation, auto-immune disease, cancer, and subsequent therapy with long-term antibiotic administration can markedly alter the composition of the intestinal microbiome (Carding et al. 2015). During periods of some illnesses increases in oxygen concentration and temperature can be observed within the inflamed tissues of the GIT (Lih-Brody et al. 1996; Kruidenier and Verspaget 2002). In turn, these changes to the intestinal environment can result in adverse changes to the microbial community structure thereby producing what is referred to as dysbiosis. Dysbiosis is described as an imbalance between the numbers of putative 'beneficial' or 'protective' bacteria species and the numbers of 'harmful' bacteria in the intestine (Tamboli et al. 2004). During periods of dysbiosis, an overgrowth of 'harmful' or pathogenic bacteria can also be observed. These increased numbers of pathogenic bacteria can then lead to increased occurrences in intestinal injury and subsequent host disease. One pathogenic bacterium associated with overgrowth during periods of dysbiosis is *Salmonella enterica*. Importantly, these bacteria have been associated with dysbiotic events and intestinal disease in people, pigs and chickens (Nurmi and Rantala 1973; van der Wolf et al. 1999; Gordon 2008). Moreover, understanding the mechanisms by which this organism can induce disease within the GIT of these animals is of critical importance for developing mitigation strategies to reduce disease.

Many different serovars, and phage-types of *Salmonella enterica* exist and these different serovars can colonize the intestine and infect mammals and poultry species. Common *Salmonella* serovars that infect livestock include *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (Simpson et al. 2018). Interestingly, some phage types, such *S. enterica* serovar Typhimurium phage type DT 104 are capable of colonizing the intestines of humans, chickens, pigs, and cattle, and these phage types are considered important pathogens for causing zoonotic disease in people (Duijkeren et al. 2002). *Salmonella* sp. are also pathogens that can colonize the GIT without establishing active infections or generating tissue injury (Sadeyen et al. 2004). However, modifications in bacterial populations within the gut, or during period of intestinal inflammation, can lead to the reduction of lactate. This reduction in lactate enables Salmonella to out-compete other autochthonous bacteria and colonize habitats within the gut and by this means generating localized intestinal and systemic infections (Gillis et al. 2018).

Intestinal Salmonellosis affecting pigs is characterized by either acute septicemia or chronic enterocolitis. This disease can present as necrotizing tissue inflammation within the mucosa and deeper submucosa of the ileum, cecum, and colon (Wilcock et al. 1976). One mechanism by which *Salmonella* sp. can exacerbate dysbiosis leading to chronic intestinal inflammation is by using tetrathionate as an

alternative electron acceptor within inflamed microenvironments. Specifically, during an intestinal infection with *Salmonella* bacteria, the host increases the production of oxygen free radicals within mucosa and the bacteria produces thiosulfate. These two products combine to form tetrathionate, a redox compound that binds electron enhancing energy production and metabolic activity of the bacteria. This mechanism provides a competitive advantage for the bacteria improving their ability to colonize the gut (Behnsen et al. 2015).

In addition, *Salmonella's* genome contains various genetic elements that encode for proteins that enhance the ability of the bacteria to induce tissue injury during periods of infection. As an example, when *Salmonella* is grown in the presence of chlortetracycline (CTC), a potential antibiotic growth promoter (defined as an antibiotic provided to animals in-feed to promote growth or enhance feed efficiency), *Salmonella enterica* is able to selectively colonize the tonsils of pigs (Brown, et al. 2017; Holman et al. 2019). The mechanism involved in tonsillar colonization remains unclear. However, it is speculated that the expression of a group of proteins referred to as *Salmonella* pathogenicity islands (ex. SPI-3, SPI-4, and SPI-5) are upregulated following exposure to CTC. It is also suggested that expression of *mgtC*, a membrane bound protein required for survival within macrophages, is also responsible for this colonization strategy during CTC therapy (Holman et al. 2018b; Choi et al. 2019). It is quite possible that these SPIs and the associated *mgtC* expressed protein are elevated during antimicrobial therapy and are responsible for the ability of pathogen to outcompete the commensal bacteria, and for these reasons selectively colonize the tonsils during periods of infection. Since *Salmonella* sp. infections can impact host intestinal health and the microbiota, further studies examining the pathogenesis of disease and the effects of *Salmonella* on growth and colonization of specific bacterial populations within the gut are warranted.

1.4 Microbiological analyses: culture-dependent and -independent methods

Understanding interactions between the host and their microbiota is a crucial consideration in microbiological research, with important implications for human and animal health and livestock performance. Currently, there is a paucity of information on the genotypes of bacteria isolated from the microbiota of healthy and diseased human and animal intestines. Until recently the evaluation of the microbiota's composition required the isolation of bacteria; however, methods such as metagenome-assembled bacterial genomes are allowing researchers to evaluate genomes without the need for isolating bacteria from samples. Such studies have been conducted in chickens examining changes to intestinal bacterial diversity following modifications to diets supplemented with fish meal. In this example, 469 draft (*in silico* aligned) genomes from chickens were evaluated (Glendinning et al. 2019). In

this investigation, metagenome-assembled bacterial genomes required deep sequencing of samples to cluster (or bin) related DNA sequences to create many different putative genomes that can be used to identify and evaluate the diversity of individual bacterial genomes within a sample (Stewart et al. 2019). Although useful, this method can be cost prohibitive. As such, the evaluation of genomes from isolated bacteria is advantageous since it both reduces costs and isolates organisms that can be cultivated and used in future *in vivo* studies. The demand for identifying novel bacterial isolates and their respective genomes has created a paradigm shift in microbiology. Research is being redirected toward developing new strategies to both isolate and identify the taxa, species, and genera of these newly isolated bacteria. These new strategies are utilized within culturomics (Seng et al. 2009; Lagier et al. 2012). Culturomics includes the culturing and archiving of live bacteria to study changes to bacterial genomes between species as well as the expression of proteins that are involved in function, such as metabolism, growth, and colonization (Lagier et al. 2012). By employing an assortment of isolation methods culturomics has been effective at isolating diverse populations of bacterial species. Using culturomics, variations in the diversity of bacteria within a sample can be evaluated with more in-depth evaluations as compared to next-generation sequencing methods. This enables more accurate measurements of bacterial strains and species within a sample. Specifically, the longer 16S rRNA gene sequences evaluated during traditional Sanger sequencing or through comparisons (SNP or *in silico*) to their genomes can assist in further speciating the isolated bacteria. Evaluated genomes can be further assessed by determining variations in single nucleotide polymorphisms (SNPs) or the presence of genomic material that express bateriocins, which are important proteins involved in antimicrobial resistance (van Heel et al. 2013; Jia et al. 2017; Zou et al. 2019).

Determining the diversity of bacteria found within collected samples (i.e. fecal, tissue, etc.) is an essential consideration in microbiota research. In particular, modifications to the host's microbiota, and corresponding changes to the metabolic activities of intestinal bacteria, can impact the function of the intestinal microbiota and the host's health. Several specific functions of the microbiota include the fermentation of indigestible carbohydrates, the production of SCFAs, and the colonization of various locations with the gut. These functional tasks are defined as a 'niche' that an organism can occupy within a specific environment or 'habitat' (Atlas and Bartha 1993). As many of the existing microorganisms have not previously been cultured, other methods are required to identify these currently unculturable microorganisms. Interestingly, research shows that the majority of bacteria within the human intestinal tract have previously been cultured. Indeed, approximately 45-97% of all bacteria within the gut were identified by culture dependent techniques. In contrast, only 22-87% of

bacteria within the intestinal microbiota of non-human hosts have been identified by culture dependent methods (Lloyd et al. 2018). Moreover, culture dependant methods for isolating bacteria species within these non-human animal species can be challenging. As an example, studies have examined potential use for culturomics methods to isolate bacteria collected from rumen fluid. For instance, Zehavi et al (2018) showed that a majority of the bacteria within rumen fluid could be cultured with traditional microbiological techniques. However, the isolation of bacterial cultures as 'pure' cultures remains difficult (Zehavi et al. 2018). Therefore, determining alternative methods in which unculturable bacteria can be isolated, grown in pure culture, and evaluated *in vitro* is needed. Expanding culturomics would enable a better understanding of the functional dynamics and interactions between different bacterial populations within the intestinal microbiota.

Methods for the isolation of anaerobic bacteria were pioneered by Dr. Robert Hungate (Hungate 1957; Chung and Bryant 1997). Importantly, prior to the development of this technique, the isolation of anaerobic bacteria in pure culture was not possible. To enable this, the researcher developed a bacterial isolation method using CO_2 gas to displace oxygen within media contained within sealed glass tubes. In this isolation technique, bacteria from samples can be isolated using 'roll tubes'. To accomplish this, bacteria are diluted in thin layers of reduced agar within glass tubes, incubated, and then pure bacterial cultures can be established (Clark 2019). This method was later improved by using an anaerobic chamber that enabled researchers to isolate bacteria using traditional direct plating methods within anaerobic environments (Aranki and Freter 1972). More recently, better isolation methods have been developed by using modified enrichment strategies to enhance bacterial growth. Such enrichment methods include, growing bacteria cultures for long periods (<8 weeks), using *in situ* isolation techniques, and growing bacteria in different media supplemented with nutrient rich products such as whole blood or rumen fluid (Seng et al. 2009; Lagier et al. 2012; Berdy et al. 2017).

Another advancement that greatly improved the isolation of uncultured bacteria is the use of the Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) analytical instrumentation. This technology can characterize and catalogue bacteria by measuring the composition of proteins and fatty acids within a bacterium (Lay 2001). Through this cataloguing method, many previously uncultured bacteria have been identified (Fykse et al. 2015). In addition, newer microbiological methods have been used to enrich single bacteria into growth of pure cultures. Examples of this include the Ichip, which isolates bacteria using an *in situ* isolation method that enables bacteria to grow within their natural environments. In this method, bacterial are individually segregated within wells of the Ichip apparatus and are able to utilize and ferment microbial by-products to support the growth of fastidious organisms.

These microbial by-products can be essential for bacteria growth and not present in media using traditional media preparations. Using the Ichip, many previously uncultured bacteria have been isolated (Berdy, et al. 2017). Indeed, the use of new technologies to isolate previously unculturable bacteria will enhance our knowledge of microorganisms that are present within the environment and populate important habitats, such as the mammalian intestine. In addition, improving our understanding of these unculturable bacteria will allow researchers to determine the effects of products, such as microbiota produced metabolites, within diets on the gut microbiota and host intestinal health.

1.5 Complex carbohydrates and metabolism

The digestion of complex carbohydrates is an important physiological process since they represent the primary energy source within the human diet (Jéquier 1994). The structures of carbohydrates are diverse and can vary from single ringed (monosaccharide) sugar units to highly complex multi-branched polysaccharides. The position and extent of chemical modification and branches, as well as the linkages that exist between monosaccharides can yield significant changes to digestibility and chemical properties of the monosaccharides (Lehninger 1985). Complex carbohydrates are defined as carbohydrates that either contain more than one monosaccharide isomer or contain more than one different glyosidic linkage between the individual monosaccharide units (ex. α1-6 or β1-4 glycosidic linkages) (Yamagaki et al. 1998).

Interestingly, although carbohydrates are ubiquitous within the environment, the digestibility of carbohydrates by bacteria within the microbiota is variable. Different bacterial species express different enzymes needed to dismantle complex carbohydrate substrates. For instance, members of *Bacteroides* spp. and *Prevotella* spp. express a suite of carbohydrate active enzymes (CAZymes) that are encoded within polysaccharide utilization loci (PULs) (Martens et al. 2011; Accetto and Avguštin 2015). PULs are defined as starch utilization system (SUS)-like clusters of genes that encode all enzymes for the catabolism and metabolism of a specific indigestible carbohydrate (ex. Homogalacturonan) (Martens et al. 2009; Luis et al. 2018). CAZyme producing bacteria are generally found in large numbers within the colons of both humans and animals and these bacteria function to hydrolyze complex polysaccharides into smaller saccharide units (Looft et al. 2012).

The quantity of CAZymes expressed by bacterial populations within the microbiota varies greatly along the distal GIT. For example, the presence of CAZymes, such as GH2 (one family of β-galactosidases) are found in greater numbers within the cecum compared to the ileum of swine (Yang et al. 2016). Specifically, bacteria such as *Prevotella* spp. and *Bacteroides* spp., which are found in greater numbers in the large intestine and may be associated with the increased concentration of GH2 enzymes

within this location. The vast metabolic potential of some bacteria has resulted from the incorporation of genomic DNA sequences from other non-terrestrial bacteria that metabolize rare carbohydrates, such as seaweed cell wall polysaccharides (i.e. porphyran). These structural polysaccharides are not present within terrestrial plants. The transfer of metabolic pathways is believed to have resulted from horizontal gene transfer from marine bacteria to gut bacteria in humans consuming seaweed (Hehemann, 2010).

The expression of enzymes within PULs that digest seaweed cell wall polysaccharides has been well studied (Kabisch et al. 2014; Neumann et al. 2015). For example, the incorporation of the Ag-PUL into the *B. uniformis* NP1 genome enables the bacteria to completely saccharify agarose, a galactan from red algae. Specific genes required to hydrolyze the polysaccharide contained within the Ag-PUL include: endo-acting agarases. including three GH16s and a GH86, required for the indiscriminate cleavage of β1-4 linkages in agarose to produce neoagarooligosaccharides of varying lengths (Figure 1.2) (Pluvinage et al. 2018).

In some cases PUL function has been linked to a 'selfish' mechanism of glycan foraging (Reintjes et al. 2019). In this mechanism, surface enzymes hydrolyse carbohydrates into smaller oligosaccharides. However, these products are rapidly transported into the periplasm of the bacteria for further depolymerization and metabolism, which reduces the amount of carbohydrates products available to other organisms in the community. In contrast, Gram-positive organisms depolymerize polysaccharides extracellularly, which presents more product loss to the community (Mattéotti et al. 2012). One particular example of selfish metabolism occurs in the Gram-negative bacterium *B. thetaiotaomicron* (*B. theta*) which is able to metabolize carbohydrates, such as yeast mannan, in progressive stages. To accomplish this, the large and structurally complex products generated at the cell surface are gradually imported into the periplasm where the majority of scarification occurs. In this way, liberated mannooligosaccahrides and mannose products are unavailable to other bacteria, providing *B. theta* with a competitive advance for colonization within a specific environment (Cuskin et al. 2015).

Gram positive bacteria, such as the *Clostridium* spp. and *Ruminococcus* spp., can degrade complex carbohydrates through different mechanisms including cellulosomes. Briefly, cellulosomes are multi-enzyme structures produce by anaerobic bacteria that are involved in the hydrolysis of recalcitrant polysaccharide plant cell walls. The higher order structure of cellulosomes is mediated by high affinity, high specificity protein interactions between dockerins and cohesions. This multi-CAZyme system, attaches cellulases and hemicellulases in proximity to the bacterial outer cell wall via a scaffold, enabling glycoside hydrolases with diverse specificities to hydrolyze the complex polysaccharide matrix of the

plant cell wall. The magnitude of such structures is evident as they form megadalton sized complexes that can be observed via transmission electron microscopy (Bayer et al, 1998).

1.5.1 Methods used to identify bacteria able to metabolize specific carbohydrates

The identification of bacteria able to degrade specific carbohydrates is of great importance to the fields of microbiology, agriculture, and healthcare. Understanding various methods used by bacteria to hydrolyze polysaccharides can lead to developments in targeted drug delivery systems (e.g. polysaccharide enriched nanoparticles), improved animal nutrition, and the development of potential treatments for enteric disease (Liu et al. 2008). In the context of agriculture, the identification of bacteria that hydrolyze indigestible carbohydrates can also increase the variety of carbohydrates livestock metabolize as an energy source (Han 1978; Haruta et al. 2002). Haruta *et al*., (2002) demonstrated that stable ruminal microbial communities could be generated containing bacteria with specific hydrolase capabilities. This modified ruminal bacterial community was capable of effectively digesting rice straw for energy production. More specifically, this microbial community was stable and maintained its community structure when sub-cultured within ruminal contents in either the presence or absence of rice straw. In another study, synthetic mixed cultures were also evaluated to determine the roles of non-cellulolytic bacteria in the metabolism of cellulose by *Clostridium straminisolvens* CSK1 for biofuels. In this investigation, the presence of aerobic and non-cellulose degrading bacteria was unable to provide the GH enzymes required for cellulose fermentation. However, these organisms were able to rapidly produce an anaerobic environment within media which is a requirement for cellulolysis. Moreover, co-administration of non-cellulolytic bacteria with *Clostridium straminisolvens* CSK1 greatly increased the activity of cellulolysis within the environment (Kato et al. 2004). Collectively, these studies show that determining communities of bacteria that occupy specialized nutrient niches is important for understanding bacteria – host interactions. Unfortunately, the identification of bacteria capable of hydrolyzing specific carbohydrates can be challenging and taxonomic recovery is highly effected by the method of identification.

Many bacteria that colonize the GIT co-colonize with other autochthonous bacteria by having specialized enzymes systems that metabolize unique complex carbohydrates. In this way, multiple bacteria coexist through differential fermentation of energy sources (Martens et al. 2009). In general, the accurate identification of bacteria able to degrade specific carbohydrates has been variable and identification of bacterial populations has been based on genotypic and phenotypic assessments. Phenotypic analyses for the identification of bacterial that metabolize specific carbohydrates is generally completed with culture media using single carbohydrates (i.e. minimal medium) or with analytic

methods using specific instrumentation (Larsbrink et al. 2014, Hartemink et al. 1996). Using hydrolysis of xyloglucan as an example, phenotypic analysis for determining the ability of the bacteria able to grow on xyloglucan can be performed either by using a minimal culture medium containing xyloglucan as the sole carbon source or by instrumentation methods, such as High-Performance Liquid Chromatography with a Pulsed Amperometric Detector, to measure by-products of xyloglucan metabolism (Hartemink et al. 1996). In contrast, the use of minimal medium culture analysis can identify common colonic anaerobic bacteria such as *B. uniformis* and *B. ovatus* that degrade xyloglucan. In this assay system, bacteria are grown within a media containing xyloglucan as a sole carbon source. The presence of growth suggests the bacteria can metabolize the carbohydrate for survival (Varel and Bryant 1974). For example, Larsbrink et al. (2014) evaluated a collection of *Bacteroidetes* spp., for the ability to metabolize xyloglucan using minimal media. In this investigation, various organisms were grown in minimal medium pure culture with xyloglucan as a sole carbon source. Indeed, various methods exist for the identification of bacteria able to metabolize specific carbohydrates and the preferential identification method is often determined by resources and technical expertise.

Conversely, instrumental analyses can evaluate bacteria within complex media, thus increasing the numbers of bacteria that can be evaluated. As an example, analytic instruments, such as the HPLC-PAD, are commonly used to determine the amount of xyloglucan hydrolysis by bacteria. This analysis can differentiate between bacteria capable of either complete or partial hydrolysis of xyloglucan (Hartemink et al. 1996). Also, the amount of xyloglucan oligomers produced can be quantified providing researchers with a more accurate assessment of bacterial metabolic activity and gives insight into the overall metabolic activities of complex bacterial communities. This method has been successfully used to determine the diversity of *Bacteroidetes* spp., *Actinobacteria* spp., and *Firmicutes* spp. within bacteria isolated from human fecal samples. At present xyloglucan is known to be initially hydrolyzed by bacteria using GH5 (sub-family 4) and GH74 enzymes expressed by different anaerobic bacteria, including *B. ovatus* and *R. flavefaciens* respectively. Importantly, the difference between the bacteria expressing enzymes with similar function (i.e. hydrolyze glycosidic linkages), underscores the need to develop methods that correctly identify xyloglucanase activity within different bacteria that have different growth and culture requirements.

Other methods, such as colourimetric methods, can also be used to identify the by-products of bacterial metabolism of specific carbohydrates. Colourimetric methods have been applied through the linking of dyes to the carbohydrates. For example, remazol brilliant blue chemically linked to xylan can identify bacteria expressing xylanolytic enzymes (ex. GH11). These enzymes degrade the carbohydrate

releasing the dye and resulting in 'clear zones' on culture plates thereby demonstrating carbohydrate hydrolysis (Sachslehner et al. 1998). A major advantage of this colourimetric method is that this method is non-destructive to the bacteria and identified bacteria can be rapidly cultured.

The congo red colourimetric assay can be used to evaluate the metabolism of xylan and cellulose. Mechanistically, congo red docks onto crystalline polymers of polysaccharides within the β-1,4-glycosidic linkages found in polysaccharides such as xylan and cellulose. Following lysis of the carbohydrate-dye linkage, the dye then disassociates from the carbohydrate generating a zone of clearing within the culture (Teather and Wood 1982; Woodcock et al. 1995, Samanta et al. 2011). The congo red colourimetric method has been used to identify a variety of different xylanolytic organisms, including: *Caldocellum saccharolyticum*, *Ruminococcus* spp., *Pseudobacteroides* spp., *B. cellulosolvens*, and the fungi *Aspergillus clavatus* (Teather and Wood 1982; Lüthi et al. 1990; Squina et al. 2009). However, one major disadvantage is that congo red is a destructive assay since it requires the flooding of the plates with dye. Therefore, to identify xylanolytic bacteria, duplicate plates are required for further bacterial isolation, thus increasing the labour associated with isolating and culturing bacteria with specific CAzy functions.

Although these phenotypic methods are useful in evaluating the specific enzyme activity of microbiota bacteria on a carbohydrate metabolism, there is a growing body of work determining the genotypes of sequenced bacteria which express hydrolytic enzymes that degrade specific carbohydrates. Moreover, genotypic evaluations of carbohydrate hydrolysis can be further advantageous as they reduce the need for laborious evaluations of carbohydrate activity *in vivo*.

1.5.2 Genotypic methods to identify bacteria able to utilize specific carbohydrates

Genotypic evaluations of individual bacteria or a bacterial communities' ability to hydrolyze complex carbohydrates can be assessed with various software programs. As an example, assessing the capability of diverse bacterial communities to hydrolyze polysaccharides can be determined with the PICRUSt program. This software program uses a database containing previously published information to predict the metabolic function of identified operational taxonomic units obtained from next generation sequencing of bacterial genomic DNA. In this program, the hypothetical metabolic activity of a microbiota sample can be estimated according to the relative abundance of genera observed (Langille et al. 2013). A limitation of the PICRUSt program is the inability to determine the metabolic activity of individual bacteria within a large bacterial community.

In contrast to the PICRUSt program, metagenomic data can be evaluated for predicted metabolic pathways, including the potential presence of carbohydrate active enzymes. An example of this type of

analysis is the SACCHARIS program (Jones et al. 2018). This program compares open reading frames within the genome to protein sequences characterized within the dbCAN database and thus predicts the function of specific carbohydrate hydrolytic enzymes (Yin et al. 2012). One advantage of the SACCHARIS program is that it can better predict the activity of glycoside hydrolases (GHs) within genome, by aligning these enzyme sequences to similar characterized proteins. The SACCHARIS program can evaluate the ecological similarity of specific proteins and identify putative characterized and non-characterized functional proteins (i.e. proteins that have not been previously studied to confirm structure function relationships) (Jones et al. 2018). Determining this information can be vital to understanding the repertoire of carbohydrate active enzymes that could be expressed by to specific bacteria, as well as activity of these enzymes within the host.

1.6 Microbial functions within the intestinal tract

The utilization of specific carbohydrates by microorganisms of the microbiota enables bacteria to compete for niches and colonize habitats within localized mucosal areas or distinct regions along the GIT. Commensal bacteria are needed to maintain a stable microbiota, ensure a healthy gut, reduce the incidence of intestinal dysbiosis and prevent enteric inflammation. A robust intestinal microbiome is maintained by bacterial communities that form stable community structures Bäckhed et al. 2012). A healthy microbiome within the GIT also benefits the host by assisting in providing a quiescent and homeostatic immunological environment within the gut (Jobin 2014). Most importantly, a healthy microbiome is frequently associated with the reduced incidence and severity of disease (Hollister et al. 2014).

The benefits of a healthy gut microbiota can often have extra-intestinal effects. For example, germ free mice challenged with the respiratory pathogen *Streptococcus pneumonia* are susceptible to developing severe pneumonia. However, the occurrence of disease is reduced in germ free mice administered a balanced microbiota from fecal microbial transplant (FMT). Extra-intestinal effects associated with this FMT include altered tumor necrosis factors α and interleukin-10 cytokine profiles as well as enhanced phagocytic activity of LPS and LTA sensitized macrophages (Schuijt et al. 2016). On the other hand, although commensal bacteria are generally beneficial to the host, on occasion they can be associated with the induction of acute and chronic disease. For example, *Streptococcus gallolyticus*, a common enteric microorganism in humans and pigs, has been associated with the induction of colon cancer, and it has been suggested that the production of bacteriocins by the microbiota reduces the numbers of commensal organisms in inflamed colons. It is postulated that a reduced bacterial population can predispose the gut to the development of precancerous lesions (Boleij and Tjalsma

2013). It is also well established that breaches in the mucosa and translocation of bacteria to extra-intestinal tissue can lead to acute septicemia and death (Allan et al. 1995). Interestingly, microorganisms have also been associated with the development of slowly progressing chronic diseases. As an example, *Enterococcus gallinarum* following mild intestinal injury, can induce autoimmunity and hypersensitivities, such as inflammatory bowel disease in genetically susceptible hosts (Fine et al. 2019).

As mentioned above, commensal bacteria are important microorganisms required to maintain a stable bacterial community structure within the intestinal microbiome. One method in which these commensal bacteria help establish a stable bacterial community is by promoting 'colonization resistance' within the GIT, a process that limits the growth of enteric pathogens and non-resident allochthonous bacteria. Colonization resistance occurs when commensal bacteria colonize specific environmental habitats by 'out competing' pathogens for nutrients, growth factors and localized mucosal space within the intestinal microenvironment (Lozupone, 2012). An example, of colonization resistance metabolism is the subsequent biotransformation of primary bile acids by the microbiota into secondary bile acids (Ajouz, et al. 2014). Briefly, bile acids are the water-soluble end products of cholesterol metabolism. Primary bile acids (e.g. cholic acid and chenodeoxycholic acid) are amphipathic compounds synthesized from cholesterol in the liver (Staels and Fonsece 2009). The compounds are important in the absorption of lipids and fat-soluble vitamins and involved in glucose metabolism and homeostasis. Notably, primary bile acids are secreted in bile and can be deconjugated and dehydroxylated by bacteria (such as *Ruminococcus* spp., and *Lacnospiraceae* spp., in the distal GIT) into secondary bile acids. These secondary bile acids increase the diversity of bile acids within of the total bile acid pool and are important in the enterohepatic circulation of bile acids (Mertens, et al. 2017). Secondary bile acids can also inhibit the growth of enteric pathogens such as *C. difficile*, which is unable to effectively colonize the distal colon in the presence of secondary bile acids (Theriot et al. 2014). This may be exacerbated by prolonged antimicrobial administration which creates disruption in the intestinal microbiota community structure causing dysbiosis and loss of specific commensal bacteria, resulting in a corresponding reduction in the amount of secondary bile acids within the gut. The loss of colonization resistance, resulting from such a reduction in secondary bile acids, would enable enteric pathogens to flourish with the gut, causing intestinal injury and disease.

The colonization of different bacteria species in different locations within the GIT is dependent on the diverse and often specialized metabolic functions of the individual bacteria. These specialized functions which exist within a distinct environment are referred to as a 'niche' and can vary substantively (Atlas and Bartha 1993). These functions include fermentation of polysaccharides with

different chemical compositions as well as the production of different quantities and types of SCFAs. In addition, a niche may also refer to an organism's ability to grow in different areas of the mucosa, such as the tightly or loosely adherent mucous layers, villi, and deep crypts (Shroeder, 2019). Many of the niches that exist within the GIT are required to ensure good intestinal and host health. For instance, as mentioned earlier, the fermentation of indigestible carbohydrates by enteric bacteria (e.g. *Eubacterium rectale*), provides the primary energy source (i.e. butyrate) for colonocytes (Purwani, et al 2012; Nagpal et al. 2018). Perturbation in the intestinal microbiota and loss of bacteria that produce butyrate following carbohydrate fermentation, will exacerbate intestinal diseases such as ulcerative colitis and pseudomembranous colitis (Shin et al. 2016). Interestingly, it was shown that rectal administration of therapeutic concentrations of butyrate attenuated the incidence of colitis in humans and it's believed that butyrate restored barrier function by improving energy metabolism within colonocytes (Fachi et al. 2019). As such, the occupation of the microbiota to fulfill niches relevant to the health of the host is essential for the generation of a healthy microbiota.

Redundancy of functions and niches utilized by bacteria in the microbiota exists since many bacteria have similar and overlapping cellular functions within the intestinal ecosystem. Although this redundancy leads to competition between bacteria, it also prevents the complete loss of an essential niche if a specific bacterial species is eliminated from the GIT. Therefore, overlapping niches are common and essential for homeostasis within the GIT and include the use of similar carbohydrates by commensal bacteria. Colonization is often based on the commensal bacteria's ability to ferment certain polysaccharides. For example, *Bacteroides* spp., *Clostridium* spp., and *Ruminococcus* spp., are capable of hydrolyzing xyloglucan as a carbon source by expressing enzymes such as the GH5 or GH74 family of enzymes that have been shown to hydrolyze glycosidic linkages within this polysaccharide. Notably these bacteria employ different strategies to ensure that hydrolases can access the carbohydrate substrate. These bacteria can link the enzyme to the outer cell wall, contain the enzyme within bacteria cellulosome (also attached to the cell wall) or release the enzymes into the external environment (Bayer et al. 1998). Each method is an example of adaptations by bacteria that assist in their ability to occupy niches. Furthermore, these adaptations also ensure the microbiota have a diverse array of methods to access and digest carbohydrates, such as xyloglucan, a necessary requirement for the sustainability of a healthy microbiota in the digestive system of herbivorous or omnivorous animals (Hartemink et al. 1996; Larsbrink et al. 2014; Ravachol et al. 2016).

A primary location for pathogenic bacteria invasion and colonization of the colon is within the colonic crypts because they are populated with proliferating germinative epithelial cells (Zaborin et al.

2017). The immaturity of these cells enables the pathogens to translocate the epithelium into the underlying lamina propria causing mucosal injury. Examples of bacteria that colonize crypts and translocate the epithelium include: *V. cholera* which establishes microcolonies within the intestine and *Lawsonia intracellularis,* which alters the Notch and WNt/β catenin signalling pathway for cellular proliferation in order to translocate the colonic mucosa via the intestinal crypt (Millet et al. 2014; Bengtsson et al. 2015; Huan et al. 2017). Although pathogens can invade the host through the crypts, commensal bacteria can colonize crypts without inciting tissue injury. For instance, certain *Bacteroides* spp., such as *B. fragilis and* Btheta, can colonize the crypts of mice. *B. fragilis* was the first *Bacteroides* spp. to demonstrate the expression of proteins known as commensal colonization factors. These proteins have different enzymatic activities required for epithelial colonization, including the hydrolysis of specific carbohydrates associated with the epithelium, such as chitobiose. These observations suggest the bacteria requires a set of enzymes that digest complex polysaccharides needed to colonize colonic crypts. In addition, these genes are preferentially upregulated when bacteria are located on the apical surface of the crypts, further supporting the hypothesis that these enzymes are required for colonization of crypts (Lee et al. 2013). In addition, although *Bacteroides fragilis* is an obligate anaerobe, the bacteria can grow in a nanoaerophilic environment, an environment that has small amounts of oxygen (<1%). This oxygen tolerance is advantageous for organisms that grow at the interface of the lumen and epithelium as this environment contains oxygen that dissociates from the nearby tissues (Baughn and Malamy 2004). These characteristics of *Bacteroides* spp., allow the microbe to compete with pathogens and colonize the crypt regions therefore providing both protective and beneficial effects for the host.

As described above, bacteria can occupy niches selected by both the nutrients (e.g. expression of CAZymes for carbohydrate digestion) present within the microenvironment and anatomical location (e.g. colonic crypts). On occasion, these niches can work synergistically to assist the colonization of a habitat by bacteria. As an example, bacteria expressing enzymes that hydrolyze rare and indigestible polysaccharides enables these bacteria to more effectively colonize the intestine-nutritional niche hypothesis (Pereira and Berry 2017). *Bacteroides* spp., commonly reside in the distal GIT of terrestrial mammals, and in general, these bacteria (i.e. Btheta) are rarely exposed to marine polysaccharides, such as porphyran, which are present in the cell wall of red algae seaweed. Therefore, these bacteria lack the enzymes required to digest this complex marine carbohydrates (Hehemann et al. 2012). In contrast, on rare occasions, bacteria such as *B. plebeius* can inherit specialized enzyme systems that effectively digest the carbohydrate porphyran isolated from red algae (Hehemann, et al. 2010). To test this nutritional niche hypothesis, CAZymes that hydrolyze porphyran were engineered into Btheta. In this investigation,

mice were orally administered wild type and engineered Btheta and fed diets supplemented with either inulin or porphyran enriched seaweed. In mice treated with Btheta in the presence of inulin, tissue samples demonstrated that Btheta was outcompeted by the autochthonous microbiota and failed to colonize the colon. Conversely, mice provided inoculums of engineered Btheta supplemented with porphyran enriched seaweed demonstrated robust bacterial growth within the intestine and the colonic crypts while other bacterial population were unable to colonize these areas (Shepherd et al. 2018). In summary, the metabolism of rare or complex carbohydrates by commensal bacteria expressing unique collection of enzymes is an important strategy to provides bacteria with a selective advantage for colonization of habitats using nutrient niches within the GIT.

1.7 Methods to study Microbiome-Inflammation interactions

When evaluating the physiological and functional processes between the microbiota and the host, different methods can be used to determine these specific interactions between bacterial populations and the host. Understanding the interplay between the microbiota and host often requires the use of animal models to determine intestinal and extra-intestinal biochemical, cellular, and physiological mechanisms involving bacterial communities and host. As examples, mice have been used to determine the biochemical interactions associated with high fat diets on the bacterial communities within the microbiota of obese and diabetic rodents (Cani et al. 2008). In addition, mice have been used to study the effects of *Salmonella* infection on mucosal phagocytic cell activity (Kaiser et al. 2012). Moreover, mice have been used to evaluate the impacts of intestinal physiological changes that are associated with the production of fermentation by-products (e.g. SCFAs). To accomplish this, mouse diets were supplemented with dietary fibre in the form of wheat bran or resistant starch, and their ability to ameliorate pathogen associated inflammation was evaluated. Through studies such as this it has been demonstrated that fermentation by-products such as SCFAs resulting from dietary fibre consumption promoted the amelioration of inflammation, possibly through the downregulation of pro-inflammatory cytokines (ex. TH1/Th17) or the increased expression of response genes, such as *RegIIIγ* (Jiminez et al. 2016). Finally, researchers have used mice models to examine extra-intestinal effects of the microbiota on the host. Foster et al (2017) has shown that the changes in intestinal bacterial populations alters central nervous system function and secretion of neurotransmitters within the brain. This work suggested that the microbiota can influence brain function through gut-brain axis (Foster et al. 2017). Although the use of animals to study the impacts of the intestinal microbiota on host physiology and intestinal disease is an excellent scientific model, the use of animals for research is often labour intensive, costly, and can face public opposition. As a result, other research alternatives, incorporating

both *in vitro* and *ex vivo* designs can assist with examining the effects of the microbiota on bacterial-host interactions within the intestine.

Historically, researchers have used various methods to evaluate the ability of bacterial populations to metabolize different nutrients. For instance, individual bacterial species within the microbiota have been evaluated using pure bacterial cultures to determine their ability to metabolize carbohydrates such as xyloglucan. However, it is often more effective to evaluate the bacterial communities as a whole using systems such as chemostats. Chemostats, are bioreactors and are the primary 'steady state' system use in microbiology to assess energy requirements for growth of either individual bacterial populations or entire bacterial communities (Guedon et al, 1999). Other methods capable of evaluating bacterial community compositions have been developed as well. Methods such as the RUSITEC (a rumen model) and mini-bioreactor arrays are artificial systems believed to be more representative of the microbial composition of these environments as compared to growth in chemostats; enabling research to evaluate more specific changes to the gut microbiota.

The RUSITEC system is an *ex vivo* model that evaluates the composition of ruminal bacterial communities. Although the rumen is not part of the distal intestine, this system was instrumental in providing fundamental information needed to develop other artificial intestinal systems. The RUSITEC is often used to evaluate the digestibility of cattle feeds and methane gas production within the rumen. This system operates by mechanically compressing (i.e. mimicking mastication) ruminal dietary content and exposing these contents to pressurized mixtures of anaerobic gases. This system has been useful in determining the effects of dietary nutrients on bacterial populations within the rumen microbiota and methane production (Lins et al. 2019).

The development of another *ex vivo* model being used to study the microbiota occurred when specific engineered strains of bacteria were evaluated for their ability to effectively colonize ruminal contents. In this study Btheta was engineered to express enzymes that hydrolyze glyosidic linkages within xylan and chondroitin sulfate. The engineered bacteria were able to successfully compete for nutrients in a bioreactor composed of rumen fluid and enriched with either xylan (a commonly digested polysaccharide by the rumen microbiota) or chondroitin sulfate (a rarely encountered sulfated carbohydrate). Interestingly, regardless of the CAZyme expressed these bacteria were rapidly displaced by the microbiota that were better established in the environment (Cotta et al. 1997).

Additionally, mini-bioreactor arrays (MBRAs) are an *ex vivo* system that were specifically designed to provide an accurate assessment of bacterial population within culture samples, and importantly provide a system that is inexpensive and reduces the need for large sample volumes. Mini-bioreactor

arrays are small (12.5ml) bioreactors that continuously replenish media and are able to generate a representative community of a host's microbiota. In this way MBRAs can be used to evaluate the impact of diet and therapies on bacteria within the microbiota from selected hosts. Notably, this method allows researchers to maintain a representative community of the original host bacterial community structure while using a small sample size to generate representative bacterial communities. At present, this method has been primarily used to measure changes in the intestinal microbiota of both healthy individuals and people with intestinal disease. This system was used to examine the microbiota in humans following prolonged antibiotic treatments, and patients with fecal transplants (Auchtung et al. 2015). Moreover, a modified version of the MBRA was designed at the LeRDC to evaluate microbiota of chickens (Figure 1.3). Specifically, this modified MBRA was used to determine the changes to the chicken cecal bacterial populations when fed either canola meal or non-starch polysaccharides isolated from the meal. In this study, bacteria were identified that express hydrolytic enzymes that effectively digested carbohydrates from canola meal (Low et al; paper submitted September 2020)). In addition, and also with our research group, the MBRAs have also evaluated changes to communities of bacteria isolated from select anatomical sections of the intestinal tract of pigs. This presently unpublished work aims to validate the use of MBRAs to generate stable communities *ex vivo* from different regions of the GIT.

1.8 Conclusions

The relationships between the microbiota and the host are diverse and complex. Consequently, methods used to evaluate these relationships vary depending on the information required, hypotheses being tested, technical expertise, and infrastructure of the research laboratories. Although different bacterial communities within the intestinal microbiota are capable of metabolizing carbohydrates as energy sources, only a select few members of these communities are well studied. Previously, research into identifying and understanding bacterial fermentation of dietary fibers was largely restricted to bacteria that were easily grown and evaluated within the laboratory. For instance, an extensive amount of knowledge is known about the fermentation profiles of *Bacteroides* spp. Interestingly, the major reason for their selection as candidate organisms for extensive evaluations (By Dr. Abigail Salyers, a founding contributor to the field of enteric microbiology) was that they were easy to grow and manipulate within a lab setting (Sonnenburg and Sonnenburg 2016 p. 26). In contrast the intricately interconnected community structure of the intestinal microbiota functions as a consortium to facilitate to complete utilization of many complex metabolic functions (i.e. niches) while colonizing a variety of enteric locations (i.e. habitats). The specific niches that individual bacteria species occupy within specific habitats includes their ability to ferment carbohydrates into short-chain fatty acids, and their ability to

metabolize specific sugars enabling them to colonize various habitats within the intestinal tract. The interactions of bacteria within specific habitats of the intestinal tract is of particular importance as it leads to the maintenance of a healthy and balanced community structure, a requirement for good intestinal health. Indeed, disruption of a balanced enteric community can lead to dysbiosis, enteric inflammation, and on occasion, death of the host. During periods of infection, bacteria such as *Salmonella enterica*, can colonize enteric habitats leading to increased numbers of pathogens within the intestinal tract and eventually leading to enteric disease. As the animal recovers from the intestinal injury, different bacterial populations are likely involved with clearance of the pathogen and resolution of the intestinal infection. These autochthonous bacteria, which often engage in mutualistic or commensalistic relationships with the host, can colonize intestinal habitats and compete for specific niches utilized by enteric pathogens (i.e. colonization resistance) thus reducing pathogen growth.

The identification of such bacterial species could be applied to improve animal health as these organisms could potentially lead to the generation of treatments to reduce pathogen growth and enteric inflammation. Employing this strategy, bacteria that colonize similar locations as enteric pathogens within the gut can lead to the generation of 'inflammation-tailored therapeutics'. These bacteria could effectively compete with pathogens during the induction and progression of an intestinal infection and potentially reduce tissue injury and improve recovery following enteric inflammation. In addition, these organisms could be co-administered with select carbohydrates (nutrient source) with engineered bacteria to deliver therapeutic agents to reduce enteric inflammation; within their native habitat (i.e. inflamed intestinal tissues). As such, a primary goal of this research was to identify the best isolation strategies to collect diverse communities of bacteria from chickens and pigs, as well as identifying bacteria populations that could effectively colonize inflamed intestinal tissues within monogastric animals. Through these strategies it may be possible to identify bacteria that specifically colonize intestinal habitats, as well as occupy unique intestinal niches that can metabolize dietary carbohydrates, such as xyloglucan.

1.9 Hypotheses

The literature cited in this chapter clearly demonstrates a need to evaluate the function of bacteria within the intestinal tract of animals and identify members of the microbiota that can effectively colonize specific enteric habitats and occupy defined niches within the intestinal tract of animals. The interactions of bacteria within specific habitats are of particular importance during periods of inflammation. Of interest is the possibility that specific populations and communities of bacteria may be able to out-compete specific pathogens by effectively colonizing specific microenvironments (i.e.

inflamed vs non-inflamed mucosa) within the gut. In this way, specific organisms could be used as 'inflammation-tailored therapeutics' to compete with pathogenic microbes in regions of intestinal inflammation. The goal of my PhD research project was to: 1) evaluate the discrete differences that are observed in traditional isolation strategies (direct plating and enrichment techniques), 2) further evaluate advantages and disadvantages of these traditional techniques as well as more modern isolation strategies (Ichip and endospore selection methods); and, 3) isolate and identify a vast array of bacteria that colonize inflamed tissues within monogastric animals that can be used as inflammation targeted bacteria.

The hypotheses to be evaluated in this thesis are:

1) The isolation of diverse populations of enteric bacteria using various microbiological techniques is required to increase the diversity of bacteria harvested from media culture collections.
2) Traditional microbiological techniques such as direct plating and media enrichment of samples produces less diverse communities of cultured bacteria as compared to *in situ* methods of isolation of bacteria.
3) Variation in the numbers and diversity of bacteria will be observed between different anatomical regions of the intestine and changes in these bacterial populations are influenced by intestinal inflammation.

1.10 Figures

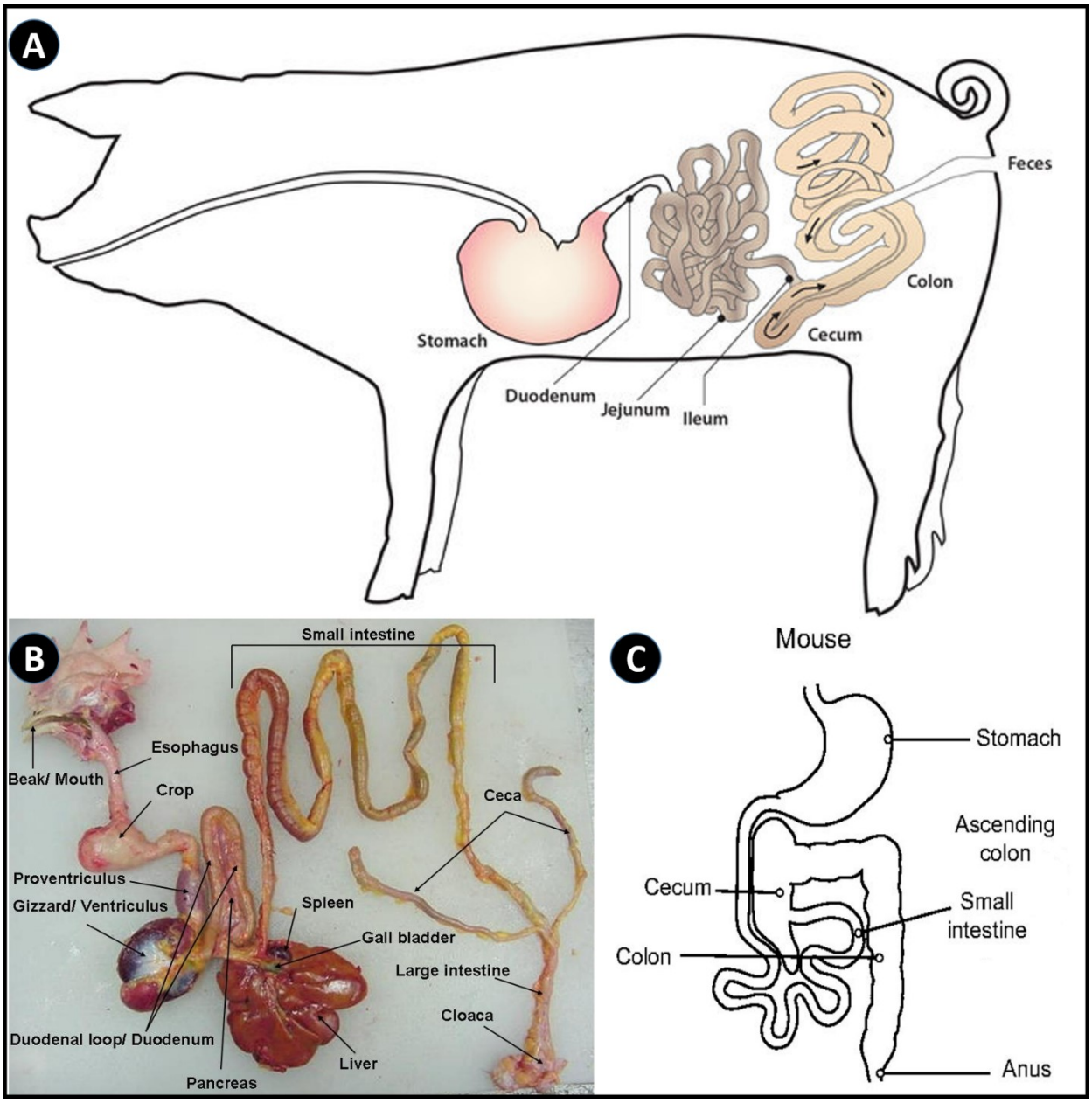

Figure 1.1 Illustrations of the intestinal tracts of: A) pigs (Holman et al. 2017); B) Chickens (Jacob 2012b); and C) mice (Nguyen et al. 2015).

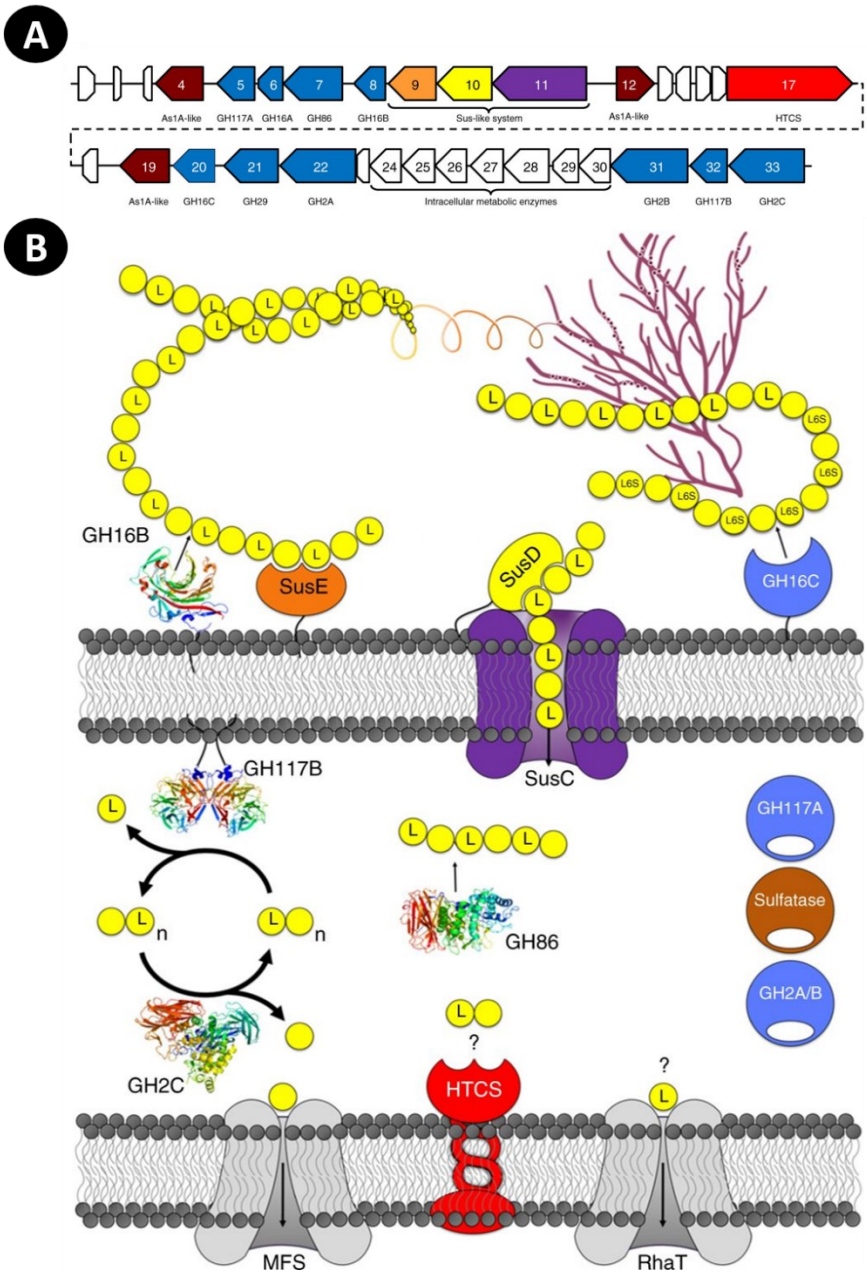

Figure 1.2 A) Schematic representation of the Ag-PUL present in *Bacteroides uniformis* NP1. Genes are sequentially positioned demonstrating their orientation within the Ag-PUL genome. Colors represent enzymes and enzyme systems; brown = sulphatases, blue = GHs, orange = SusE-like, yellow = SusD-like, purple = SusC-like, red = response regulator, and white = hypothetical proteins (not-currently classified) B) Illustration of the agarolytic pathway as outlined by genes expressed from the Ag-PUL (Pluvinage et al. 2018).

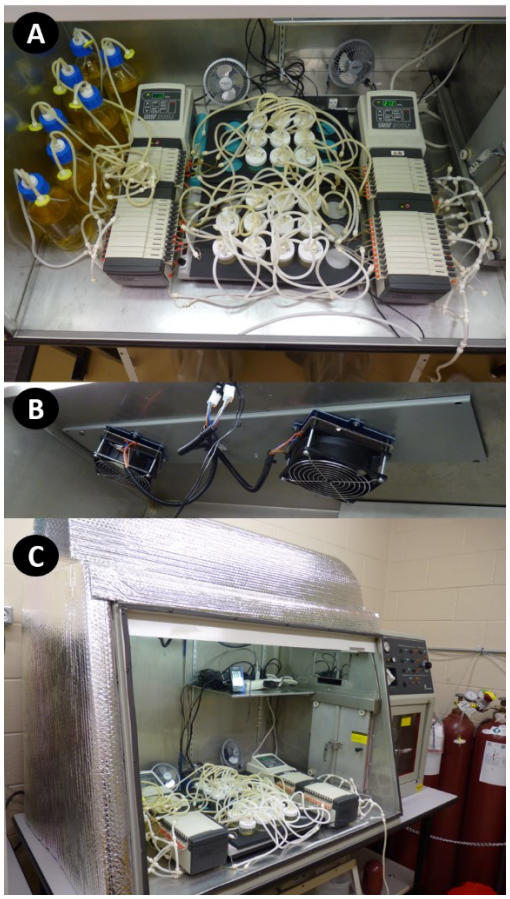

Figure 1.3 Images of the artificial intestine based on the mini bioreactor arrays Britton lab (Auchtung et al. 2015) and used at the Lethbridge Research and Development Centre for research in swine and poultry studies. A) Components of the artificial intestine bioreactor used for isolating bacteria. B) Location of heated elements used to maintain specific temperatures for bioreactor activity within the artificial intestine. C) Presence of an insulated membrane around the chamber.

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Chapter 2 Application of culturomics to characterize diverse anaerobic bacteria from the gastrointestinal tract of broiler chickens in relation to environmental reservoirs[1]

2.1 Introduction

Characterization of the microbiota is an increasing focus in the study of animal welfare and animal production (Clavijo and Flórez 2018). The majority of research studying the microbiota in poultry has focused on culture-independent analyses of samples obtained from the gastrointestinal tract (GIT), predominantly the cecum. For example, bird density was shown to affect the prevalence of bacterial operational taxonomic units associated with decreases in specific butyrate producers in the intestine, such as *Clostridium* spp., which convert lactic acid into butyric acid (Kridtayopas *et al*. 2019). In addition, when studying specific members of the microbiota of poultry it has been found that members of the Lactobacillus spp. elicit equal numbers of positive and negative interactions within the GIT of chickens (Zou *et al*. 2018). A focus of our research group is to identify ways to enhance the richness of comprehensive culture-dependent evaluations of bacterial diversity in chickens to not only support the culture-independent results but also to provide bacteria for future whole genome and functional analyses.

Many of the bacteria within the GIT of human beings and livestock are identifiable *in situ* using DNA-based methods but are exceptionally difficult to isolate due to their sensitivity to oxygen, fastidious nutrition, and slow rates of growth (Lloyd et al. 2018). Although limited effort has been expended on the comprehensive recovery of anaerobic bacteria from chickens, the recognized need to obtain bacteria to elucidate their function toward enhancing bird health (e.g., alternatives to antibiotics) has generated a renewed interest in culturomics (Lagier et al. 2016; Ferrario et al. 2017). The foundation for culturomics in chickens lies in past work, largely by Salanitro et al. (1974). In their work, bacteria within the ceca of cockerels (White Cornish cockerel × White Rock hens) were isolated using roll tubes; the isolated bacteria included members of the genera *Bacteroides*, *Propionibacterium*, *Eubacterium*, and *Clostridium*. More recently, culturomic approaches have been applied to avian breeds to elucidate the impacts of diet and stressors on the GIT microbiota (Park et al. 2017; Zou et al. 2018). To realize the goal of generating a collection of GIT bacteria from chickens that are representative of the GIT microbiota, it is important to develop and evaluate anaerobic isolation strategies.

Traditional anaerobic isolation strategies primarily used media prepared in reduced carbon dioxide atmospheres, as outlined in the Hungate method (Hungate 1957; Salanitro et al. 1974). Recent efforts

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have adopted nitrogen atmospheres, as media preparation and buffering requirements are less rigorous (Salanitro et al. 1974; Browne et al. 2016). Although both atmospheres are effective, the gaseous environment of the large intestinal lumen differs from that of the isolation atmospheres used; the luminal atmosphere consists of N_2 (64% ± 21%), CO_2 (14.0% ± 7.0%), CH_4 (8.8% ± 9.0%), H_2 (19.0% ± 9.9%), and O_2 (0.69% ± 0.49%) (Levitt 1971). Using a nitrogen predominant atmosphere, enrichment methods using complex media, often containing blood or rumen fluid, and incubated for 24 hours or longer, have been successfully employed (Lagier et al. 2012). Direct-plating strategies using a variety of media have also been successfully used (Ferrario et al. 2017). Both isolation strategies possess advantages as well as inherent weaknesses, and it is important to better understand their utility for culturomic studies of the chicken microbiota.

Characterizing the microbiota of the chicken GIT is an area of increasing interest in agricultural research; however, the vast majority of efforts have focused on culture-independent methods to characterize bacterial communities. Although the application of culture-independent methods is effective at cataloguing communities, the technologies currently available suffer from low taxonomic resolution and the inability to logistically elucidate bacterial genomics and function. An additional issue encountered is the high variability of bacterial communities within the developing bird. In a recent study, the diversity of bacterial communities was evaluated across animal trials (Stanley et al. 2013). They observed that even when birds were raised under similar conditions and on the same diet, a large degree of variation was observed within the cecal microbiota. In another study using culture-independent analysis, a succession of bacteria was observed in the fecal material of developing chicks, including a shift in the community structure from one dominated by class *Alphaproteobacteria* to one dominated by classes *Bacteroidia* and *Clostridia* (Jurburg et al. 2019). These studies were able to ascertain limited information on activity. The application of comprehensive anaerobic isolation methods is necessary to obtain representative bacteria isolates for ancillary evaluations to ascertain function. The primary objective of the current study was to evaluate different direct-plating and long-term enrichment strategies in nitrogen and carbon dioxide atmospheres for their ability to recover representative and diverse anaerobic bacteria from the GIT of chickens housed in a controlled production setting. In addition, culturable bacteria within the production environment were compared with those in the GIT.

2.2 Materials and methods

2.2.1 Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care guidelines. Before commencement of the research, the project was reviewed

and approved by the Agriculture and Agri-Food Canada (AAFC) Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol Review 1622). Animals and husbandry Ross 308 broiler chickens were used in this study. Chicks (1-day-old) were obtained from a local hatchery and were reared in a controlled production environment. In this regard, chicks were housed in two stainless steel pens (2.23 m^2 per pen with 10 birds per pen for a stocking density of 0.22 m^2 per bird). Birds were provided free access to a nonmedicated starter diet (Hi-Pro Feeds, Lethbridge, Alberta) and water. To establish a microbiota representative of a production setting, chicks were exposed to fresh litter that was obtained from a commercial broiler barn. Ambient room temperature was maintained at 22 ± 2 °C and birds were provided free access to brooders for the first 20 days. Throughout the rearing period, birds were subjected to a 12 h light: 12 h dark photoperiod. At 31 days of age, five arbitrarily selected birds from two different cages were selected, anesthetized with isoflurane, and humanely euthanized under anesthesia by cervical dislocation.

2.2.2 Sample collection and processing

Within 5 min of euthanization, a laparotomy was preformed, the GIT was exposed, and the crop, jejunum, ileum, and one arbitrarily selected cecum were ligated using zip ties sterilized by autoclaving. The segments were excised from the GIT adjacent to the ties thereby ensuring no infiltration of air into the intestine. The segments were placed in sterile urine containers, immediately placed on ice, and then transferred to the laboratory complex. The containers were placed into a Thermo Forma anaerobic chamber (ThermoFisher Scientific Inc., Waltham, Massachusetts) containing a 85% N_2 : 10% CO_2 : 5% H_2 atmosphere (i.e., within 60 min of bird euthanization) and lids were removed. In addition, environmental samples of poultry litter and drinking water were collected in sterile urine containers and processed similarly to intestinal samples. Within the anaerobic chamber, the samples in containers were maintained on frozen icepacks for 30 min to allow de-gassing. Each GIT segment was then removed from the container and longitudinally opened with a sterile scissor. A 1 cm^2 piece of tissue with residual digesta was excised and transferred to 5 ml of reduced Columbia broth (CB; Difco, Frankin Lakes, New Jersey) in a 15 ml culture tube with a screw-top cap. Each sample was gently agitated in CB for 30 s to remove nonadherent digesta (i.e., containing luminal bacteria). The washed tissue was then carefully transferred to another 5 ml aliquot of CB to isolate mucosa-associated bacteria. Both tubes were then vortexed for 30 s (high setting). The poultry litter and drinking water samples were processed by diluting the sample 10 times in reduced CB (m/v and v/v, respectively), and a sterile 10 µL loop was used to streak the diluted samples onto desired media.

2.2.3 Anaerobic media preparation

All liquid anaerobic media were prepared by autoclaving at 121 °C for 5 min to heat and reduce the media, after which time the media were transferred into an anaerobic chamber charged with either a 85% N_2 : 10% CO_2 : 5% H_2 atmosphere or a 90% CO_2 : 10% H_2 atmosphere, and allowed to cool. When cool, media were agitated to displace excess oxygen, and 1 g/L L-cysteine monohydrochloride (Sigma–Aldrich) was added as an indicator of oxygen status. Media to be used in the 90% CO_2 atmosphere were buffered with 40 ml of 8% (m/v) sodium carbonate (Sigma–Aldrich), and pH was confirmed using pH paper (pH Hydrion Vivid 6-8, Micro Essential Laboratory Inc., Brooklyn, New York) before use. Media were then autoclaved for 30 min at 121 °C at 105 kPa, and pH was confirmed. To visualize that the media remained reduced, resazurin (1 µg/ml; Sigma–Aldrich) was added before autoclaving. An endospore viability test using *Geobacillus stearothermophilus* was used to ensure the efficacy of autoclaving.

Solid media used for this study included Columbia agar (HiMedia Laboratories PVT Ltd., Mumbai, India) supplemented with 10% sheep's blood (CBA); Columbia agar supplemented with 10% sheep's blood and 100 g/ml gentamicin (CBAG); de Man–Rogosa–Sharpe agar (MRS; Oxoid Ltd., Hampshire, UK); and minimal medium supplemented with agarose (Agarose I, VWR International, LLC, Radnor, Pennsylvania) (MMA) as done previously (Pluvinage et al. 2018). MMA consisted of 8.5 mmol/L NH_4SO_4, 9.4 mmol/L Na2CO3, 4.1 mmol/L L-cysteine free base, 100 mmol/L KH_2PO_4 (pH 7.2), 1.4 µmol/L $FeSO_4$·7 H2O, 5 ng/ml vitamin B_{12}, 1 µg/ml vitamin K_3, 15.4 mmol/L NaCl, 0.24 mmol/L $CaCl_2$, 98 µmol/L $MgCl_2$·$6H_2O$, 50 µmol/L $MnCl_2$·$4H_2O$, 42 µmol/L $CoCl_2$·$6H_2O$, and 1 µg/ml resazurin (Pluvinage et al. 2018). Liquid media used in this study included a modified Dehority's medium (recipe in Table 2.1) supplemented with 0.5% xylan (Sigma–Aldrich) or 0.5% porcine mucus III (M-2378, Sigma–Aldrich, Burlington, Massachusetts) (Lin et al. 1999); and CB with 10% sheep's blood. Sheep's blood was obtained from animals maintained at the AAFC LeRDC (Approved Animal Use Protocol ACC 1631).

2.2.4 Bacterial isolation

Enrichment cultures were created by transferring 20 µL of sample into 10 ml of sterile liquid media contained within a 15 ml screw-cap tube within an anaerobic chamber containing a 85% N_2 : 10% CO_2 : 5% H_2 atmosphere. This small volume (20 µL) was designed to provide small numbers of bacteria for enrichment to produce variation among enrichment vials. Tubes were then sealed and incubated outside of the chamber at 37 °C for 12 weeks (maintenance of the reduced status was confirmed visually during the incubation period via the presence of the resazurin indicator within the medium). This long enrichment period was chosen to ensure the fast-growing organisms were supplanted by bacteria able

to grow on indigestible carbohydrates and metabolites contained within the media. To isolate bacteria from the enrichment cultures, tubes were transferred into an anaerobic chamber containing a nitrogen or carbon dioxide atmosphere, opened, vortexed for 15 s, and contents were streaked onto agar media in 100-mmdiameter Petri dishes using a 10 µL transfer loop (Simport, Beloil, Quebec). Cultures were incubated at 37 °C for 7 days, and biomass from individual colonies was streaked for purity on CBA in 60-mm-diameter Petri dishes, and the process was repeated to ensure a pure culture was isolated. Glycerol stocks were created by scraping biomass from the surface of CBA and suspending the biomass in reduced CB containing 40% glycerol. Stocks were stored at –80 °C until needed.

2.2.5 Bacterial identification

Bacteria were rejuvenated from glycerol stocks on CBA at 37 °C in the atmospheres in which they were originally isolated until sufficient biomass was produced. Biomass was scraped from the agar surface, placed in CB, and sedimented by centrifugation (13,200g for 10 min). DNA was extracted from bacterial biomass in CTAB extraction buffer (100 ml of 1 mol/L Tris–HCl, pH 8.0; 50 ml of 0.5 mol/L EDTA, pH 8.0; 73 g of NaCl; 20 g of CTAB (hexadecyltrimethylammonium bromide); and diluted to 1 L with Optima water, ThermoFisher Scientific) to which 10 mg/ml lysozyme (Thermo, Waltham, Massachusetts) and 1 µL at 1.7 U/L RNase (Sigma–Aldrich) was added. A 300 µL aliquot of the CTAB extraction buffer was added to biomass for each bacterial sample and incubated at 37 °C overnight (16 h). DNA was extracted using a phenol–chloroform procedure conducted using an Autogen 740 instrument (Autogen, Inc., Holliston, Massachusetts) according to the manufacturer's recommendations. Amplicons of the 16S rRNA gene were generated using the primers 27F and 1492R as reported previously (Puhl et al. 2009). Amplicons were purified using a QIAquick PCR purification kit (Qiagen N.V., Hilden, Germany) and sequenced by Eurofins genomics (Toronto, Ontario) using the 27F primer. Sequences were trimmed at the 25 and 825 bp sites.

2.2.6 Analysis of composition of microbiomes (ANCOM)

Detection of the differential abundance of taxa isolated with the culturomics methods was done with the ANCOM (Mandal et al. 2015), which was performed on taxa characterized at the genus level. Tissue and isolation methods were the main variables used in the analyses. The R code for ANCOM 2.0 was obtained from the website https://github.com/sidhujyatha/ANCOM (accessed on 2 March 2020). The code was run with R version 3.6.1 (R Core Team 2013), and the following parameters were used: no adjustment (adjustment = FALSE), no repeated measures (repeated = FALSE), main variables were set to either Tissue or Method, no repeat variable (repeat.var = NULL), taxa-wise multiple correction (multcorr = 2), a significance level of 0.05 (sig = 0.05), and fraction of taxa with a proportion of zeroes greater than

0.90 were not included in the analysis (prev.cut = 0.90). The comparison test returned the W statistic for each taxon as well as whether the taxa were differentially abundant under a series of percentile cutoffs (0.9, 0.8, 0.7, 0.6).

2.2.7 Linear discriminant analysis effect size (LEfSe) analyses

Bacterial taxonomy was assigned using the ribosomal database project (RDP) with the following settings selected: strain = both (Type and Non-Type); source = isolates; size = both (>1200 and <1200); quality = good; taxonomy = nomenclature; and KNN matches = 1 (Cole et al. 2014). Taxonomic comparisons were made using functions of the *vegan* package in R (version 3.4.3) (Oksanen et al. 2019) and phylogenetic relationships were analyzed using the LefSE tool on the Galaxy platform (http://huttenhower.sph.harvard.edu/galaxy/) (Segata et al. 2011). Dendrograms were generated from the 16S rRNA gene sequence information using the MEGA-X software package (Kumar et al. 2018), and alignments were made using the *MUSCLE* package and dendrograms were generated using an UPGMA (unweighted pair group method with arithmetic mean) tree with a bootstrap value of 500. The Newick alignment was then transferred to FigTree version 1.4.4 to generate the dendrogram (Rambaut 2012; Pohlert 2018).

2.2.8 Experimental design and statistics

Bacteria recovered using the different isolation strategies were statistically compared among birds and the environmental samples. Evaluations of diversity among isolation strategies and tissues was conducted using the *vegan* package in R (Oksanen et al. 2019), and statistical inferences were determined using the Kruskal–Wallis rank sum test in R (R Core Team 2013) and the Nemenyi test with 2 approximations (Pohlert 2018) to identify significant changes to Shannon's indices. False discovery rates were determined via the *q* value program in R (Storey et al. 2019). The presence of over-represented bacterial isolates was determined by comparing the abundance of bacterial isolates using the LEfSe package of the galaxy program (Segata et al. 2011) and the ANCOM package (Mandal et al. 2015).

2.3 Results

2.3.1 Bacterial isolation

During this study, 899 bacterial isolates were obtained from the GIT of broiler chickens raised in a controlled production setting. Isolated bacteria included 75 unique taxa contained within either the *Firmicutes* or *Proteobacteria*, and these two phyla accounted for 57% and 42% of the collection, respectively (Figure 2.1). The predominant genera isolated were *Lactobacillus*, *Escherichia*/*Shigella*, *Proteus*, and *Enterococcus* spp., which collectively accounted for 85% of the total bacteria isolated, and represented 25%, 22%, 19%, and 17% of isolated bacteria, respectively (Table 2.2). Other taxa identified

in the collection included members of *Clostridiales* spp., and of the 75 RDP identified taxa, 31 were found to be within this taxonomic order.

2.3.2 Bacterial diversity by isolation method

The α-diversity (Shannon's index) of bacteria was evaluated to compare methods. Of note, the highest diversity observed was for direct plating on CBAG (Shannon's index = 2.8) in the nitrogen atmosphere, and on CBA in both carbon dioxide and nitrogen atmospheres (Shannon's index = 2.5 for both atmospheres) (Table 2.3). For enrichment isolations, enriching with mucus yielded the most diverse bacterial communities with a Shannon's index of 2.3, compared with indices of 0.8 and 2.2 for the enrichments with xylan and blood, respectively.

To further evaluate bacterial diversity by direct plating methods, box plots were generated and showed that most methods yielded similar results with the exception of the selective medium MRS, which yielded significantly less bacterial diversity (Figure 2.2). The relative abundance of the *Lactobacillus*, *Escherichia*/*Shigella*, and *Enterococcus* spp. was lower in enrichments relative to direct plating; however, the abundance of *Proteus* spp. was higher for the enrichment method (Table 2.2). Comparing the Shannon's diversity of bacteria using a Kruskal–Wallis rank sum test in R (R Core Team 2013) confirmed that the different isolation methods applied yielded different community compositions ($P = 0.021$), likely due to changes ($P = 0.048$) in bacteria recovered on MRS versus CBAG (Table 2.4). P values generated from the Nemenyi test with χ^2 approximations were compared using the *q* value package in R, resulting in a *q* value of 0.480 associated with the comparisons between MRS and CBAG (Storey et al. 2019).

The Euclidian distances of bacteria provided by RDP (Cole et al. 2014) were used to compare the diversity of taxa by isolation method. When comparing the similarity of operational taxonomic unit identities, direct plating of bacteria onto MMA provided a unique cluster as compared with all other isolation methods; in contrast, bacteria isolated by enrichment and direct plating onto CBAG clustered separately from bacteria isolated with the other methods or media evaluated (Figures. 2.3–2.4).

In addition, examination of taxa recovered by the different isolation methods employed indicated that direct plating onto MRS was more commonly associated with the isolation of *Lactobacillus* spp., whereas *Escherichia*/*Shigella* spp. were commonly isolated from samples plated on MMA. As many of the recovered bacteria were *Lactobacillus*, *Escherichia*/*Shigella*, *Proteus*, or *Enterococcus* spp., these bacteria were removed from further analysis to examine less frequently isolated taxa in more detail (Figure 2.5). This ancillary analysis revealed that *Clostridium* spp., such as *C. perfringens*, were commonly isolated on CBAG. As well, *Clostridium* spp., such as *C. thiosulfatireducens* and *C. cochlearium*, were

commonly recovered from enrichment broths supplemented with mucus (Table 2.5). *Ruminococcus* spp. were commonly isolated on CBA in both the N_2 and CO_2 atmospheres. In contrast, bacteria within the Firmicutes phylum were not commonly isolated by direct plating on MMA or MRS, nor from enrichments supplemented with xylan (Figure 2.5).

2.3.3 Bacterial diversity by GIT location

The composition and diversity of bacteria throughout the chicken GIT and associated with mucosa and luminal contents were evaluated. The greatest α-diversity (Shannon's index) was observed in the cecum (Shannon's index = 2.9), and diversity of bacteria in digesta was higher than bacteria associated with mucosa (Shannon's index = 2.9 and 2.5, respectively; Table 2.6).

Global diversity did not differ ($P = 0.07$) among GIT sites as determined by the Kruskal–Wallis rank sum test. Furthermore, no differences ($P \geq 0.56$) in diversity among sites were observed using a post-hoc test (Table 2.7). The composition of bacterial taxa recovered from the crop and cecum were unique, whereas taxa from the ileum and jejunum samples grouped together (Figures. 2.3 and 2.6). Bacterial taxa recovered from luminal digesta and associated with mucosa were similar within the small intestine (i.e., ileum and jejunum). *Escherichia*/*Shigella* were most commonly isolated from the cecum, whereas *Lactobacillus* spp. were most often isolated from the ileum and jejunum (Figure 2.7). In addition to the abundant genera recovered throughout the GIT, *Ruminococcus* and *Clostridium* spp. were commonly isolated from the ileum as well as the cecum, which was also dominated by *Lachnospiraceae* spp.

2.3.4 ANCOM

To evaluate the differential abundance of bacteria, ANCOM was performed on the count data of the taxa classified at the genus level using tissue and isolation methods as the main variables. Only *Proteus* was differentially abundant (W = 6) among the tissues evaluated, where it was found to be more abundant at the 0.9, 0.8, 0.7, and 0.6 cutoffs in the cecum.

When analyzing differential abundance by isolation method, there were five taxa that were differentially abundant at the 0.9, 0.8. 0.7, and 0.6 cutoffs (W = 7). They were *Enterococcus* (MMA), *Escherichia*/*Shigella* (MMA), *Proteus* (CBAG and CBA-N_2), *Lactobacillus* (CBA-CO_2, CBA-N_2, and MRS), and *Ruminococcus* (CBA-CO2 and CBA-N2). Furthermore, three additional taxa were differentially abundant but only at the 0.7 and 0.6 cutoffs (W = 5). They were *Clostridium sensu stricto* (CBAG and enrichments containing mucus), unclassified *Lachnospiraceae* (CBAG, CBA-CO_2, and CBA-N_2), and unclassified *Erysipelotrichaceae* (CBAG).

2.3.5 LEfSe analyses

To further compare the abundance of bacterial taxa by isolation method, LEfSe analysis was applied. Bacteria within the *Lactobacillales* and *Enterobacteriales* were most commonly recovered by direct plating onto MRS and MMA, respectively. Bacteria within the *Clostridiales* and Proteus spp. were most commonly isolated by enrichment containing blood and xylan, respectively (Figure 2.8). The LEfSe method was also used to compare sample locations and confirmed that *Lactobacillales* spp. were most commonly recovered from mucosal surfaces from the crop, whereas *Enterococcus* spp. were commonly isolated from mucosal surfaces from the jejunum. *Escherichia*/ *Shigella* and various Enterobacteriaceae were the predominate bacteria isolated from drinking water and litter, respectively (Figure. 2.9).

2.3.6 Bacterial diversity in environment samples

Bacteria were isolated from drinking water and soiled litter. In total, 18 bacterial isolates were collected and identified from each of the water and litter samples. One *Clostridium sensu stricto*, three *Enterococcus* spp., and 14 *Escherichia*/*Shigella* spp. were isolated. No *Proteus* spp. were isolated from water. The litter sample was dominated by *Proteus* spp. (11) and to a lesser degree by *Escherichia*/ *Shigella* spp. No *Clostridium* spp. or *Enterococcus* spp. were isolated from litter.

2.4 Discussion

Few culturomics studies have been conducted on the GIT of chickens. A recently completed study examined the diversity of culturable bacteria from ceca of free range and feral layers (Ferrario et al. 2017). They used a variety of selective and nonselective isolation media and collected 1000 bacterial isolates, which is similar in magnitude to the current study. They observed that bacteria belonged to four phyla and 15 genera. In contrast, we recovered bacteria assigned to two phyla and 20 genera from the GIT broilers housed in a controlled production setting. Salient differences between the two studies were the increased isolation of *Bacteroidetes* spp. isolated from layers by Ferrario et al. (2017) including *B. salanitronis*, *B. barnesiae*, and *B. gallinarum*. In contrast, no *Bacteroidetes* spp. were isolated from broilers in the current study. Furthermore, the diversity of *Lactobacillus* spp. was much higher in layers (16 species) compared with broilers (two species). Lactobacillus isolated from broilers were identified as *L. taiwanensis* and *L. reuteri* (new classification: *Limosilactobacillus reuteri*); *L. taiwanensis* was not recovered from layers (Zheng et al. 2020). *Lactobacillus* spp. of chickens have been reported to impart a buffering effect (termed the “Marmite” effect) in the chicken cecal microbiota, as it is associated with equal positive and negative responses. As such, a greater diversity of *Lactobacillus* spp. in the chicken intestinal contents in feral birds may improve the resilience of the cecal microbiota and host immunity during changing diets and pathogen exposures over time (Zou et al. 2018). The current study recovered

a much greater diversity of *Clostridium* spp. from broilers (six species) than the previously conducted study in which layers were examined. *Clostridium* spp. isolated from broilers were *C. amygdalinum*, *C. cochlearium*, *C. difficile*, *C. paraputrificum*, *C. perfringens*, and *C. ramosum*. Only *C. butyricium* was isolated from layers. In addition, four potentially new *Clostridium* spp. (sequence similarity <97% to those from the RDP database) were recovered from broilers. A possible reason for the difference between the two studies may be due to the different isolation methods applied, the samples that were evaluated, or both (Ferrario et al. 2017). Feral birds are likely to eat a variety of foods, including arthropods and worms, that would be absent from the high-grain diets fed to broilers in the current study. As such, it is possible that the diversity of *Lactobacillus* spp. that were more abundant in feral layers was a result of the birds' diet as well as the variation in bird breeds (Martinez-Fleites et al. 2006; Zou et al. 2018).

In the current study, a variety of media and two anaerobic atmospheres were used. The diversity of the bacteria recovered differed among the media used ($P = 0.021$), and this was most conspicuous for MRS agar; this medium was effective at isolating *Lactobacillus* as was expected, as it was developed for this purpose (De Man et al. 1960). However, when comparing the q values associated with this medium the presence of a false positive is likely ($q = 0.480$), and as such, this result should be noted with care. Interestingly, CA amended with sheep's blood and maintained in either the nitrogen or carbon dioxide atmosphere was as effective as MRS agar for isolating *Lactobacillus* spp. In contrast, the minimal medium without supplements was found to differentially isolate *Escherichia*/*Shigella* spp., according to both ANCOM and LEfSe analyses, as well as *Enterococcus* spp., according to ANCOM analysis, possibly due to the ability of these bacteria to cross feed and survive on purines and phenolics that were contained in this medium (Seth and Taga 2014; LaSarre et al. 2017). The gentamicin-supplemented blood medium was effective at isolating *Clostridium sensu stricto*, according to both LEfSe and ANCOM analyses, and the diversity of bacteria recovered on CBAG was comparable to enrichment cultures. Gentamicin was chosen as a selective agent because of its ability to select for *Bacteroides* spp. when used in media, such as the *Bacteroides* Bile Esculin agar. Gentamicin is also an effective way to select for members of *Clostridium* spp. and exclude faster growing organisms, such as *Lactobacillus* spp. and *Escherichia* spp (Livingston et al. 1978; Carroll et al. 1983).

Enrichment is a standard method for bacterial culturomics, and this strategy has been used with success in other studies (e.g., Lagier et al. 2016). In the current study, we recovered 61 bacterial isolates by enrichment that included 13 unique bacterial taxa according to the RDP database. Although the diversity of bacteria recovered using enrichment was not conspicuously different from that using direct

plating (e.g., using CBAG), the dominant taxa isolated by direct plating (i.e., *Lactobacillus*, *Enterococcus*, and *Escherichia*/*Shigella* species) differed from enrichment. An examination of taxa recovered using different enrichment media via LEfSe analysis indicated that the xylan-supplemented enrichments favored the isolation of *Proteus* spp. Enrichments supplemented with mucus favored the isolation of *Clostridium* spp. Both LEfSe and ANCOM analysis supported this conclusion and showed that members of the *Clostridium sensu stricto* taxon were preferentially recovered in enrichments supplemented with mucus. The observed predominance of *Clostridium* spp. in the mucus enrichments may be a result of the mucosome possessed by these bacteria (Woodhams et al. 2014; Hirano et al. 2016). The relative absence of *Clostridium* spp. from enrichments containing xylan may indicate that this carbon source is not well metabolized by members of the chicken microbiota, although the germination status of this organism within birds is uncertain. This is supported in production settings where xylan is considered an anti-nutritive component of chicken diets, and it has been shown to yield no useful energy for the birds (Baker 1977). Our findings indicated that the different methods and media used profoundly influenced the types of bacteria isolated, indicating that comprehensive isolation of diverse anaerobic bacteria from the GIT requires the use of a combination of methods.

The composition of bacteria isolated from the ileum and jejunum luminal digesta were similar, as were bacteria isolated from the crop (luminal digesta and mucosa associated). Bacteria isolated from the cecum, both mucosa-associated and within luminal digesta, were most similar in composition to both samples and differed from other sampled locations within the GIT. Bacteria isolated from litter contaminated with feces were similar to cecal communities, indicating that communities of culturable bacteria excreted in feces into the environment are generally representative of the distal GIT of chickens. The correlation of communities between litter and the GIT has been studied previously. For example, when birds are raised on clean and fresh litter, more *Lactobacillus* spp. in the ileal microbiota were observed, which was consistent with the isolations from this study. Additionally, previous research has found that the cecal communities of birds are highly affected by the litters upon which they were raised (Torok et al. 2009; Cressman et al. 2010). In the current study, *Proteus* spp. were most commonly associated with isolations from the cecum, as determined by ANCOM analysis. In addition to ANCOM evaluation, LEfSe also showed that *Escherichia*/*Shigella* spp. and *Enterobacteriales* spp. were most commonly isolated from litter and drinking water. In contrast, the crop was much more commonly associated with *Lactobacillus* spp., which supports the conclusions of previous reports (Hinton et al. 2000; Guan et al. 2003). Interestingly, the mucosal samples from the jejunum were most commonly found to harbour *Enterococcus* spp.

The diversity of bacteria recovered was lower than we expected for each method and associated sample. This can be attributed to the isolation prevalence of *Escherichia*/ *Shigella* spp., *Enterococcus* spp., *Lactobacillus* spp., and *Proteus* spp. The highest diversity of bacteria was obtained by direct plating onto CBAG, and the lowest diversity was obtained by direct plating onto MRS agar, as well as enrichment using a xylan supplement. Direct plating on an agar medium containing 5% blood is common in culturomics; however, the incorporation of gentamicin is not (Lagier et al. 2016). Enrichment broths containing 5% blood are also relatively common, but the supplementation of enrichment media with xylan is not. Notably, our findings indicate that amendment with xylan was not effective, whereas the addition of mucus was beneficial in increasing the diversity of bacteria recovered. The reduced efficiency of xylan was not expected, and is possibly linked to an inability of the microbiota in these birds to utilize this complex carbohydrate (Baker 1977).

Although bacteria isolated from environmental samples differed from GIT samples, according to LEfSe analyses, no bacteria were preferentially found in environmental samples when using ANCOM analyses, likely due to the lower counts of bacteria associated with these samples. Concerning these results, LEfSe evaluations determined that the ceca and litter samples were found to be similar when comparing the Euclidian distances of the isolated bacteria. Previous studies using culture-independent methods have shown that cloacal swabs are more representative of the litter microbiota than are ileal or cecal swabs (Torok et al. 2009). Notably, the composition of the re-used litter bacterial community has been linked to the presence of alkaliphilic and halotolerant bacteria such as *Faecalibacterium prausnitzii* (Wang et al. 2016). We used a controlled production setting in an attempt to reduce potential confounding variables encountered in a production barn. One salient difference between our study and those conducted in actual production settings is density; we provided 0.22 m^2 per bird compared with 0.01 m^2 per bird that is typical of a production barn (Wang et al. 2016). Thus, in our study, birds may have had more access to litter, thereby influencing the GIT bacterial community composition. Additionally, we did not isolate any *Bacteroidetes* spp., which may be due to the production setting and animal diets, as well as the breed and age of the birds used (Zou et al. 2018; Jurburg et al. 2019). If the goal is the isolation of *Bacteroidetes* spp. from chickens, the evaluation of multiple chicken breeds may be advisable. Additionally, the use of media, such as the *Bacteroides* Bile Esculin agar could prove useful as well as increasing the concentration of gentamicin in the medium to 200 g/ml, which has been an effective strategy to isolate *Bacteroides* spp. from communities containing *Escherichia coli* (Livingston et al. 1978; Valentine et al. 1988). Future studies looking to maximize the diversity of bacteria isolated from chickens could use a bacterial grouping strategy to identify rare taxa

and to reduce overevaluation of abundant bacterial taxa. Such methods could include MALDI–TOF (matrix-assisted laser desorption – time of flight) analyses, as it has been done previously to yield many newly discovered taxa (Tandina et al. 2016) or PCR-based methods such as rep-PCR (repetitive extragenic palindromic PCR) prior to sequencing (Mishra et al. 2014).

2.5 Tables and Figures

Table 2.1 Dehority's Medium

Ingredient	Amount
Carbohydrate	5 g/L
Trypticase	4.5 g/L
Yeast Extract	0.5 g/L
Mineral 1[a]	40 ml/L
Mineral 2[b]	40 ml/L
Hemin (0.01%)[c]	10 ml/L
VFA solution[d]	10 ml/L
Resazurin solution (25 mg/ml)	1 ml/L
L-Cysteine HCl	1 ml/L
Clarified Rumen Fluid[e]	50 ml/L

[a]Mineral 1 = K_2HPO_4 (6 g/L)
[b]Mineral 2 = KH_2PO_4 (6g/L), $((NH_4)_2)SO_4$ (6 g/L), NaCl (12 g/L), $MgSO_4$ (2.45 g/L), $CaCl_2 \bullet 2H_2O$ (1.69 g/L)
[c]Hemin was dissolved in 1 M NaOH and then diluted to appropriate concentration
[d]VFA solultion = acetic acid (6.85 ml/L), propionic acid (3 ml/L), butyric acid (1.85 ml/L), isobutyric acid (0.5 ml/L), 2-methyl butyric acid (0.55 ml/L), N-valeric acid (0.55 ml/L), and isovaleric acid (0.55 ml/L)
[e]Clarified rumen fluid was prepared by centrifuging to rumen fluid to remove debris and gently decanting in a fresh vial for storage at -20 ^{o}C.

Table 2.2 Relative abundance of bacterial genera isolated from the gastrointestinal tract of broiler chickens by direct plating (DP) and enrichment.

Genus	DP (%)	Enrichment (%)	Grand Total (%)
Anaerostipes	0.0	4.9	0.4
Anaerotruncus	0.3	0.0	0.3
Clostridium IV	0.1	0.0	0.1
Clostridium sensu stricto	3.6	18.0	4.7
Clostridium XI	0.1	8.2	0.8
Clostridium XlVa	0.8	0.0	0.8
Clostridium XVIII	0.1	4.9	0.5
Eisenbergiella	0.0	6.6	0.5
Enterococcus	19.1	3.3	17.8
Erysipelotrichaceae incertae sedis	0.1	1.6	0.3
Escherichia/Shigella	23.3	11.5	22.4
Flavonifractor	0.4	0.0	0.4
Lactobacillus	26.9	0.0	24.8
Proteus	18.1	41.0	19.9
Ruminococcus II	3.0	0.0	2.8
Staphylococcus	0.3	0.0	0.3
Unclassified Enterobacteriaceae	0.3	0.0	0.3
Unclassified Erysipelotrichaceae	1.8	0.0	1.7
Unclassified Lachnospiraceae	1.4	0.0	1.3
Unclassified Ruminococcaceae	0.1	0.0	0.1

Table 2.3 Shannon diversity of bacteria recovered by isolation method.

Name	Method	Medium	Atmosphere	Shannon
MMA[a]	Direct Plating	MMA	N_2	2.2
CBAG[b]	Direct Plating	CBAG	N_2	2.8
CBA[c]-CO_2[d]	Direct Plating	CBA	CO_2	2.5
CBA-N_2[e]	Direct Plating	CBA	N_2	2.5
MRS[f]	Direct Plating	MRS	CO_2	1.9
Blood	Enrichment	Blood	N_2	2.2
Mucus	Enrichment	Mucus	N_2	2.3
Xylan	Enrichment	Xylan	N_2	0.8

[a]Minimal medium supplemented with 0.5% agarose.
[b]Columbia blood agar supplemented with 100 µg/ml gentamycin sodium sulfate.
[c]Columbia agar supplemented with 10% sheep's blood.
[d]Carbon dioxide atmosphere.
[e]Nitrogen atmosphere.
[f]de Man Rogosa and Sharpe agar.

Table 2.4 Pairwise comparisons using Nemenyi-test with Chi-squared approximation for independent samples generated using the Shannon's indices of isolates obtained from different isolation methods when isolating from samples of the chicken intestinal tract.

Media	**MMA**[a]	**CBAG**[b]	**CBA**[c]**-CO_2**[d]	**CBA-N_2**[e]
CBAG	0.78	--	--	--
CBA-CO_2	0.98	0.98	--	--
CBA-N_2	0.95	0.99	1.00	--
MRS[f]	0.54	0.05	0.22	0.14

[a]Minimal medium supplemented with 0.5% agarose
[b]Columbia blood agar supplemented with 100 µg/ml gentamycin sodium sulfate.
[c]Columbia agar supplemented with 10% sheep's blood.
[d]Carbon dioxide atmosphere.
[e]Nitrogen atmosphere.
[f]de Man Rogosa and Sharpe agar.

Table 2.5 Relative abundance of bacterial species isolated from the gastrointestinal tract of broiler chickens by direct plating and enrichment.

Species	Direct Plating	Enrichment
Anaeromassilibacillus senegalensis	1	0
Anaerostipes caccae	0	2
Anaerotruncus colihominis	2	0
Blautia hominis	6	0
Clostridium amygdalinum	5	0
Clostridium cochlearium	0	4
Clostridium difficile	0	2
Clostridium indolis	0	1
Clostridium innocuum	1	1
Clostridium mangenotii	1	3
Clostridium paraputrificum	0	3
Clostridium perfringens	26	2
Clostridium ramosum	1	3
Clostridium thiosulfatireducens	0	2
Ecoli/Shigella	166	7
Eisenbergiella tayi	0	4
Enterococcus faecalis	4	0
Enterococcus faecium	131	0
Enterococcus gallinarum	3	2
Escherichia fergusonii	5	0
Eubacterium desmolans	1	0
Eubacterium sp. Marseille-P3177	4	0
Flavonifractor plautii	3	0
Lactobacillus reuteri	134	0
Lactobacillus taiwanensis	61	0
Massiliomicrobiota timonensis	14	0
Proteus mirabilis	132	25
Pseudoflavonifractor capillosus	1	0
Ruminococcus torques	22	0
Staphylococcus aureus	2	0
Total isolates	**726**	**61**

Table 2.6 Shannon diversity by sample type.

Sample	Location	Shannon's Index
Crop	Lumen	2.4
Crop	Mucosa	1.4
Jejunum	Lumen	2.7
Jejunum	Mucosa	2.4
Ileum	Lumen	2.8
Ileum	Mucosa	2.3
Cecum	Lumen	2.9
Cecum	Mucosa	2.5
Drinking water	Environment	1.8
Litter	Environment	2.1

Table 2.7 Pairwise comparisons using Nemenyi-test with Chi-squared approximation for independent samples generated using the Shannon's indices of isolates from different tissue segments comprising both luminal and mucosal samples of the chicken intestine.

Sample	Cecum-lumen	Cecum-mucosa	Crop-lumen	Crop-mucosa	Ileum-lumen	Ileum-mucosa	Jejunum-lumen
Cecum-mucosa	0.97	--	--	--	--	--	--
Crop-lumen	0.56	0.96	--	--	--	--	--
Crop-mucosa	0.76	0.98	1.00	--	--	--	--
Ileum-lumen	1.00	0.99	0.64	0.81	--	--	--
Ileum-mucosa	0.80	1.00	1.00	1.00	0.87	--	--
Jejunum-lumen	1.00	1.00	0.83	0.92	1.00	0.98	--
Jejunum-mucosa	0.80	1.00	1.00	1.00	0.87	1.00	0.98

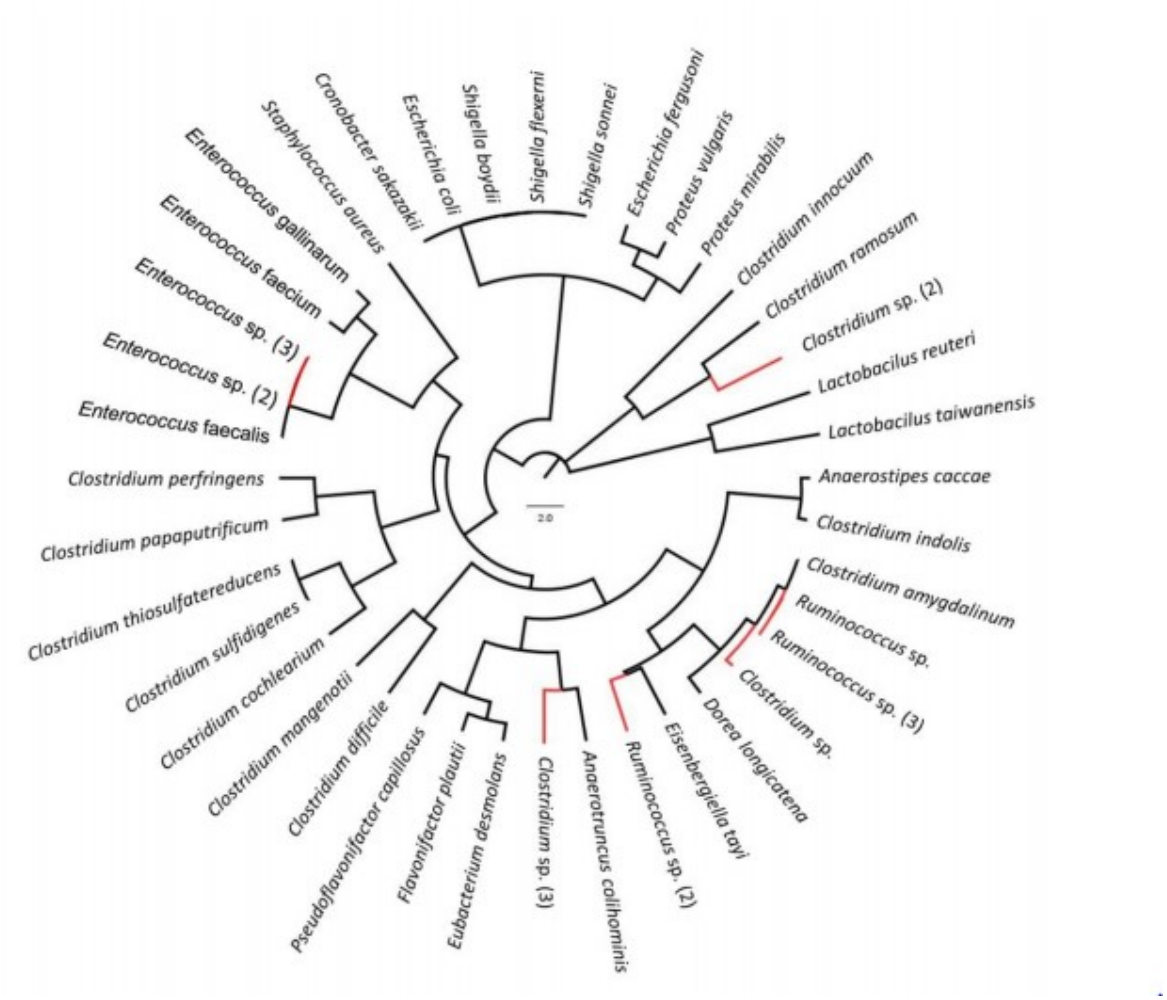

Figure 2.1 Phylogram showing the diversity of bacterial species isolated from broiler chickens and their environment. The occurrence of previously uncultured bacteria (<97% similarity to bacterial sequences contained in the ribosomal database project) is shown in red.

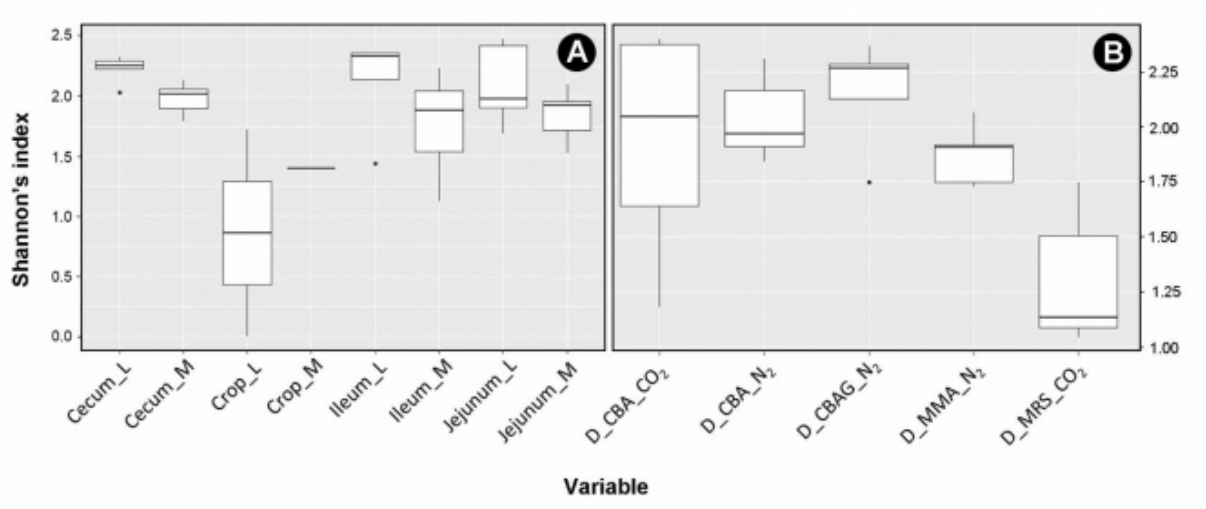

Figure 2.2 Box plot of Shannon's diversity of bacteria by sample (A) and method (B). Bacteria were isolated from digesta in the intestinal lumen (L) or associated with mucosa (M) by direct plating (D). Media used were Columbia agar with 10% sheep's blood (CBA), Columbia blood agar with 100 g/ml gentamicin (CBAG), minimal medium with 0.5% agarose (MMA), and de Man–Rogosa–Sharpe agar (MRS). Atmospheres were either carbon dioxide (CO_2) or nitrogen (N_2) predominant. The center lines in the box plot represent the median value, the size of the box plot represents the distribution within a confidence of 95%, and the vertical lines and dots associated with the box plot represent the total spread of data. Five birds were examined.

Figure 2.3 Dendrograms of Euclidean distances observed among isolated bacterial communities within digesta in the intestinal lumen (L), associated with intestinal mucosa (M), in drinking water, or within litter. Bacteria were isolated by direct plating (D) or enrichment (E). Solid media used were Columbia agar with 10% sheep's blood (CBA), Columbia blood agar with 100 g/ml gentamicin (CBAG), minimal medium with 0.5% agarose (MMA), and de Man–Rogosa–Sharpe agar (MRS). Enrichment media used were a modified Dehority's medium supplemented 10% sheep's blood (Blood), 0.5% porcine mucus III (Mucus), and 0.5% xylan (Xylan). Atmospheres were either carbon dioxide (CO_2) or nitrogen (N_2) predominant.

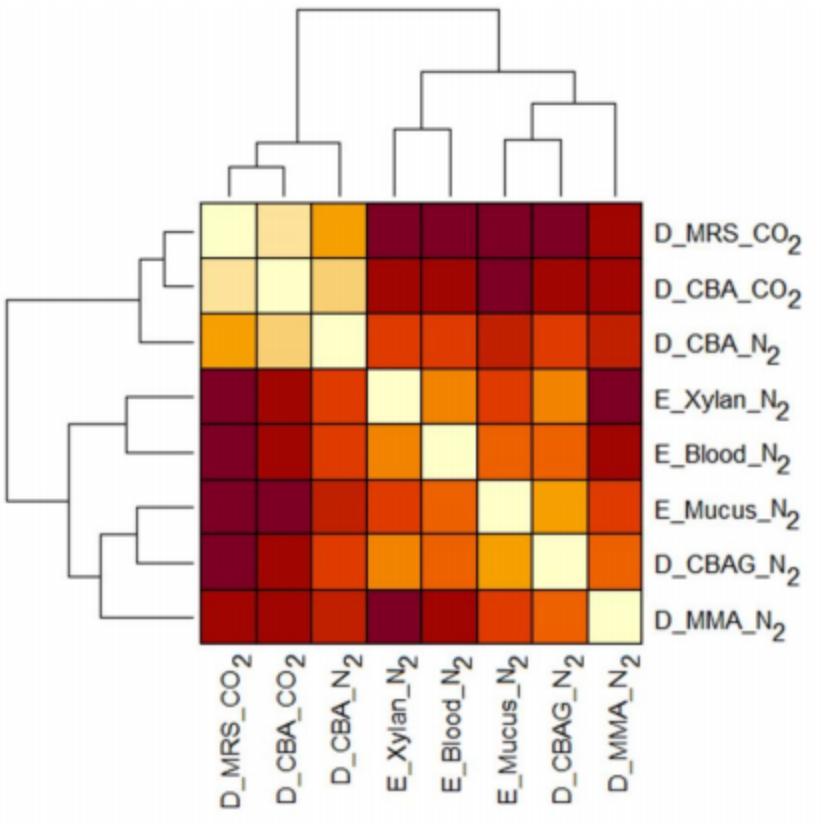

Figure 2.4 Heatmap of Euclidean distances of isolated bacterial communities isolated by direct plating (D) or enrichment (E). Solid media used were Columbia agar with 10% sheep's blood (CBA), Columbia blood agar with 100 g/ml gentamicin (CBAG), minimal medium with 0.5% agarose (MMA), and de Man–Rogosa–Sharpe agar (MRS). Enrichment media used were a modified Dehority's medium supplemented 10% sheep's blood (Blood), 0.5% porcine mucus III (Mucus), and 0.5% xylan (Xylan). Atmospheres were either carbon dioxide (CO_2) or nitrogen (N_2) predominant. The heatmap and dendrogram were generated using the *heatmap* and *Hclust* (*vegan* package) functions within the *vegan* package of R (Oksanen et al. 2019).

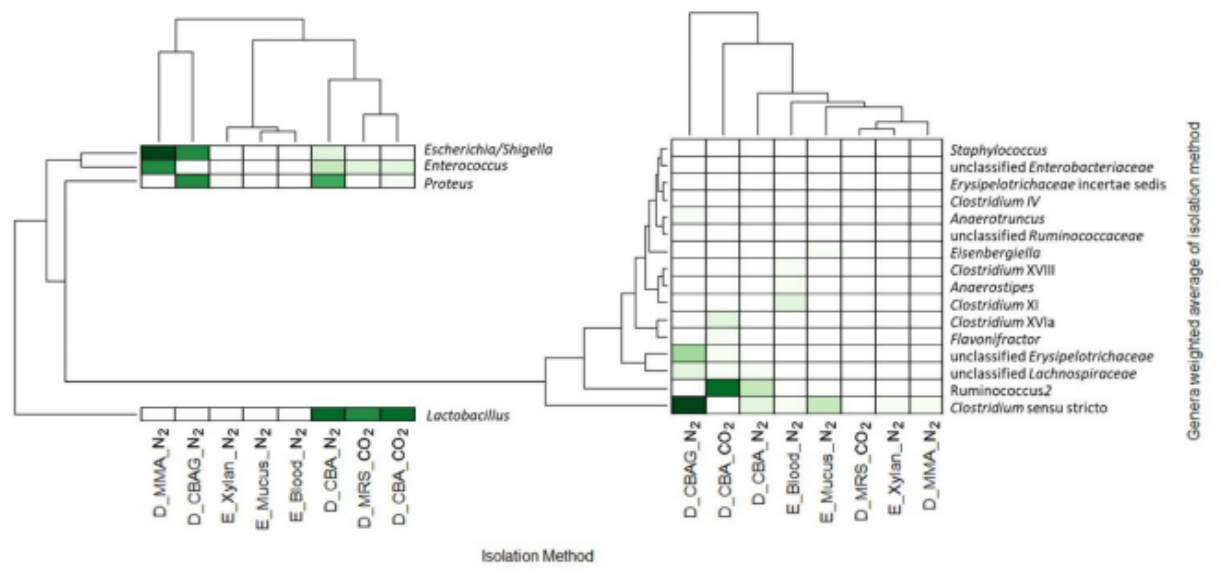

Figure 2.5 Heatmap of Euclidean distances and relative abundance of prominent isolated bacterial taxa. Bacteria were isolated by direct plating (D) or enrichment (E). Solid media used were Columbia agar with 10% sheep's blood (CBA), Columbia blood agar with 100 g/ml gentamicin (CBAG), minimal medium with 0.5% agarose (MMA), and de Man–Rogosa–Sharpe agar (MRS). Enrichment media used were a modified Dehority's medium supplemented 10% sheep's blood (Blood), 0.5% porcine mucus III (Mucus), and 0.5% xylan (Xylan). Atmospheres were either carbon dioxide (CO_2) or nitrogen (N_2) predominant. The heatmap and dendogram were generated using the *heatmap* and *Hclust* (vegan package) functions in R (Oksanen et al. 2019).

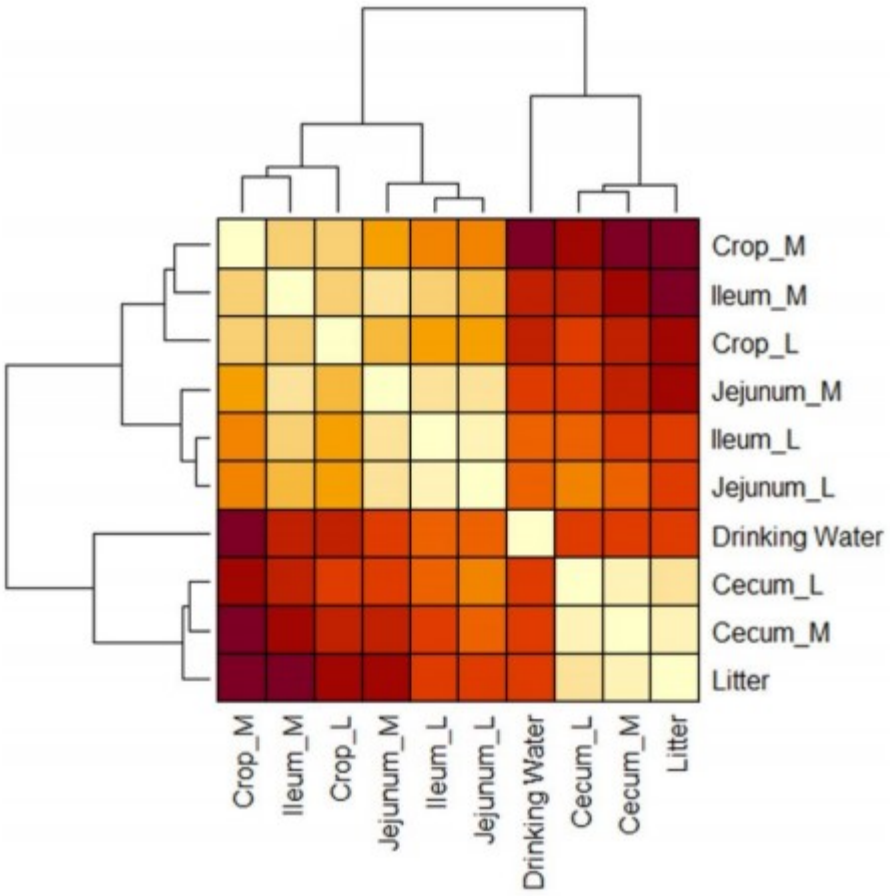

Figure 2.6 Heatmap of Euclidean distances of bacterial communities isolated from digesta in the intestinal lumen (L), associated with intestinal mucosa (M), in drinking water, or within litter. The heatmap and dendrogram were generated using the *heatmap* and *Hclust* (*vegan* package) functions in R (Oksanen et al. 2019).

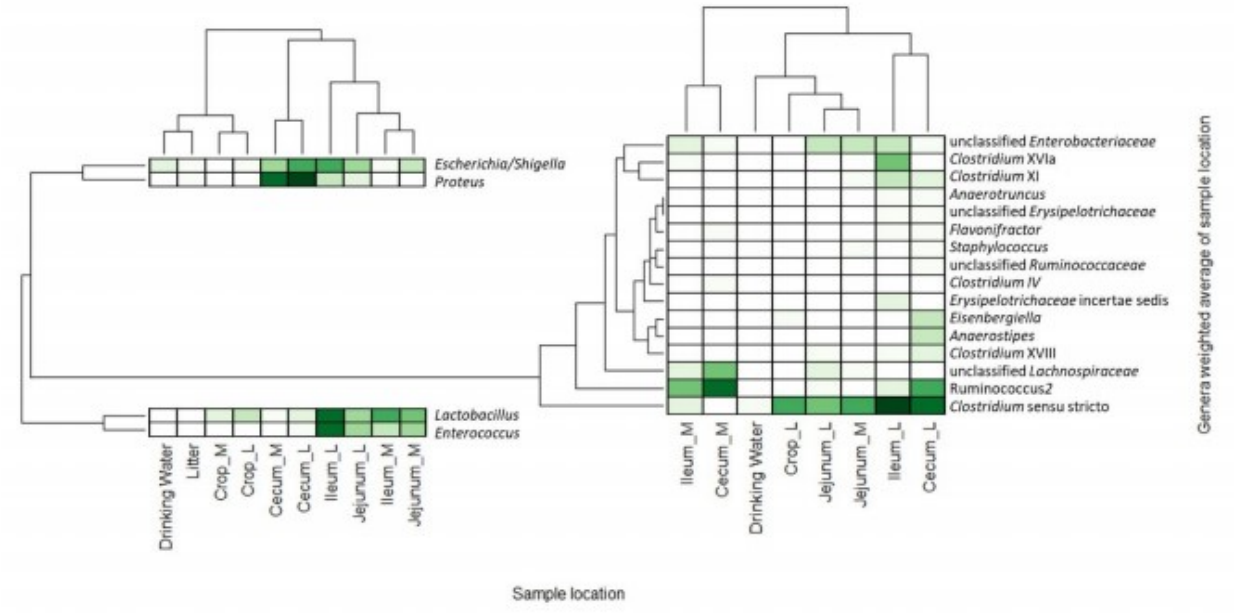

Figure 2.7 Heatmap of Euclidean distances and relative abundance of prominent isolated bacterial taxa. Bacteria were isolated from digesta in the intestinal lumen (L), associated with intestinal mucosa (M), in drinking water, or within litter. The heatmap and dendrogram were generated using the *heatmap* and *Hclust* (*vegan* package) functions in R (Oksanen et al. 2019).

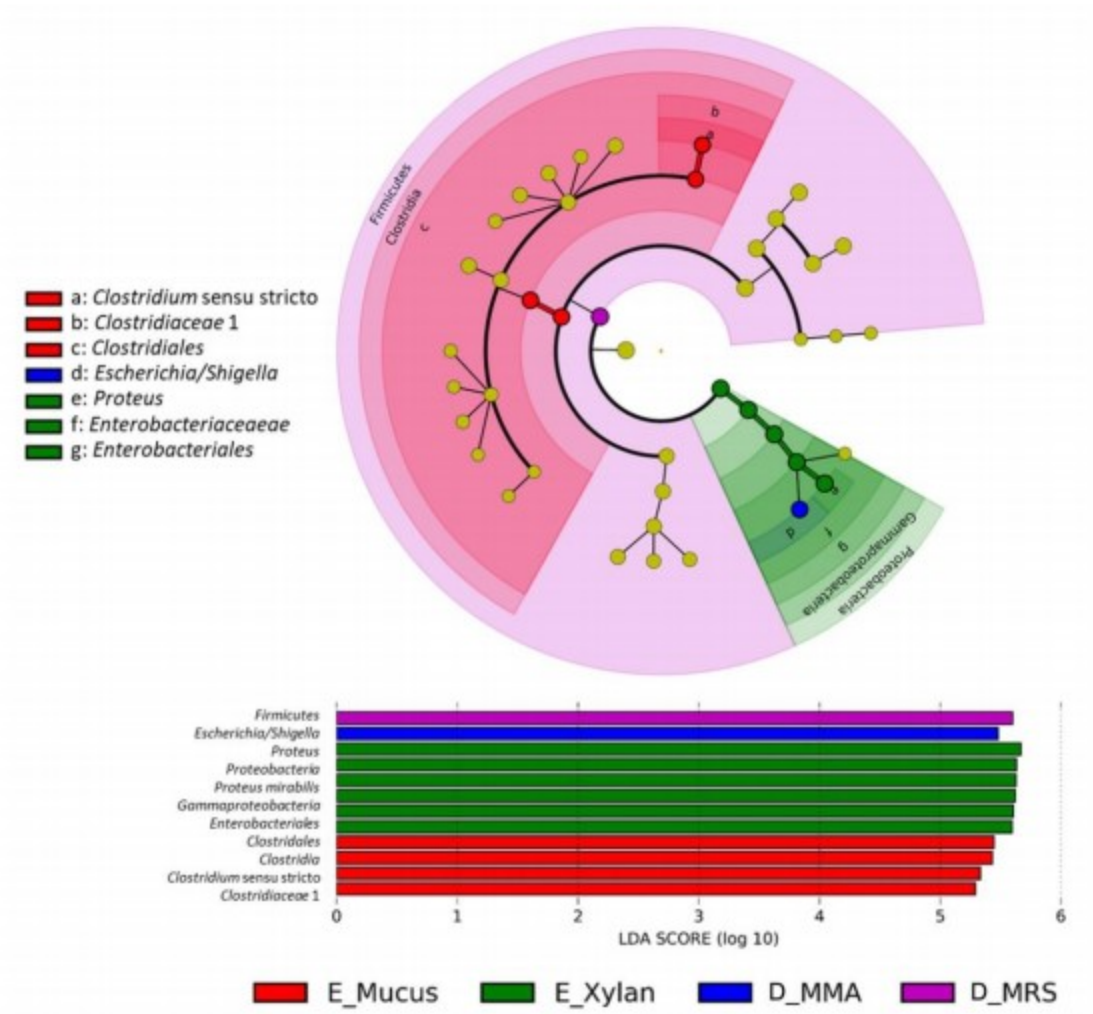

Figure 2.8 Cladogram showing isolation methods for which changes in the abundance of the bacteria taxa isolated were observed, particularly for direct plating (D) and enrichment (E). Taxonomic levels in the cladogram range from superkingdom (in the centre) to genus (at the perimeter). Media in which changes were observed were Dehority's medium supplemented with 0.5% porcine mucus III (E_Mucus), Dehority's medium supplemented 0.5% xylan (E_Xylan), minimal medium with 1.5% agarose (D_MMA), and de Man – Rogosa – Sharpe agar (D_MRS). The colour of the taxonomic markers, cladogram background, and associated histogram bars illustrate the methods that yielded differences in bacterial abundance as indicated by LEfSe (linear discriminant analysis effect size) analysis; taxa that were not affected by the isolation method are shown as yellow markers. The cladogram was generated using the online version of Galaxy (available from https://huttenhower.sph.harvard. edu/galaxy).

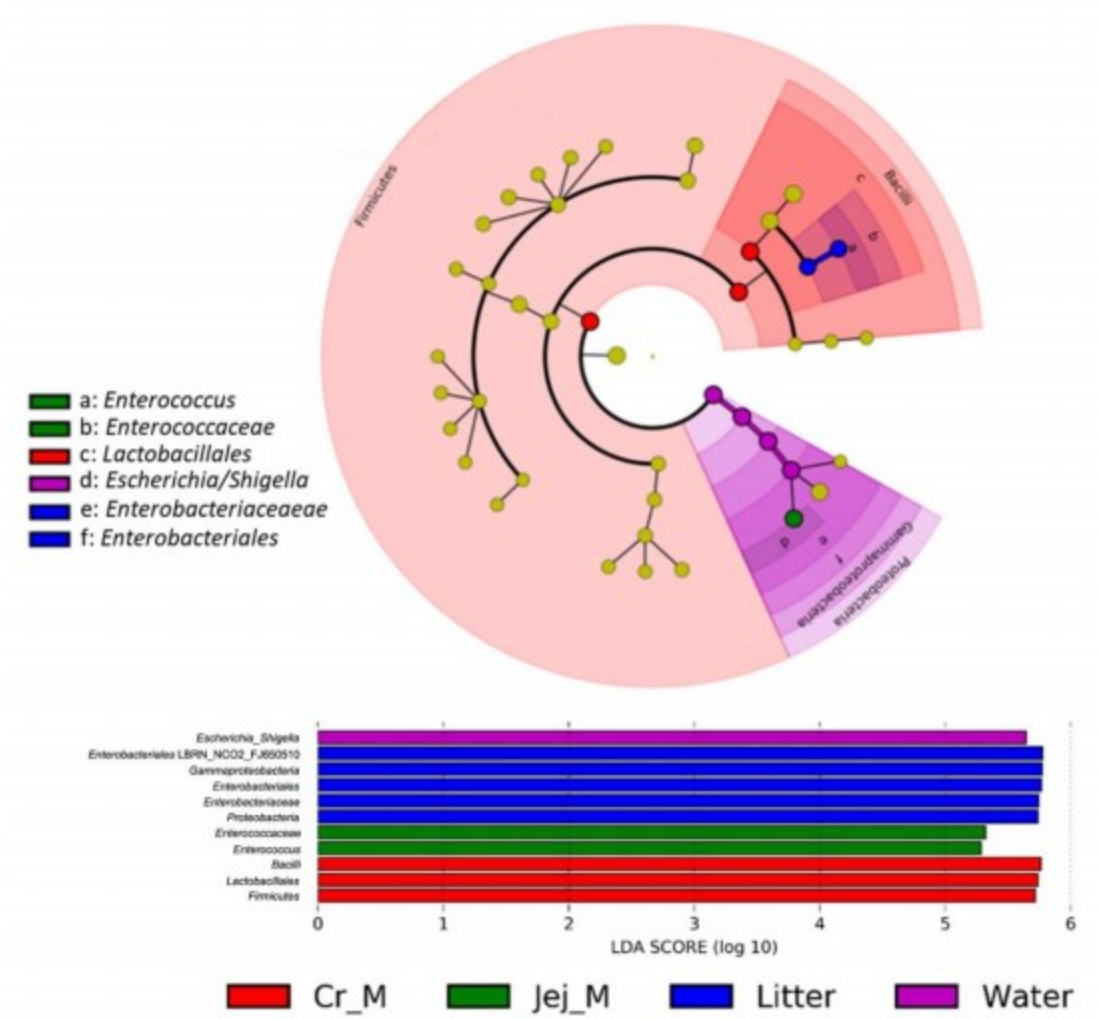

Figure 2.9 Cladogram showing samples for which changes in the abundance of the bacteria taxa isolated were observed. Taxonomic levels in the cladogram range from superkingdom (in the centre) to genus (at the perimeter). Samples in which changes were observed were from the intestinal mucosa of the crop (Cr_M), intestinal mucosa of the jejunum (Jej_M), litter, and drinking water. The colour of the taxonomic markers, cladogram background, and associated histogram bars illustrate the sample types that yielded differences in bacterial abundance as indicated by LEfSe (linear discriminant analysis effect size) analysis; taxa that were not affected by sample type are shown as yellow markers. The cladogram was generated using the online version of Galaxy (available from https://huttenhower.sph.harvard.edu/galaxy).

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Chapter 3 Comprehensive isolation of bacteria from mammalian intestines requires a combination of methods[2]

3.1 Introduction

The intestinal microbiome plays a key role in host health via a multitude of mechanisms (Zeevi et al. 2019). One essential function of the microbiota is the metabolism of foods not digested by host-produced enzymes (e.g. complex carbohydrates). For instance, the human genome contains a total of 17 putative digestive related carbohydrate active enzymes, compared to >15,000 carbohydrate active enzymes contained within the enteric microbiota (El Kaoutari et al. 2013). Colonization resistance is another crucial function imparted by microorganisms in the intestine. In this regard, various bacteria competitively occupy habitats and niches required for pathogen invasion, such as within intestinal crypts (Lee et al. 2013) and metabolism of carbohydrates (Shepherd et al. 2018). To fully elucidate bacterial function, it is critical that bacteria are isolated, characterized, and preserved for experimentation. In this way, candidate organisms can be utilized for the development of innovations in industry and healthcare.

Culture-independent analyses (i.e. next generation sequencing) have greatly advanced our understanding of the diversity of bacteria in natural habitats, including mammalian intestines. However, most research has focused on characterizing the fecal microbiota (Goodwin et al. 2016), which is not necessarily representative of the autochthonous bacterial taxa found along the intestinal tract of most mammals (Zehavi et al. 2018). Further, the information obtained via traditional next-generation sequence analysis is limited, in particular with respect to taxonomic resolution and elucidation of bacterial functional (Dubourg et al. 2014; Holman et al. 2017; Wang et al. 2019). Most importantly, bacteria are not recovered thus eliminating the option to further study and use these organisms for *in vivo* research and industrial applications.

Swine are an ideal monogastric mammalian model for microbiological studies as they are an important livestock species, as well as an ideal model for human beings. Pork production in 2019 accounted for $23.4 billion dollars towards the gross domestic product of the USA with over a quarter of this value due to the export of pork or pork products (Queck-Matzie 2019). Pigs are a particularly good human model as they are of comparable size, possess a similar genome, and have a similar diet to people (Houpt et al. 1979; Rothschild and Ruvinsky 2011). In addition, their high fecundity and tractability facilitate their use as models to study the mammalian immune system (Meurens et al. 2012). Despite the importance of swine as a livestock species and mammalian model, research investigating

[2] A version of this chapter has been submitted to Applied and Environmental Microbiology

the culturable diversity and characterization of the microbiota of pigs is relatively stagnant. The culturable microbiota of pigs was originally evaluated in 1979 (Russell 1979); however, the application of culturomics to characterize the enteric microbiota has largely been supplanted by culture-independent evaluations in recent years (Pena Cortes et al. 2018; Pollock et al. 2019; Sun et al. 2016). Sequence-based analysis of the intestinal microbiota of pigs identified that species of bacteria within the *Alloprevotella*, *Blautia*, *Clostridium*, *Lactobacillus*, *Prevotella*, *Roseburia*, *Ruminococcus*, and the RC9 gut group genera comprise the core microbiota (Holman et al. 2017). This has not been confirmed using culturomics. Importantly, bacterial abundance does not necessarily equate with dominant functions (e.g. niche utilization), and taxa that occur at low densities can occupy critical niches important for host health. As such, it is important that bacteria associated with beneficial niche and habitat utilization in pigs are isolated and preserved for functional assessments.

Isolation methods with many variations of methodologies and media have historically been used to isolate bacteria from microbiologically complex substrates (Lagier et al. 2015). These methods primarily involve variations on enrichment (Lagier et al. 2015; Ziemer 2014), direct plating (Fenske et al. 2020), membrane filtration (e.g. Ichip) (Nichols et al. 2010), and the differential isolation of endospore-forming taxa (Browne et al. 2016). Fenske et al. (Fenske et al. 2020) evaluated direct plating methods including the use of antibiotics, and heat treating samples to evaluate changes to the diversity of enteric bacteria from feral and industrially raised pigs. However, the comparative examination of methods other than direct plating and enrichment methods is currently lacking.

Using a swine model, I hypothesized that the different isolation techniques applied will recover unique assemblages of bacteria, and that the methods can be performed in concert to maximize the estimation of the diversity of enteric bacteria recovered. Furthermore, by employing a combination of methods, a collection of bacteria that is highly representative of the diversity of taxa present in the intestine can be recovered. To test these hypotheses, the objectives of this study were to: (i) harvest intestinal segments from pigs (i.e. ileum, cecum, ascending colon, and spiral colon) implementing methods that prevent infiltration of ambient oxygen; (ii) isolate bacteria using direct plating, long-term enrichments, a modified Ichip method, and strategies that select for endospore-forming taxa; (iii) contrast isolation in a carbon dioxide- and nitrogen-predominant atmospheres; (iv) apply bioinformatics methods to compare the efficacy of individual isolation methods/conditions; and (v) acquire a comprehensive collection of enteric bacteria that is representative of the diversity of the enteric microbiota for downstream analyses and applications (i.e. as bioresource for functional analyses).

3.2 Materials and methods

3.2.1 Ethics approvals.

Approval to utilize piglets in the study was obtained from the Agriculture and Agri-Food Canada (AAFC) Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (ACC) before commencement of the experiment (Animal Use Protocol #1512). In addition, approval to collect blood from sheep for using in microbiological media (Animal Use Protocols #1922) and to collect rumen fluid from cannulated cattle (Animal Use Protocol #1909) were also obtained from the LeRDC ACC in advance.

3.2.2 Animals and husbandry.

Twenty-four castrated large white Landrace cross piglets (6-wk-of-age) were used in the experiment. The piglets were not exposed to antibiotics at any point, and the dam was not administered antibiotics for at least 1 yr before parturition. Piglets were transferred to the Livestock Containment Unit and allowed to acclimatize for 1 wk within the facility before commencement of the experiment. Animals were provided a minipellet ration diet that was free of antibiotics (Proform Pig Starter 2; Hi-Pro Feeds, Okotoks, AB). Feed was provided daily, and piglets were permitted to eat and drink ad libitum. Straw was used for bedding, and toys were provided for environmental enrichment.

3.2.3 Intestinal sample collection and processing.

Samples were collected from live pigs under general anesthesia. Piglets were initially sedated with ketamine (Vetalar, Fort Dodge, IA) and xylazine (Xylamax, Bimeda, Cambridge, ON) at doses of 22 mg kg^{-1} and 2.2 mg kg^{-1}, respectively. Animals were placed in dorsal recumbancy on a v-trough surgical table, intubated, and general anesthesia was established with isoflurane (Abbott Laboratories, North Chicago, IL) at 1500 ml min^{-1} O_2. The abdomen was disinfected with Stanhexidine (Omega Laboratories Ltd., Montreal, QC), 70% ethanol, and Prepodyne (West Penetone Inc., Ville D'Anjou, QC). A longitudinal ventral laparotomy was established. Segments of intestine (≈10 cm-long) were collected from the ileum, cecum, ascending colon, and spiral colon. To ensure integrity of the intestinal segment (e.g. to prevent infiltration of air into the segment) and to minimize release of digesta, double ligatures were established at the two ends of the segment, and the segment was then excised from the intestine by cutting between the two ligatures. Care was taken to ligate mesenteric blood vessels immediately prior to intestinal segment removal to ensure maintenance of blood flow to adjacent intestinal tissue. The segments were placed in an anaerobic jar (3.5 liter anaerobic jar; HP0011A, Oxoid, Nepean, ON) within 3 to 5 min of removal from the animal, the ambient atmosphere in the jar was removed by vacuum, replaced with N_2 gas, and the segments were transported to the laboratory for processing. Before

placement in anaerobic chamber, samples were placed on ice. The time from sample removal from the animals to its placement in the anaerobic chamber was less than 20 min.

3.2.4 Sample processing.

Ligated intestinal samples were transferred into an anaerobic chamber (Forma 1025 anaerobic chamber, Forma Scientific, Inc., Marietta, OH) containing a predominantly N_2 atmosphere (i.e. 85% N_2, 10% CO_2, and 5% H_2). Once in the chamber, ligatures were aseptically removed, and the intestinal segment was incised to expose the mucosal surface and luminal contents. A 1-cm^2 sample of the intestinal wall was removed and transferred into 5 ml of reduced Columbia broth (CB; HiMedia Laboratories LLC, West Chester PA) contained in a 50 ml Cellstar® polypropylene tube (Greiner Bio One International, Kremsmünster, Austria) (Figure 3.1). The sample was gently inverted 30 times to separate the ingesta from the mucosa. The intestinal segment was then transferred to a new 50 ml tube containing reduced CB, and both tubes were vortexed (high setting for 30 sec). The resultant suspension was used to isolate bacteria through various methods. The tubes containing samples were then placed into an anaerobic jar (Oxoid), the lid sealed, and the jar with tubes was transferred into a separate anaerobic chamber with a predominant CO_2 atmosphere (i.e. 90% CO_2 and 10% H_2). Once in the CO_2 atmosphere chamber, the enteric samples were processed to obtain luminal or mucosal samples for processing as done previously (Moote et al. 2020). Samples were processed for isolation as outlined below.

3.2.5 Bacteriological media.

All media used during this study were reduced before use. Media used included a basal Dehority's medium (Dehority and Grubb 1976) supplemented with xylan or porcine mucus type III (Table 3.1), Columbia broth supplemented with 10% blood, *Bacteroides* Bile Esculine agar with and without 100 µg L^{-1} gentamicin (Table 3.2). To create liquid media, media prepared without the addition of cysteine was autoclaved for 5 min. Once autoclaved, warm media were transferred into a N_2 atmosphere chamber and vigorously agitated to displace oxygen from the media. When media cooled, it was decanted into a media bottle containing the desired cysteine content (1 g L^{-1}) to remove any residual oxygen, the bottle was sealed with a stopper, removed from the chamber, and autoclaved for 30 min at 121°C and 100 kPa. Media prepared for use in the CO_2 atmosphere were prepared in the same fashion; however, 40 ml L^{-1} of 8% sodium carbonate (Sigma-Aldrich Canada, Oakville, ON) was added before autoclaving to prevent acidification.

3.2.5.1 Enrichment broths.

Broths for enrichment were prepared in a similar fashion to liquid media, with the exceptions that after the first 5-min autoclave cycle, 10 ml of the medium was transferred into 15-ml glass tubes (Fisher Scientific, Ottawa, ON). Tubes were sealed with a screw cap, and oxidization was detected by the addition of resazurin (1 µg ml^{-1} final concentration) (Table 3.3). For enrichment cultures containing blood, 10% whole sheep blood was added to liquid media containing resazurin, dispensed into glass tubes, and autoclaved; after autoclaving, the curdling of blood allowed visualization of resazurin oxidation.

3.2.5.2 Agar media.

Media containing agar (1.5% agar; BD Difco, Fisher Scientific) and resazurin (1 µg ml^{-1}; Sigma-Aldrich Canada) were autoclaved for 30 min at 121°C and 100 kPa (Table 3.4). Molten media were dispensed into Petri dishes, and immediately transferred directly into the N_2 and CO_2 atmosphere chambers, and maintained in the chambers for 10 d before use.

3.2.6 Isolation strategy.

A number of isolation methods were used. These included: (i) direct plating; (ii) long-term enrichment; (iii) use of an Ichip adapted for enteric isolations; and (iv) selection of endospore-forming taxa via ethanol treatment and Tyndallization, followed by direct plating and enrichment (Figure 3.1). In all instances, isolations were conducted under strict anaerobic conditions (CO_2 and/or N_2 atmospheres) at 37°C.

3.2.6.1 Direct plating.

The bacterial suspension from intestines in CB was streaked (10 µl) onto various agar media (Table 3.5) within the N_2 or CO_2 atmosphere chambers, and cultures were incubated for 7 d.

3.2.6.2 Enrichment broths.

To generate enrichment cultures, 20 µl of the bacterial suspension from intestines in CB was added to 10 ml of enrichment media (Table 3.6). Cultures were maintained in the N_2 or CO_2 atmosphere chambers for 12 wk.

3.2.6.3 Ichip.

A modified version of Ichip described previously (Berdy et al. 2017) was used (Figure3.2-3.4; Table 3.7). Instead of a three-dimensional printed manifold, we generated a diffusion chamber from a pipette tip holder as done previously (Berdy et al. 2017). We also used a membrane containing 0.02 µm-diameter pores (Sterlitech, Kent, WA), which was sealed to the apparatus bottom using Silicone II sealant (General Electric Company, Faifield, CT). Ichips were then autoclaved in a sealable container and placed in the N_2 and CO_2 atmosphere anaerobic chambers for 24 h before use. Phosphate buffered

saline (1X PBS; 0.01 M sodium phosphate; pH 7.4) amended with 0.5% agarose was reduced by autoclaving material for 5 min at 121°C and then it was quickly transferred into an anaerobic chamber where it was vigorously mixed, and L-cysteine HCl (1 g L^{-1}) was added to assist in its reduction. Once cooled, the liquid was transferred into a clean bottle, sealed with a rubber septum cap, and autoclaved. A 50 µl aliquot of the intestinal sample diluted in reduced PBS with agarose was stained with 50 µl of a solution of trypan blue (0.4%; Sigma-Aldrich Canada). Stained bacterial cells in PBS were enumerated visually using a Petroff-Hausser chamber (VWR International, Randor, PA) at a 100X magnification under bright field and oil emersion using a Zeiss Axioskop 3 (Carl Zeiss Canada Ltd., Toronto, ON). Within the anaerobic chamber, the appropriate volume of the intestinal sample was added to reduced PBS to obtain a target density of one bacterium in 200 µl. The diluted sample (200 µl) was then dispensed into individual chambers of the Ichip apparatus. The top of the chamber was then securely sealed with a BioRad plate seal (Bio-Rad Laboratories, Hercules, CA). The apparatus was placed in fresh rumen fluid obtained from fistulated cattle ensuring that the porous membrane was submerged; the rumen fluid was used as source of nutrients, including rare nutrients and co-factors (Berdy et al. 2017; Caldwell et al. 1966). Ichips were maintained for 12 wk, and the rumen fluid was replaced at 2-wk intervals. After the incubation period, the Ichip was removed from the rumen fluid, residual fluid was removed, the Ichip surface sanitized with 70% ethanol, and the top membrane was carefully removed. A 10 µl subsample was taken from each chamber, and streaked onto reduced Columbia agar (CA; HiMedia Laboratories, Mumbai, India) amended with 10% whole sheep blood (CBA).

3.2.6.4 Isolation of endospore-forming bacteria – ethanol.

The basic method of Browne et al. (2016) (Browne et al. 2016) was used with some modifications. In this regard, intestinal samples were stored at -80°C until processing. In addition, ethanol was filtered before placement in the anaerobic chambers, the YCFA medium used by Browne et al. (Browne et al. 2016) was replaced with CBA medium and cultures were incubated for 7 d (i.e. instead of 3 d), and a 12-wk enrichment was used to isolate bacteria. Frozen samples were thawed in the anaerobic chamber containing a N_2 atmosphere, placed in 1 ml of reduced CB, vortexed (high setting for 30 sec), 0.5 ml of the suspension was transferred to a new sterile vial, and 0.5 ml of reduced 70% ethanol was added to the suspension (1:1 ratio). The ethanol suspensions were incubated for 3-h. Bacteria were recovered by direct plating and enrichment. For isolation using direct plating, 10 µl subsamples were streaked onto CBA containing 0.1% sodium taurocholate (Sigma-Aldrich Canada), and maintained in a N_2 atmosphere anaerobic chamber. In addition, 20 µl subsamples were added to 10 ml of reduced enrichment broth in Hungate tubes. The enrichment media used were Dehority's medium amended with 0.5% xylan (xylan

from beech wood; Sigma-Aldrich Canada) or mucin (porcine mucus III; Sigma-Aldrich Canada), as well as CB with 5% sheep blood. After the enrichment period in the N_2 atmosphere, 10 µl from each culture was streaked onto CBA, and maintained in the N_2 atmosphere as described above.

3.2.6.5 Isolation of endospore-forming bacteria – Tyndallization.

Tyndallization of samples was conducted in concert with the ethanol treatment method. Intestinal samples were placed in a 1.0 ml glass vials (9 mm tubes with 11 mm caps, Fisher Scientific) within the N_2 atmosphere anaerobic chamber. The vials were sealed to maintain samples in a reduced state, removed from the anaerobic chambers, and placed at 100°C for 30 min. Vials were then transferred back to the anaerobic chambers, and bacteria were isolated by direct plating and enrichment as described above for the ethanol treatment.

3.2.7 Recovery and storage of bacteria.

Based on colony morphology, representative colonies were selected, streaked for purity on CBA and maintained in the atmosphere in which they were isolated. Colonies were then streaked for biomass on CBA. After ≥7 d, biomass was removed from the surface of the medium, and transferred to tubes containing 1.5 ml of reduced CB with 40% glycerol. The tubes were then sealed and removed from the anaerobic chambers, snap-frozen on dry ice, and transferred to -80°C for medium-term storage (Table 3.8). In addition, biomass was placed in tubes, and stored at -80°C for subsequent extraction of genomic DNA.

3.2.8 Identification of bacteria.

Biomass for extraction of genomic DNA was thawed at room temperature, after which 300 µl of lysis buffer (Autogen Plant Lysis Buffer 102; Holliston, MA) supplemented with 10 mg ml^{-1} lysozyme (Thermo Fisher Scientific Inc.; Waltham, MA) was added to the biomass for cell lysis. Cells were lysed at 37°C overnight, and then placed at 50°C for 1 h. Genomic DNA was then extracted using an Autogen 740 (AutoGen Inc, Holliston, MA) according to the manufacturer's recommendations. The 16S RNA gene was amplified using the 27F and 1492R primers as previously described (Puhl et al. 2009) and the 16S gene was partially sequenced by Eurofins Genomics (Toronto, ON) using the 27F primers as previously described (Cole et al. 2014; Moote et al. 2020). Sequence chromatograms were visualized, assessed for quality, and trimmed using Geneious software (Geneious v5.3.9; San Diego, CA). Trimming was conducted to ensure chromatogram peaks were of good signal intensity and generally formed individual peaks. Trimmed sequences in fasta format were queried against the RDP [Ribosomal Database Project] database using the sequence match program (SeqMatch; http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Cole et al. 2014) with following settings

selected: strain = both (Type and Non-Type); source = isolates; size = both (>1200 and <1200); quality = good; taxonomy = nomenclature; and KNN matches = 1. Sequences are available in Zeonodo: https://doi.org/10.5281/zenodo.3728174. Representative isolates were accessioned in the Intestinal Bacterial Collection (IBaC) at LeRDC and appropriately stored.

3.2.9 Data analyses.

Bacterial taxa were collated with intestinal location, intestinal sample type (i.e. mucosa-associated or ingesta), isolation atmosphere, isolation method, and isolation medium. Bacterial diversity was determined by Shannon's index of diversity, and community composition was determined using the *vegan* and *pvclust* packages in R (Oksanen et al. 2007; R-Core-Team 2013). Analyses were conducted similarly to that done by Moote et al. (Moote et al. 2020). Detection of the differential abundance of taxa isolated was done via the *LEfSe* methods (Segata et al. 2011). *LEfSe* analyses were done using the *LefSE* tool on the Huttonhower Lab Galaxy instance (http://huttenhower.sph.harvard.edu/galaxy/) (Segata et al. 2011). Dendrograms were generated from the 16S rRNA gene sequences using the *MEGA-X* software package (Kumar et al. 2018), multiple sequence alignments were made using the embedded *MUSCLE* tool, and dendrograms were generated using UPGMA linkage with a bootstrap value of 500. The Newick alignment file was then transferred to FigTree version 1.4.4 to generate the dendrogram (Rambaut 2012). Experimental statistics were evaluated using the *vegan* package in R (Oksanen et al. 2007), and statistical inferences were determined using the Kruskal-Wallis rank sum test in R (R-Core-Team 2013) and the Nemenyi test with χ^2 approximations (Pohlert 2018) to identify significant changes to Shannon's indices. False discovery rates were determined via the q value program in R (Storey et al. 2019).

3.3 Results

3.3.1 A diverse collection of enteric bacteria was obtained from the intestines of pigs.

Overall, 1,523 bacterial isolates were recovered and identified, which represented 234 taxa including 80 genera, and seven phyla (Figure 3.5). Phyla represented were *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Lentispiracea, Proteobacteria,* and *Synergistetes*. The collection was dominated by members of the *Firmicutes* and *Proteobacteria*, which represented 70.8% and 16.9% of the taxa isolated, respectively.

3.3.2 Atmosphere had a minimal influence on the diversity of bacteria isolated.

All isolation methods were conducted within N_2- and CO_2-predominant atmospheres (referred to as N_2 and CO_2 atmospheres hereafter), with the exception of endospore-forming bacteria selection methods (i.e. the Tyndallization and ethanol exposure methods) which were conducted in an N_2

atmosphere to be consistent with that of Browne et al. (Browne et al. 2016). No difference ($P \geq 0.992$) in the diversity of recovered bacteria was observed between methods conducted in the N_2 and CO_2 atmospheres. The UPGMA (Unweighted Pair Group Method with Arithmetic mean) of Euclidian distances were compared using the *pvclust* package in R (Oksanen et al. 2007; Suzuki and Shimodaira 2006), and similarities were observed among isolation methods regardless of the atmosphere used, with the exception of direct plating (Figure 3.6). Bacterial taxa recovered using the Ichip, enrichment, and direct plating methods clustered into discrete groups regardless of isolation atmosphere ($P \geq 0.950$). However, bacterial taxa isolated by direct plating onto CBA or Dehority's agar containing 5% porcine mucus or 5% xylan within the CO_2 atmosphere clustered with taxa isolated by enrichment ($P = 1.000$). In contrast, these collections formed distinct clusters if isolated within the N_2 atmosphere ($P = 0.890$). Given the similarity in isolation of bacteria in the two atmospheres, data were combined across atmosphere and presented as such hereafter.

3.3.3 The isolation strategies used influenced the diversity and composition of bacteria recovered.

Shannon's indices differed ($\chi^2 = 72.2$; $P > 0.010$) among isolation methods and media as determined by the Kruskal-Wallis rank sum test in R (R-Core-Team 2013). However, a post-hoc test (Thorsten 2018) showed no difference ($P \geq 0.160$) among the methods, and this was further supported by a q-test ($q = 1$) (Storey et al. 2019). Comparison of bacterial diversity by isolation strategy (i.e. direct plating, enrichment, Ichip, Tyndallization, and ethanol treatment) across media revealed differences ($P < 0.001$) among the five strategies employed. This was attributed to the low diversity of bacteria recovered by the methods designed to isolate endospore-forming taxa. The diversity of bacteria isolated by long-term enrichment, direct plating, and Ichip methods did not differ ($P \geq 0.989$), yielding a Shannon's index (SI) of ≤ 2.5, ≤ 2.2, and ≤ 1.5, respectively (Figure 3.7; Table 3.9). The diversity of bacteria isolated using the two methods that select for endospore-forming taxa (i.e. ethanol treatment and Tyndallization) did not differ ($P \geq 0.786$) (Figure 3.7), yielding a SI of≤ 0.8 and ≤ 1.3, respectively. The diversity of bacteria isolated using the Tyndallization method was lower than for the enrichment ($P = 0.005$), direct plating ($P = 0.033$), and Ichip ($P = 0.070$) methods. A trend for a lower SI was observed for the ethanol treatment method relative to enrichment ($P = 0.149$).

3.3.4 Bacterial taxa recovered using individual isolation strategies differed.

The evaluation of Euclidian distances (Oksanen et al. 2007) revealed that the different isolation strategies recovered dissimilar bacterial taxa (Figure 3.8). The application of *pvclust* (Suzuki and Shimodaira 2006) confirmed this observation (Figure 3.9). Bacterial taxa isolated by long-term enrichment and direct plating methods clustered together ($P = 0.870$), whereas bacteria recovered using

the Ichip formed a unique clade ($P < 0.050$). Bacteria recovered by Tyndallization and the ethanol treatment clustered together regardless of the isolation method employed post treatment ($P = 1.000$). An examination of the 25 most frequently isolated taxa revealed that *Bacillus* spp. were most commonly isolated using the Tyndallization and ethanol treatment methods (Figure 3.10). In contrast, *Lactobacillus* spp. were most abundantly isolated using the ethanol method. The use of long-term enrichments, direct plating, and the Ichip method resulted in the isolation of diverse genera not within the top 25 taxa recovered (i.e. "other bacteria"). Direct plating methods were commonly associated with the isolation of *Escherichia*/*Shigella* spp. However, the inclusion of gentamicin into CBA for direct plating assisted in the isolation of *Bacteroides* spp. A comparison of the specific recovery of bacteria phyla revealed that the enrichment methods recovered bacteria from all seven phyla (Figure 3.11). Direct plating, the Ichip method, and endospore-selection methods recovered bacteria from six, four, and three phyla, respectively.

To further evaluate the taxa selection by isolation strategy, Linear discriminant analysis Effect Size (*LEfSe*) analysis was applied at a class level of resolution (Segata et al. 2011). *LEfSe* determined that the direct plating method was most successful for isolation of *Erysipelotrichia* and *Gammaproteobacteria* (Figure 3.12). The enrichment methods (enriched on blood, mucus, or xylan) were associated with an increased abundance of *Clostridia*, whereas the Ichip method was associated with increased isolation of *Actinobacteria*. It is noteworthy that of the methods applied to select for endospore-forming taxa, the ethanol treatment strategy yielded more associations of bacteria within the class *Bacilli*, and notably *Lactobacillus* spp., whereas, *Bacillus* spp. were more frequently isolated by Tyndallization.

3.3.5 The isolation methods examined yielded different communities and differing proportions of novel species, genera, and families.

Bacteria representing new species, genera and families were determined through evaluating their similarities to 16S rRNA sequences contained in the RDP; specifically, new species were defined as those being >95 and ≤97% similar, genera being >92 and ≤95% similar, and new families being ≤92% similar to sequences contained in the RDP database. The Ichip method was found to yield the most bacterial taxa belonging to novel families (24.1%) (Figure 3.13). Long-term enrichment yielded large numbers of novel bacteria species, and enrichments containing blood, xylan, and mucus recovered 39.9, 31.1, and 26.8% novel species, respectively. Direct plating on CBA (DP-Blood) was the most effective direct plating medium for isolating novel taxa, and 24.5% of the recovered isolates were identified as novel species. Strategies designed to select taxa that form endospores yielded the least numbers of novel species, with

≤ 21.8% and ≤ 7.2% novel taxa isolated with the ethanol treatment and Tyndallization methods, respectively.

3.3.6 Diversity did not differ between bacterial communities isolated along the gastrointestinal tract.

There were no differences ($\chi^2 = 1.12$; P = 0.773) in diversity of bacteria isolated from the ileum, cecum, ascending colon, and spiral colon (Figure 3.14;Table 3.10). This observation was confirmed by evaluating q-values (q ≥ 0.99) (Storey et al. 2019).

3.3.7 Recovered bacterial taxa differed among intestinal locations.

A comparison of approximately unbiased P-values for UPGMA clustering using the *pvclust* function in R (Suzuki and Shimodaira 2006) revealed that bacterial taxa recovered from the cecum, ascending colon, and spiral colon of pigs were similar to each other (P = 0.990) (Figure 3.15), but were located within a completely different clade compared to isolates obtained from the ileum. *Escherichia*/*Shigella* were frequently isolated from the ileum, whereas *Clostridium sensu stricto*, *Eubacterium*, and *Bacteroides* spp. were more commonly recovered from the large intestine (Figure 3.16). Ancillary analysis of bacterial class abundance using *LEfSe* analyses (Segata et al. 2011) revealed that bacteria belonging to *Bacilli*, *Fusobacteriia*, and *Gammaproteobacteria* were more frequently isolated from the ileum (Figure 3.17). Members of the *Actinobacteria*, *Bacteroidia*, and *Deltaproteobacteria* were more commonly recovered from the cecum, while *Clostridia*, *Negativitiutes*, and *Synergista* were most frequently isolated from the ascending colon.

3.4 Discussion

Diverse bacteria comprise the intestinal microbiota of mammals, and the comprehensive isolation of bacterial taxa is required to fully ascertain their function (Ghimire et al. 2020). In the current study, strict anaerobic conditions and a variety of isolation strategies were applied, including a modified Ichip method, direct plating, and long-term enrichments, along with methods designed to isolate endospore-forming bacteria. The hypothesis erected was that a combination of isolation methods used in concert is necessary to comprehensively recover bacteria from the intestines of swine as a model mammal. The findings of the study supported this hypothesis, and individual techniques yielded objectively different bacteria. In total, 80 genera and 194 species of bacteria were isolated from the porcine intestinal tract, with 24% of the bacteria representing taxa that were previously undescribed when compared to taxa within the RDP database (Cole et al. 2014).

The gaseous atmosphere within the intestinal lumen varies in composition spatially. Gas within the GIT originates from both exogenous and endogenous sources (Mego et al. 2015). In a resting state, the GIT is comprised of ≤10% O_2 (in the proximal GIT), 20-90% N_2, 10-30% CO_2, ≤50% H_2, and ≤10% CH_4

(Calloway et al. 1966). Any residual O_2 that is present within the lumen of the large intestine is rapidly utilized by facultative anaerobes (Lozupone et al. 2012) resulting in an ecosystem that is devoid of O_2 (Hillman et al. 1993). Many enteric bacteria in their vegetative states are exceptionally sensitive to O_2 exposure (Browne et al. 2016). Although substrates such as digesta can remain in a reduced state for a period of time following removal from the intestine (Albenberg et al. 2014), exposure to O_2 in ambient atmosphere can result in death of bacteria, thereby reducing the diversity of bacteria recovered (Browne et al. 2016). For this reason, we employed methods to preclude any exposure of bacteria to O_2. Specifically, we ligated intestinal segments in living animals under general anesthesia, excised the segments and placed the segments in an atmosphere devoid of O_2, transferred the segments to an anaerobic chamber within 60 min of removal of the segments, and processed the samples within a CO_2 or N_2 atmosphere. In contrast to our approach, other researchers such as Fenske et al. (2020) collected luminal contents by squeezing digesta into tubes containing 40% anaerobic glycerol followed by flash freezing of the samples. Although they did successfully isolate a diverse assemblage of bacteria, the opportunity for O_2 infiltration coupled with freezing of samples, could result in the death of bacterial cells (Fenske et al. 2020), particularly of fastidious bacterial taxa present at low cell densities. The care that we extended in precluding exposure to O_2 likely contributed to the high diversity of bacteria that we isolated from the intestine of pigs.

Traditionally the isolation of anaerobic bacteria from the distal intestine of animals has relied on the use of atmospheres that are either CO_2- or N_2-predominant. For example, the original isolation of enteric bacteria from pigs was conducted in an atmosphere comprised of 80% N_2, 10% H_2, and 10% CO_2 (Russell 1979). Fenske et al. (2020) also used a single atmosphere to isolate bacteria from the intestines of pigs. The impacts of atmosphere on bacterial selection as a function of the isolation method employed has not been extensively examined, and we hypothesized that using multiple anaerobic atmospheres for direct plating and enrichment of bacteria would substantially increase the diversity of bacteria recovered. Furthermore, we predicted that the isolation of fastidious bacteria would be favored in the CO_2 atmosphere as a high CO_2 content atmosphere occurs in the intestine (Calloway et al. 1966). However, the composition of bacteria recovered between the CO_2 or N_2 atmosphere using enrichment methods was largely similar. This may be attributed to the production of CO_2 during bacterial fermentation, which would have generated a predominant CO_2 atmosphere within the enrichment broths, thereby negating the impact of the initial N_2 atmosphere on the enrichment medium (Bond and Levitt 1978; Calloway et al. 1966). Consistent with this conclusion, we observed that bacterial taxa isolated on agar media in Petri dishes differed between the N_2 and CO_2 atmospheres, which has been

observed previously (Caspari and Macy 1983). Genera that were isolated more frequently using direct plating within the CO_2 atmosphere included members of the *Clostridium sensu stricto* and *Clostridium* XIVa groups, as well as *Eubacterium*, *Bacteroides*, and *Christensenella* spp. Our findings support the use of a single atmosphere for conducting isolations using the Ichip and enrichment methods (i.e. the isolation method and not atmosphere was associated with the composition of bacteria isolated); however, isolations using direct plating should employ both atmospheres.

Enrichment isolation is a frequently used method to isolate bacteria from microbiologically-complex samples. A major limitation of enrichment isolation is that fast-growing taxa and subtypes within taxa can be differentially favored, which can result in a reduced diversity of bacteria recovered. In the current study we used long-term enrichments, and we observed that this strategy recovered the most diverse collection of bacteria. This result was unexpected as more modern methods, such as the Ichip or those that select for endospore-forming bacteria were anticipated to outperform traditional isolation methods such as enrichment (Berdy et al. 2017; Browne et al. 2016). A possible explanation for the high diversity of bacteria that we recovered using enrichment is the extended duration of the enrichment step that we employed (12-wk) coupled with the diversity of media used. It is noteworthy that the 12-wk enrichment period that we used is substantially longer than the ≤6 wk period typically reported in the literature (Lagier et al. 2016), and previous research using an 8-wk enrichment period for cattle feces recovered a similarly high diversity of bacteria (Ziemer 2014). However, the 8-wk enrichment period employed by Ziemer et al. (Ziemer 2014) recovered novel bacteria at a frequency rate of 98%, which was higher than the 39% rate in the current study; this may be attributed to his utilization of continuous culture as compared to the static enrichment (Ziemer 2014). The media used for enrichments in the current study were supplemented with blood, mucus, or xylan. These amendments were selected for individual reasons including: (i) their role as an anti-nutritive component of the pig diet that has been shown to be effective at isolating novel bacteria during enrichments (i.e. xylan) (Tapingkae et al. 2008; Ziemer 2014); (ii) their routine use for isolating bacteria (i.e. blood) (Diakite et al. 2019; Zheng 1987); and (iii) because they provide limiting glycans within the intestinal environment (i.e. mucus) (Quintana-Hayashi et al. 2018). Enrichment has been used to isolate novel bacteria in culturomics applications, and the high diversity of bacteria that we recovered from the intestine of pigs using long-term enrichments validates the effectiveness of this strategy, and emphasizes the importance of extending the duration of the enrichment period.

Many bacterial taxa within the *Firmicutes* form endospores, and these taxa are difficult to isolate due to poor endospore germination coupled with the predominance of non-endospore-producing

bacteria, which obscure colonies emanating from endospores. The use of methods that select for endospore-forming bacteria has been promoted as a way to improve the isolation of novel taxa (Browne et al. 2016). We applied the ethanol treatment method described by Browne et al. (Browne et al. 2016) as well as Tyndallization. The Tyndallization method was originally developed as a method to kill endospores in foods (Gould et al. 1968). The method works by killing vegetative cells and stimulating the germination of endospores by heat exposure at atmospheric pressure. The Tyndallization method sterilizes foods via the use of a second heat treatment to kill recently germinated vegetative cells. To prevent sterilization of samples in the current study, samples were processed after the first heat treatment. Both of the methods employed in the current study differentially kill vegetative cells; specifically, ethanol denatures proteins (Toca-Herrera et al. 2004) and impacts lipid membranes (Gold et al. 1992), whereas exposure to high temperatures at ambient pressures kills vegetative cells by denaturation of proteins and enzymes, such as DNA polymerase (Kiefer et al. 1997; Speck and Adams 1976). We observed that both of the methods, regardless of the post-isolation strategy applied, isolated similar bacterial taxa, and overwhelmingly isolated bacteria known to produce endospores. Most notably, *Bacillus licheniformis* was frequently isolated and represented ≈50% of the bacteria recovered.

Thermostable bacteria, such as *Bacillus* spp.*,* have long been observed to withstand, germinate, and proliferate after initial heat treatment (Curran and Evans 1945). These organisms have been shown to be extremely heat stable and as such, their outgrowth within these cultures was expected (Coorevits et al. 2011). In contrast, the use of ethanol has been shown to inhibit endospore germination, suggesting that *Bacillus* spp. isolated may have been present as both endospores and vegetative cells (Curran and Knaysi 1961). We observed that diversity of bacteria isolated following ethanol treatment was higher than following Tyndallization suggesting that the heat treatment did not appreciably stimulate endospore germination. Evidence indicates that *B. anthracis* forms endospores upon exposure to oxygen as an extra-intestinal persistence mechanism (e.g. upon defecation from the host), and within the large intestine exists primarily as vegetative cells (Roth et al. 1955). In contrast, the sporulation of other anaerobic bacteria (e.g. *C. ramosum*), has been linked to incubation time, and their sporulation has been linked to the presence of specific amino acids, namely L-alanine, L-arginine, and L-phenylalanine (Hitzman et al. 1957). It is noteworthy that *Lactobacillus johnsonii*, a non-endospore-forming bacterium was commonly isolated using the ethanol method, but was absent from Tyndallized samples. A tolerance to ethanol has been observed within *Lactobacillus* spp. (Gold et al. 1992), which may explain the isolation of *L. johnsonii* using the ethanol method. Although we did not isolate *Lactobacillus* spp. from heat treated samples, Fenske et al. (Fenske et al. 2020) frequently isolated

lactobacilli (e.g. *Lactobacillus reuteri*) from Tamworth pigs after exposure of digesta samples to heat followed by direct plating. It is noteworthy that they also isolated *Lactobacillus* spp. through traditional direct plating strategies, such as when using De Man, Rogosa and Sharpe (MRS) agar. In contrast to the report of Browne et al. (Browne et al. 2016), the diversity of bacteria that we recovered using endospore-selections was comparatively low. Fenske et al. (Fenske et al. 2020) similarly observed that the application of the heat treatment method resulted in a change in the community of bacteria isolated compared to other traditional direct plating strategies. In the current study, bacterial isolates were selected based on colony morphology, and were subsequently identified by sequencing the 16S rRNA gene of isolated bacteria. This may have influenced the diversity of bacteria that we observed for the ethanol exposure and Tyndallization methods. In this regard, the utilization of methods that allow for the screening of a larger number of isolates, such as MALDI-TOF, may identify novel endospore-forming taxa that are present at low frequencies (Seng et al. 2010). Although the diversity of bacteria isolated by endospore-selective methods was low relative to other methods examined in the current study, new taxa were recovered at rates of 21.8% and 7.1% for the ethanol and Tyndallization methods, respectively. This illustrates the importance of using methods that select for endospore-forming taxa, which may be enhanced with the use of characterization strategies such as the MALDI-TOF (De Bruyne et al. 2011; Seng et al. 2010). Importantly, our findings show that relying exclusively on methods that select for endospore-forming taxa alone is insufficient to isolate bacteria that are representative of the taxa present in the intestine of pigs and other animals.

The Ichip method has been shown to efficiently isolate novel taxa from soil and other environments, including from the oral cavity of human beings (Ling et al. 2015; Sizova et al. 2012). A salient advantage of the Ichip method is that slow-growing and nutritionally fastidious bacterial taxa are allowed to proliferate in the enrichment medium independent of other faster growing or less fastidious bacteria, thereby facilitating their isolation. We adapted the Ichip method for the isolation of enteric bacteria by modifying the enrichment material for their isolation. The use of rumen fluid has not been previously used with the Ichip, and this supplement/additive is a source of rare-occurring vitamins and co-factors, and other nutrients that are required for growth of fastidious bacteria (Caldwell and Bryant 1966; Lagier et al. 2016). Fresh rumen fluid can be easily harvested in relatively large quantities from cannulated cattle, and nutrients are abundant in the liquid fraction (i.e. as compared to pig feces or digesta obtained from the large intestine). Furthermore, nutrients from the distal GIT of pigs (e.g. cecum, ascending colon, and spiral colon) would be expected to contain fewer nutrient factors, and feces even less due to their assimilation by microorganisms and the host. Using the Ichip method,

relatively diverse bacterial taxa were isolated from the intestines of pigs, but the bacteria isolated represented only four of the seven phyla recovered in the study. Within these four phyla however, 54 bacterial species were previously uncultured relative to taxa within the RDP database (Cole et al. 2014). Using the Ichip method, 26% of recovered bacterial isolates from the intestines of pigs were previously uncultured taxa. Although the value of the Ichip method for isolating novel bacteria from a variety of substrates has been previously demonstrated (e.g. soil and the human oral cavity) (Berdy et al. 2017; Ling et al. 2015; Sizova et al. 2012), we applied the Ichip to isolate bacteria from intestines. Although a number of novel taxa were isolated using the modified Ichip in the current study, the method was substantially more labor intensive as compared to other methods such as long-term enrichment. Moreover, used on its own, the Ichip method did not reveal the full diversity of bacteria present in the intestine of pigs. Thus, the method should be used in combination with other isolation strategies.

The direct plating method is commonly used to isolate bacteria from the intestines of animals. We observed that the composition of the bacteria recovered by direct plating conducted in the CO_2 atmosphere was similar to long-term enrichment; however, the diversity of bacteria isolated by direct plating was reduced. As discussed above, we observed that the direct plating method was more affected by the atmosphere than any other method, and the composition of bacteria isolated by direct plating within the CO_2 atmosphere was similar to that obtained by long-term enrichment. The direct plating method possesses many advantages, but also disadvantages. Prominent advantages are the relative ease of conducting the method, the ability to incorporate specific selective factors into the media (Fenske et al. 2020), and that colony forming units are generated, which can facilitate the isolation of multiple taxa from the same culture. Salient disadvantages of the method are that fast-growing taxa often prevent the isolation of slow growing bacteria, and growth factors from the sample matrix are introduced onto the medium, which can subsequently obscure the selective nature of growth factors introduced into the medium (e.g. carbohydrates), particular at low dilutions. In an attempt to optimize the isolation of slower growing bacteria, we maintained cultures for 7 d. We also utilized a variety of growth factors in media. In this regard, direct plating onto media containing 100 μg ml^{-1} gentamicin sulfate altered the composition of bacteria collected, largely because of the selection of *Bacteroides* spp. This is expected as the use of gentamicin at 200 μg ml^{-1} is often used for this purpose (Livingston et al. 1978). It is noteworthy that we chose to use a lower concentration of gentamicin in an attempt to isolate members of the *Firmicutes* phylum, such as *Clostridium* and *Megasphaera* spp., which was also observed previously in chickens (Moote et al. 2020). Although the diversity of bacteria recovered by direct plating in the current study was found to be lower than the enrichment or Ichip methods, the use

of a variety of media resulted in the isolation of bacteria representing six of the seven phyla recovered. Our findings show that direct plating recovered unique taxa, but if the goal is to isolate bacteria that represent the diversity of taxa present in the intestine, then relying on direct plating alone is inadequate.

Characterization of bacteria using next-generation sequence (NGS) analysis has shown that the structure of communities differs between the distal small intestine and large intestine of mammals (Crespo-Piazuelo et al. 2018; Quan et al. 2019). Using culturomics, it was also observed that the composition of isolated bacterial taxa differed in the ileum in comparison to the cecum, ascending colon, and spiral colon of pigs. This variation in bacterial structure is due in part to the higher concentrations of O_2 in the small intestine (Hillman et al. 1993). We observed that the cultural microbiota of the ileum of swine was predominated by bacteria within the *Proteobacteria*, which is consistent with NGS results (Isaacson and Kim 2012). The growth of *Proteobacteria* in the small intestine is favored by their ability to utilize O_2 as a terminal electron acceptor (i.e. many taxa are facultative anaerobes), coupled with their relative tolerance to acidic conditions (Benjamin and Datta 1995; Litvak et al. 2017). We also isolated *Firmicutes* bacteria from the ileum. Bacteria belonging to the *Firmicutes* are more prevalent in the ileum than the jejunum, possibly as a result of the introduction of bacteria into the ileum from the cecum via the ileal-cecal junction (Brown et al. 2018). At present it is unclear if *Firmicutes* taxa are autochthonous in the ileum, and if they have a important functional role in the distal small intestine of pigs. Although the merits of metagenomics approaches are recognized, the comprehensive isolation of bacteria from the GIT is crucial to elucidate their ecological role in the intestine, including niches that individual taxa, and subtypes within taxa, occupy.

The establishment of anaerobic bacterial collections requires considerable microbiological expertise and a specialized infrastructure to isolate, characterize, and store diverse taxa. Moreover, the maintenance of culture collections requires a significant expenditure in operational funds. Over the past decade, emphasis has shifted away from culture-dependant methods towards the characterization of enteric communities using NGS analysis (Lagier et al. 2012). It is noteworthy that the structure of the intestinal microbiota of pigs infected with *Salmonella enterica* Typhimurium examined by NGS and culturomic characterization did not correspond, and that bacteria possibly conferring colonization resistance were not detected by NGS (Bescucci et al. 2020). More recently, research has been directed to utilizing advanced methods to elucidate function such as transcriptomics and metabolomics. Although these methods have provided key information on community function, access anaerobic bacterial culture collections allows complementary research to further ascertain function. For example,

genetic variation among strains in relation to function, and empirical evaluation using gnotobiotic animal models to provide information that cannot be obtained using metagenomics methods. However, the use of metagenomic assembled genomes is closing this gap as it enables the "binning" of specific nucleotide segments to generate and construct genomes *in silico* enabling the identification of specific genes associated with bacterial taxa (Wang and Gänzle 2019). Crucial to empirical functional assessments is access to bacteria that represent the diversity of autochthonous taxa that exist in the intestine, including from specific locations/habitats. This requires the concerted application of culturomic methodologies. The findings of the current study show that diverse bacteria can be successfully recovered from the intestines of pigs (i.e. as a monogastric mammalian model). However, reliance on a single or limited number of isolation methods is insufficient, and it is necessary to utilize a number of strategies in concert. In this regard, we applied strict anaerobic microbiological methods, and used a number of isolation strategies, including direct plating, enrichment, Ichip, and ethanol treatment and Tyndallization to differentially recover endospore-forming taxa. Using these methods, we recovered in excess of 200 species of bacteria belonging to seven phyla, including <107 previously undescribed taxa. There are 500 to 1000 bacterial species present in the GIT of human beings (Rajilic-Stojanovic and de Vos 2014; Sankar et al. 2015; Zou et al. 2019). Although a relatively limited number of taxa are thought to contribute disproportionately to numbers of bacterial cells present in the intestine, bacterial abundance is often not correlated with function (Hajishengallis and Lamont 2016; Rolig et al. 2015). This emphasizes the importance of continuing efforts to isolate, characterize, and store bacterial taxa present in the GIT as a bioresource for functional analyses. It is noteworthy that the cost of completing the isolations, identifications, and subsequent storage of bacteria in the current study is in line with the cost of completing advanced molecular-based approaches (i.e. metagenomic assembled genomes). Even with rapid advancement of culture-independent technologies, and the expected decrease in cost of conducting these analyses, the acquisition of actual bacteria is necessary to fully elucidate function toward innovation achievement (i.e. in conjunction with "omic" approaches).

3.5 Tables and figures

Table 3.1 Dehority's medium.

Ingredient[a]	Amount
Carbohydrate	5 g L^{-1}
Tryptone	4.5 g L^{-1}
Yeast extract	0.5 g L^{-1}
Mineral 1[b]	40 ml L^{-1}
Mineral 2[c]	40 ml L^{-1}
Hemin (0.01%)[d]	10 ml L^{-1}
SCFA solution[e]	10 ml L^{-1}
Resazurin solution (25 mg ml^{-1})	1 ml L^{-1}
L-Cysteine HCl	1 ml L^{-1}
Clarified rumen fluid[f]	50 ml L^{-1}

[a]To prepare as a solid medium, add agar (15 g L^{-1}).
[b]Mineral 1: K_2HPO_4 (6 g L^{-1})
[c]Mineral 2: KH_2PO_4 (6 g L^{-1}); $((NH_4)_2)SO_4$ (6 g L^{-1}); NaCl (12 g L^{-1}); $MgSO_4$ (2.45 g L^{-1}); and $CaCl_2 \cdot 2\ H_2O$ (1.69 g L^{-1})
[d]Hemin was dissolved in 1 M NaOH and then diluted to the appropriate concentration
[e]SCFA (short chain fatty acid) solution: acetic acid (6.85 ml L^{-1}); propionic acid (3 ml L^{-1}); butyric acid (1.85 ml L^{-1}); isobutyric acid (0.5 ml L^{-1}); 2-methyl butyric acid (0.55 ml L^{-1}); N-valeric acid (0.55 ml L^{-1}); and isovaleric acid (0.55 ml L^{-1})
[f]Clarified rumen fluid was prepared by centrifuging to rumen fluid to remove debris and gently decanting in a fresh vial for storage at -20°C.

Table 3.2 *Bacteroides* bile esculin agar.

Ingredient[a]	**Amount**
Tryptic soy agar	45 g L^{-1}
Oxgall	20 g L^{-1}
Esculin	1 g L^{-1}
Ferric ammonium citrate	0.5 g L^{-1}
L-Cysteine HCl	1 g L^{-1}
Hemin (0.01%)	10 ml L^{-1}
Gentamicin sodium salt (100 mg ml^{-1})	1 ml L^{-1}

[a]Adapted from Livingston et al. (Livingston et al. 1978).

Table 3.3 Preparation of enrichment media.

Step	Procedure
1. Prepare medium	Prepare the medium to the desired formulation. DO NOT add cysteine at this step.
2. Autoclave	Autoclave at 121°C at ≥100 kPa for 5 min to boil the medium.
3. Reduce medium	Gently remove the medium from autoclave and very tightly seal media bottle. Transfer the medium into the anaerobic chamber. Watch the medium during transfer, as the lid can loosened from the media bottle in the interchange if not adequately sealed. Open the media bottle in chamber and transfer the medium between two bottles, using a funnel that has been in the interchange for more than 24 h. Repeat this process a number of times (≈10 times). Repeated mixing of this hot liquid in an anaerobic environment will ensure the reduction of the medium as bubbles allow the escape of oxygen from the solution. When satisfied, transfer the medium into a 1 L wide mouth jar containing 1 g L^{-1} L-cysteine HCl and allow the medium to cool in this container. Once the medium has cooled (≈1 h), use a 60 ml syringe to transfer 10 ml of the medium into 15-ml glass tubes and cap with an appropriate lid. Both Hungate style lids and traditional lids with no septum can be used. However, when using traditional lids, ensure the lid is sealed tightly as there is no septum to ensure that air is not transferred into medium.
4. Re-autoclave medium	Remove sealed tubes of the medium from anaerobic chamber and transfer to autoclave. Heat the medium at 121°C at ≥100 kPa for 30 min to sterilize.
5. Cool medium	Cool the medium on the bench top overnight. Ensure the medium is anaerobic by verifying no color change has occurred (i.e. resazurin turning pink).
6. Storage	Media prepared this way can be stored out of the chamber nearly indefinitely.
	Notes
Hungate lids	Hungate lids are the gold standard; however, they are difficult to remove within the chamber, and it is difficult to ensure sterility, as the septum can separate from the tube. This is a main reason why using the traditional caps is recommended. The advantage of Hungate lids are that samples can be injected into the tubes directly using a syringe.
CO_2 atmosphere	When preparing the medium for use in a CO_2 atmosphere, add an appropriate amount of 8% Na_2CO_3 to achieve the desired pH. This will buffer the medium ensuring that it is not too acidic once reduced. Use 40 ml L^{-1} of 8% Na_2CO_3. It is important to buffer media as pH can change depending on the CO_2 chamber and gas mixture.
Measuring pH	The easiest way to pH liquid media is to use pH paper, such as Fisherbrand pH Test Paper Rolls (Fisher Scientific, Hampton, NH). Twenty four h after the medium has been in the chamber, aseptically place a small aliquot of the medium onto a ≈2 cm piece of pH paper, and determine the pH based on the color chart provided with the paper. It is important to evaluate the pH because the CO_2 in the chamber will diffuse into the medium and it will become acidic. The use of Na_2CO_3 will buffer the medium, but the pH should always be confirmed as above.

Table 3.4 Preparation of agar media.

Step	Technique
1. Prepare medium	Prepare the medium to the desired recipe, pH, and then add agar and L-cysteine.
2. Autoclave	Autoclave at 121°C at ≥100 kPa for desired length of time.
3. Pour medium	Pour the medium on the bench top.
4. Transfer medium	Transfer the medium directly within the anaerobic chamber before it becomes solid as this will assist in the reduction of the medium as it cools.
	Notes
CO_2 atmosphere	When preparing media for use in a CO_2 atmosphere, add an appropriate amount of 8% Na_2CO_3 to the medium to achieve the desired pH before autoclaving. This will buffer the medium ensuring that it is not too acidic once reduced. Use 40 ml L^{-1} of 8% Na_2CO_3. It is important to pH medium as this can change depending on the CO_2 chamber and gas mixture.
Measuring pH	The easiest way to pH agar media is to use pH paper, such as Fisherbrand pH Test Paper Rolls (Fisher Scientific, Hampton, NH). Let the medium equilibrate in the chamber for 24 h. Then place a ≈2 cm piece of pH paper directly upon the surface of the medium until a color change occurs, and determine the pH based on the color chart provided with the paper. It is important to evaluate the pH of media because the CO_2 in the chamber will diffuse into the medium and it will become acidic. The use of Na_2CO_3 will buffer the medium, but the pH should always be confirmed as above.

Table 3.5 Agar media (all containing 1.5% agar) used to isolate bacteria by direct plating.

Medium	Abbreviation
Columbia broth with 10% sheep's blood	Blood
Bacteroides bile esculin agar	BactBileEsc
Bacteroides bile esculin agar with 100 µg ml^{-1} gentamicin	BBEGent
Dehority's agar with 0.5% xylan	Xylan
Dehority's agar with 0.5% porcine mucus	Mucus

Table 3.6 Liquid media used to enrich bacteria.

Medium	Abbreviation
Columbia broth with 10% sheep's blood	Blood
Dehority's medium with 0.5% xylan	Xylan
Dehority's medium with 0.5% porcine mucus type III	Mucus

Table 3.7 Creation of the Ichip apparatus, maintenance of the Ichips in rumen fluid, and isolation of bacteria from the Ichip apparatus. Adapted from Berdy et al. (Berdy et al. 2017).

Step	Technique
1. Score Ichip apparatus	To assist with adherence of glue to the Ichip apparatus, gently score an epMotion pipette tip holder (i.e. Ichip apparatus) (Figure 3.2) with 150 grit sandpaper.
2. Clean Ichip	Use ethanol and a clean cloth to wipe clean the Ichip.
3. Set membrane	Cut the 0.02 µm membrane (Sterlitech, Kent, WA) to fit the bottom of the Ichip apparatus. The membrane should be attached to the surface of the apparatus with "feet" (Figure 3.3). Use a popsicle stick to gently spread silicone (Silicone II sealant; General Electric Company, Faifield, CT) over the holder. Ensure full coverage of the apparatus with a relatively thin layer of silicone. Obtain the pored membrane, which is sandwiched between two pieces of tissue paper. Remove the paper from one side to expose the membrane, and carefully adhere the exposed membrane to the siliconed surface of the apparatus. Secure the membrane to the apparatus using a wood splint ensuring integrity of the bond with the apparatus. To facilitate this, start at one end of the apparatus, and carefully move along the apparatus exerting sufficient pressure to ensure a good seal but not to damage the membrane. The membrane should be set as smoothly as possible. Take care to ensure that the 'feet' do not prevent full adherence of the membrane to the apparatus surface (Figure 3.3). Allow the silicone membrane bond to set over night. Once dry, place the Ichip holder membrane side up and autoclave in a shallow sealable container. Once autoclaved, the Ichip can be stored.
4. Preparation of samples	Dilute the intestinal sample in reduced phosphate buffered saline (0.01 M sodium phosphate; pH 7.4) amended with 0.5% agarose. Remove an aliquot and mix add trypan blue (0.4%) at a 1:1 ratio. Load the stained sample into a Petroff-Hausser chamber, and count the density of stained cells using a microscope at 100X magnification under bright field. Within the anaerobic chamber, add the appropriate volume of the intestinal sample to reduced PBS so that 200 µl contains a target density of one bacterium.
5. Inoculation	Pipette 200 µl of the diluted sample into each chamber of the Ichip. Exercise care to not to damage the membrane.
6. Sealing the Ichip	Once the sample has been added to the chambers, seal the top of the Ichip with a BioRad Microseal 'B' PCR plate sealing film (i.e. top membrane; BioRad Laboratories, Hercules, CA). Place two Ichips into an incubation container (Figure 3.4). Label the incubation container, and the top membrane of each Ichip.
7. Rumen fluid	Filter freshly collected rumen fluid through cheese cloth outside of the anaerobic chamber. In the anaerobic chamber, carefully add rumen fluid to the incubation container, ensuring that the bottom membrane is submerged in the fluid (Figure 3.4). Refresh rumen fluid on a weekly basis.

8. Removal of samples from the Ichip	After 12 weeks, remove the Ichips from rumen fluid using a metal forceps, place the Ichips on paper towel, and allow residual rumen fluid to absorb into the paper. Use ethanol to clean/sanitize the surface of the Ichip. Carefully detach the BioRad membrane from the top of the Ichip. Using a 1-µl loop, remove liquid from each well, streak in duplicate onto Columbia blood agar (CBA). **Note:** do not process samples from the chambers at the outer periphery of the Ichip to guard against possible contamination of the chambers by ruminal bacteria (see peripheral chambers that are not fully sealed in Fig 3.4).
9. Incubate and culture	Incubate CBA cultures for 7 d at 37°C. **Note:** the goal is to generate streaks with a single colony morphology. Process cultures that produced more than one colony morphology with caution (i.e. either the sample contained ≥ two cells of different taxa, or the chamber became contaminated). Streak individual colonies onto CBA in small Petri dishes, to ensure purity.
10. Storage	Generate glycerol stocks of these isolates by flooding CBA with Columbia broth containing 40% glycerol and suspend biomass. Collect suspension and store in 2-ml snap cap tubes at -80°C until identified and accessioned into a collection.

Table 3.8 Preparation of a glycerol medium for low temperature storage of anaerobic bacteria.

Step	Technique
1. Prepare medium	Place required Columbia broth medium (17.5 g for 500 ml final volume) in a 1 L media bottle. Add 300 ml deionized H_2O to the powdered medium. Add 200 ml glycerol (final concentration 40% v/v). Add resazurin to the medium (0.001 g L^{-1}).
2. Autoclave	Autoclave at 121°C at ≥100 kPa for 5 min to boil the medium, and ensure the medium is fully dissolved.
3. Reduce medium	Gently remove the medium from the autoclave, and tightly seal the caps of the media bottles. Transfer the media bottles into the anaerobic chamber. Watch the medium during transfer as the lids can loosen within the interchange (i.e. if the caps are not adequately tightened ensuring a complete seal). Open the media bottles in the chamber, and transfer the hot medium to an empty media bottle using a funnel. Repeat the transfer between the bottles ≈10 times. **Note:** transferring the hot medium in the anaerobic atmosphere will ensure the reduction of medium as bubbles allow the escape of oxygen from the solution. When satisfied, transfer the medium into a 500 ml bottle containing 0.5 g L^{-1} cysteine to further reduce the medium. When cooled (≈1 h at room temperature), tightly seal the bottle using a PYREX media bottle septum (Fisher Scientific, Hampton, NH).
4. Re-autoclave medium	Remove sealed media bottles from the anaerobic chamber, and autoclave at 121°C at ≥100 kPa for 30 min to sterilize.
5. Storage	Store the medium within the anaerobic chamber. Although the septum should be relatively airtight, maintain the medium in the anaerobic chamber as a precaution.
	Notes
CO_2 atmosphere	When preparing media for use in a CO_2 atmosphere, add an appropriate amount of 8% Na_2CO_3 to the medium to achieve the desired pH. This will buffer the medium ensuring that it is not too acidic once reduced. Use 40 ml L^{-1} of 8% Na_2CO_3. It is important to check the pH of media as this can change depending on the CO_2 chamber and gas mixture.
Measuring pH	The easiest way to pH liquid media is to use pH paper, such as Fisherbrand pH Test Paper Rolls (Fisher Scientific, Hampton, NH). Twenty four h after the medium has been in the chamber, aseptically place a small aliquot of the medium onto a ≈2 cm piece of pH paper, and determine the pH based on the color chart provided with the paper. It is important to evaluate the pH because the CO_2 in the chamber will diffuse into the medium and it will become acidic. The use of Na_2CO_3 will buffer the medium, but the pH should always be confirmed as above.

Table 3.9 Shannon's index of diversity of bacteria isolated using different isolation strategies and media in nitrogen-and carbon dioxide-predominant atmospheres.

	N_2 atmosphere[a]			CO_2 atmosphere[b]			N_2 & CO_2 atmosphere		
Method[c]	Mean[d]	SD[e]	n[f]	Mean[d]	SD[e]	n[f]	Mean[d]	SD[e]	n[f]
DP-BactBileEsc	0.56	0.64	17	0.52	0.35	7	0.70	0.58	24
DP-BBEGent	1.14	0.85	38	0.37	0.63	5	1.34	0.82	43
DP-Blood	1.20	0.72	29	0.73	0.54	20	1.48	0.62	49
DP-Mucus	1.29	0.72	41	1.01	0.81	19	1.53	0.65	60
DP-Xylan	0.86	0.89	24	0.98	0.55	19	1.34	0.90	43
Enrich-Blood	1.05	0.54	87	2.03	0.40	111	2.28	0.29	198
Enrich-Mucus	1.55	0.86	133	2.31	0.36	151	2.47	0.90	284
Enrich-Xylan	1.31	0.74	91	1.92	1.00	128	2.07	1.09	219
EtOH-DP-Blood&Bile	1.30	0.67	142	---	---	--	1.30	0.67	142
EtOH-Enrich-Blood	1.08	0.43	41	---	---	--	1.08	0.43	41
EtOH-Enrich-Mucus	0.68	0.80	39	---	---	--	0.68	0.80	39
EtOH-Enrich-Xylan	1.09	0.72	56	---	---	--	1.09	0.72	56
Ichip-Blood	1.96	0.70	146	1.33	0.88	28	2.17	0.50	174
Tyn-DP-Blood&Bile	0.82	0.63	85	---	---	--	0.82	0.63	85
Tyn-Enrich-Blood	0.45	0.31	23	---	---	--	0.45	0.31	23
Tyn-Enrich-Mucus	0.34	0.37	13	---	---	--	0.34	0.37	13
Tyn-Enrich-Xylan	0.66	0.04	5	---	---	--	0.66	0.04	5

[a]Nitrogen-predominant atmosphere.
[b]Carbon dioxide-predominant atmosphere.
[c]DP, direct plating; BactBileEsc, *Bacteroides* bile esculin agar; BBEGent, *Bacteroides* bile esculin agar with gentamicin; Blood, Columbia medium with 10% sheep's blood; Mucus, modified Dehority's medium with 5% porcine mucus III; Xylan, Dehority's medium with 5% xylan; Enrich, 12-week enrichment culture; EtOH, samples exposed to ethanol (70%); Blood&bile, Columbia medium with 10% sheep's blood and 0.1% taurocholic acid; and Tyn, samples exposed to 100°C (i.e. Tyndallization).
[d]Mean Shannon's index among replicate animals.
[e]Standard deviation of Shannon's index.
[f]Number of isolates evaluated

Table 3.10 Shannon's index of diversity of bacteria isolated from the intestines of pigs.

	Shannon's Index		
Site[a]	**Mean[b]**	**SD[c]**	**n**
Ile	1.84	1.10	419
Cec	2.01	0.74	330
Asc	1.73	0.94	266
Spi	2.03	1.01	483

[a]Ile, ileum; Cec, cecum; Asc, ascending colon; and Spi, spiral colon.
[b]Mean Shannon's index among replicate animals.
[c]Standard deviation of the Shannon's index.

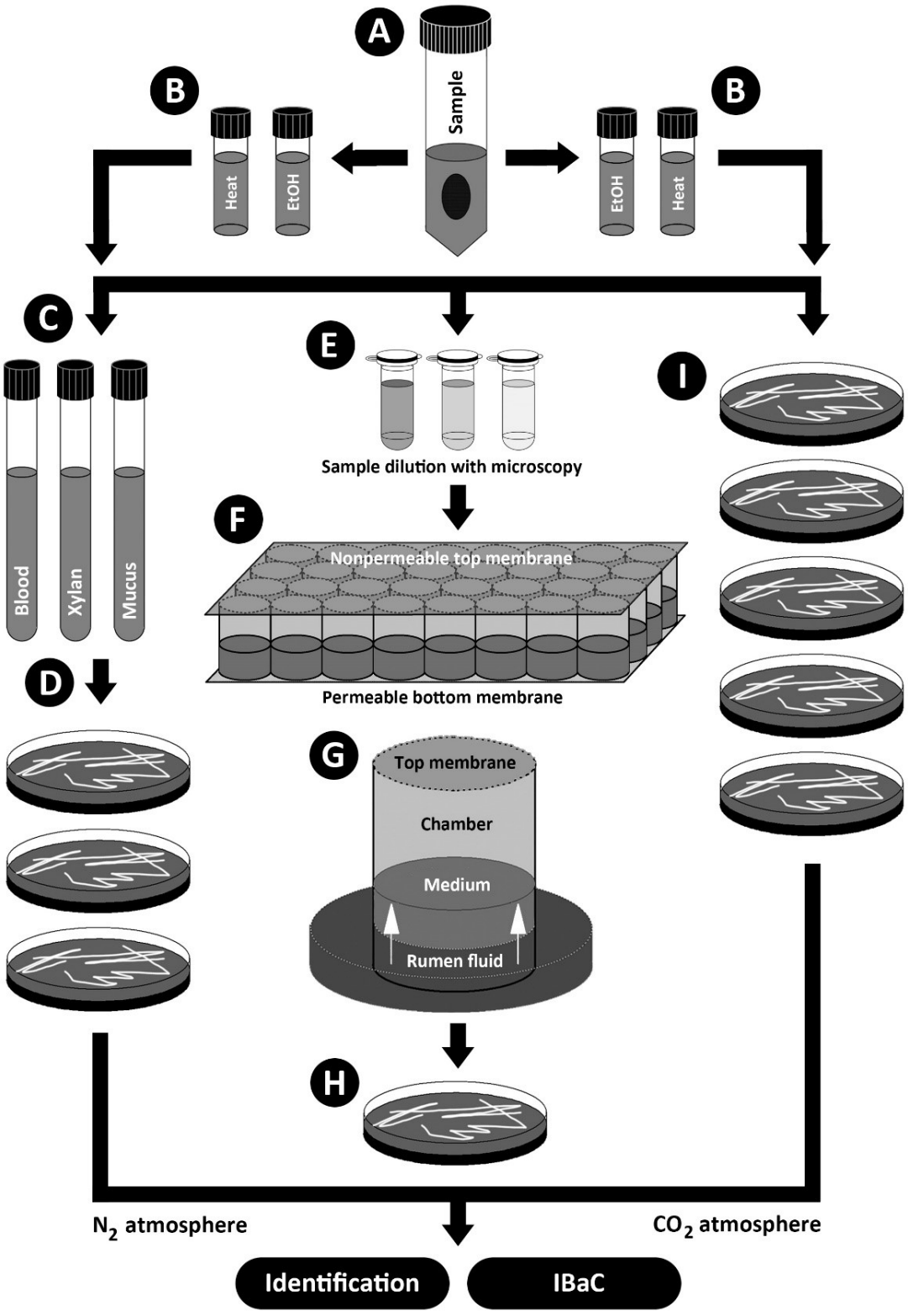

Figure 3.1 Methods used to isolate anaerobic bacteria from the intestines of pigs. (A) Intestinal segments ligated and removed from live animals, transferred to a strict anaerobic environment, liberation of mucosal and luminal contents by washes in reduced Columbia broth washes, followed by

homogenization. (B) Aliquots were exposed to ethanol or heat, and processed by enrichment and direct plating, along with samples not exposed to ethanol or heat. (C) Long-term enrichments (12 wk) into basal media amended with blood, xylan, or mucus at 37°C in anoxic atmospheres. (D) Streak plating of the enrichment broths onto basal media containing blood, xylan, or mucus. (E) Dilution of the bacterial suspension within the homogenate to elimination (determined by light microscopy using a Petroff-Hausser chamber). (F) Addition of the appropriate dilution into a Columbia basal medium containing blood within individual cells of the Ichip apparatus, followed by adherence of the top non-permeable membrane to the apparatus. (G) Placement of the Ichip apparatus into fresh rumen fluid; the rumen fluid served as a source of nutrients (e.g. cofactors and vitamins), which were able to diffuse into the medium within the Ichip chamber via 0.02 µm diameter pores in the bottom membrane (i.e. but prevent the passage of bacteria). (H) After 12 wk, the medium in individual Ichip chambers was streaked onto Columbia blood agar. (I) Streak plating of the homogenate onto variety of agar media (see manuscript text and supplemental materials for a description of media used). Cultures were maintained for 7 d at 37°C in anoxic atmospheres. Nitrogen predominant (85% N_2, 10% CO_2, and 5% H_2) and carbon dioxide predominant (i.e. 90% CO_2 and 10% H_2) atmospheres were used, with the exception of the ethanol and Tyndallization methods, which were conducted in the nitrogen predominant atmosphere. Individual bacterial colonies on agar media were streaked for purity, and identified by sequencing the 16S rRNA gene with comparison to bacterial sequences within the Ribosomal Database Project (RDP). Isolates were accession into the Intestinal Bacterial Collection (IBaC) at the Lethbridge Research and Development Centre, which included storage of bacteria over liquid nitrogen and by lyophilization.

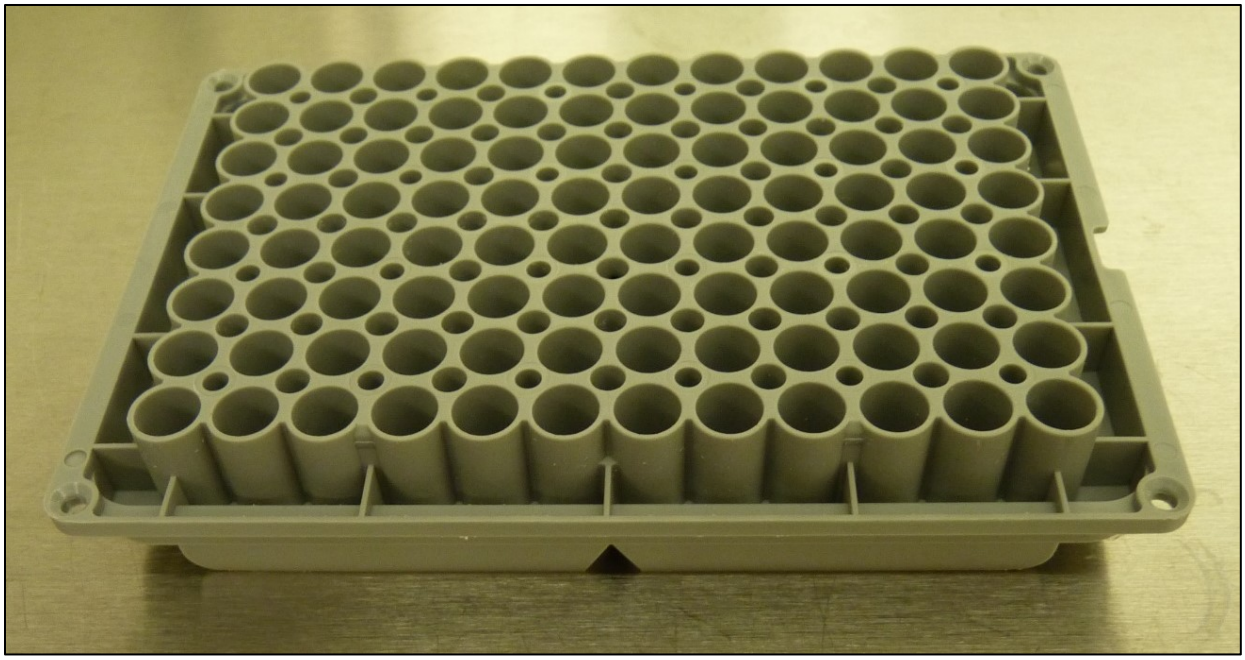

Figure 3.2 Photograph of the epMotion pipette tip holder (i.e. top side up) used to generate a 96-well Ichip apparatus.

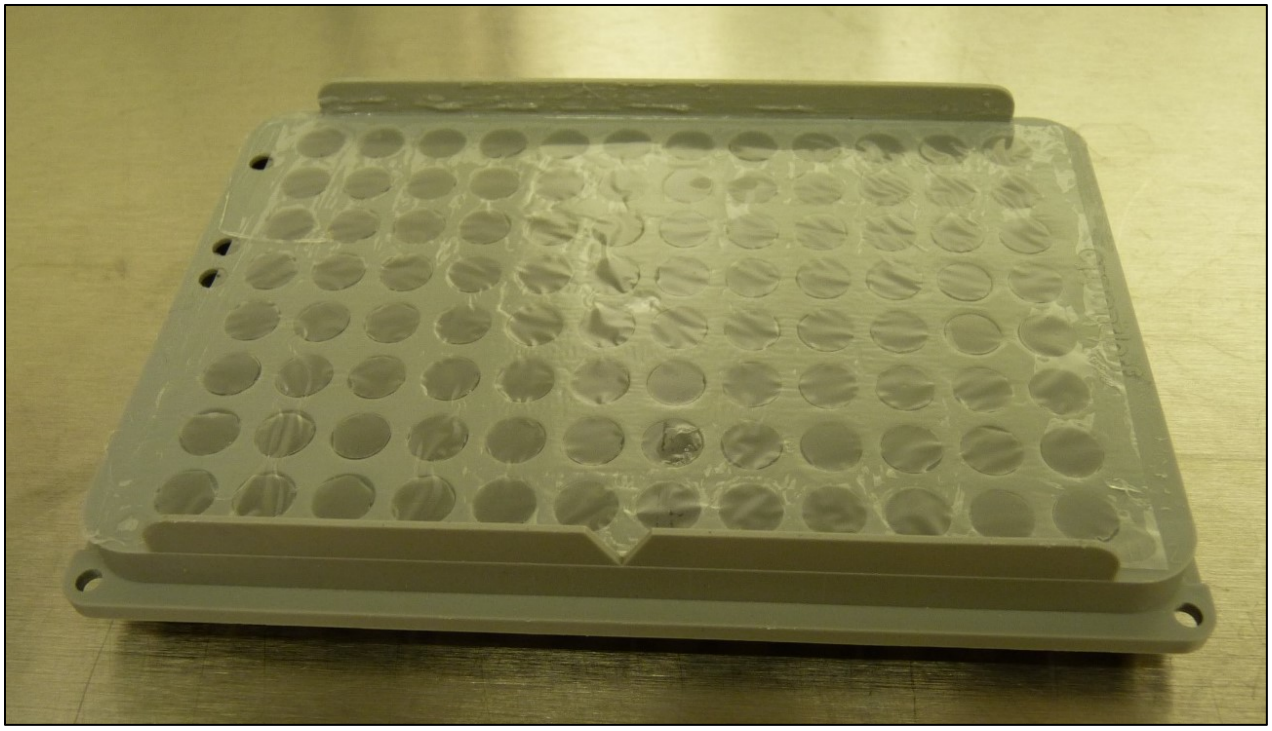

Figure 3.3 Photograph of the epMotion pipette tip holder (i.e. bottom side up) with the membrane containing 0.02 µm-diameter pores (Sterlitech, Kent, WA) adhered to the apparatus bottom using Silicone II sealant. Note the "feet" of the Ichip apparatus, which elevates the apparatus from the incubation container facilitating the infiltration of nutrients from the rumen fluid into the Ichip chambers.

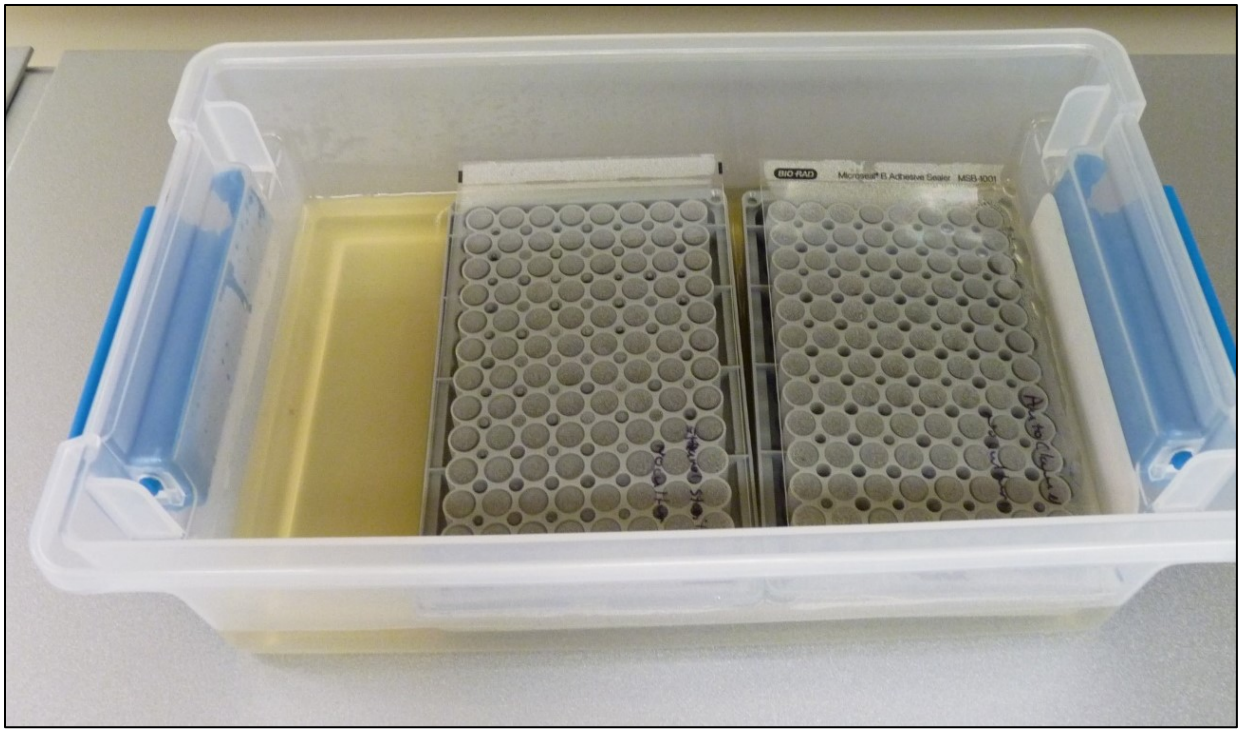

Figure 3.4 Photograph of the two labelled and sealed Ichips within an incubation container containing clarified rumen fluid. Note that the top membrane is labelled.

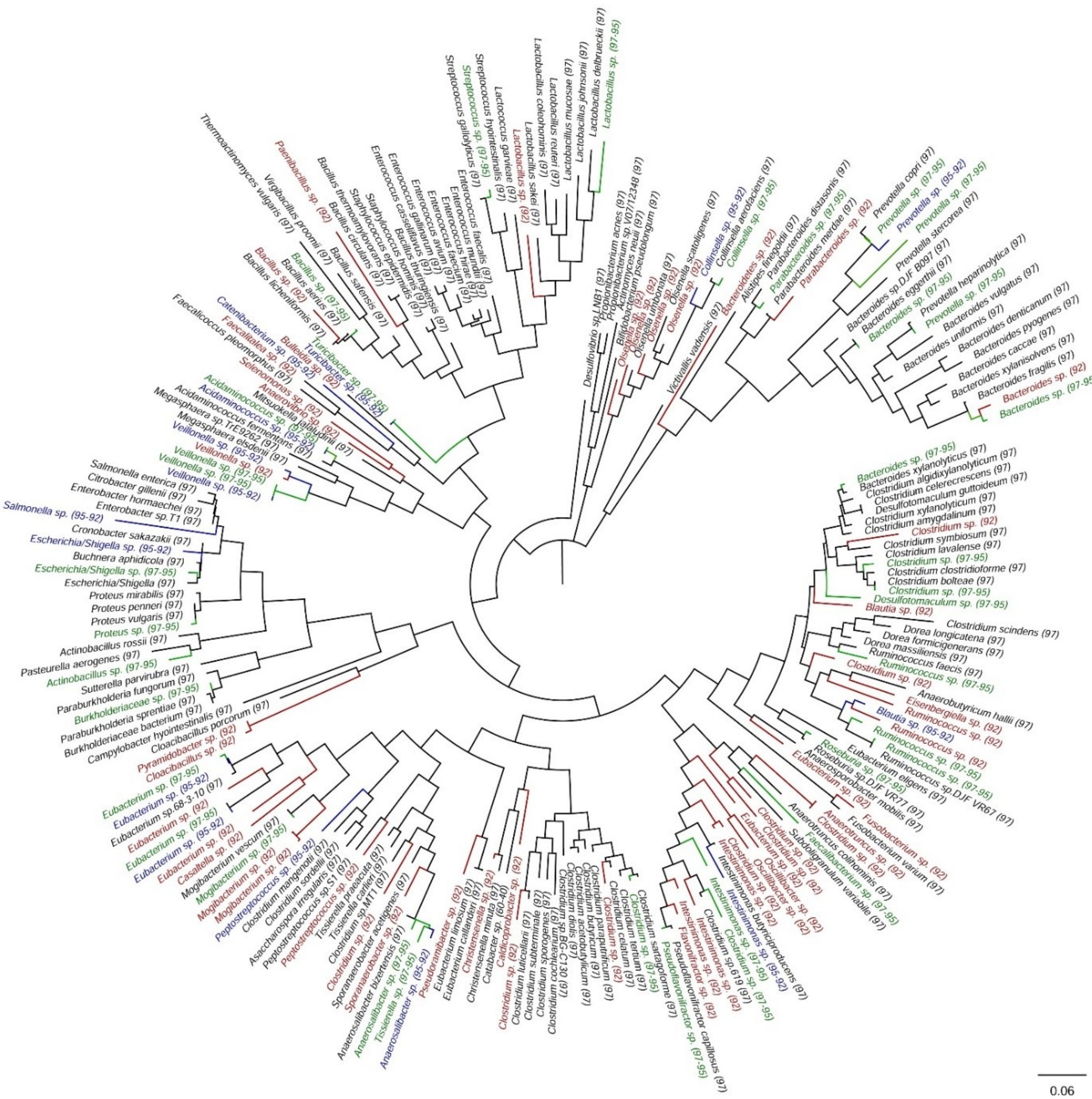

Figure 3.5 Phylogram of the bacterial species isolated from the ileum, cecum, ascending colon, and spiral colon of pigs. The bacteria that were identified as potential new species (green; >95 and ≤97% similarity), genera (blue; >92 and ≤95% similarity) and families (red; ≤92% similarity) compared to 16S rRNA gene sequences of the Ribosomal Database Project database (Cole et al. 2014).

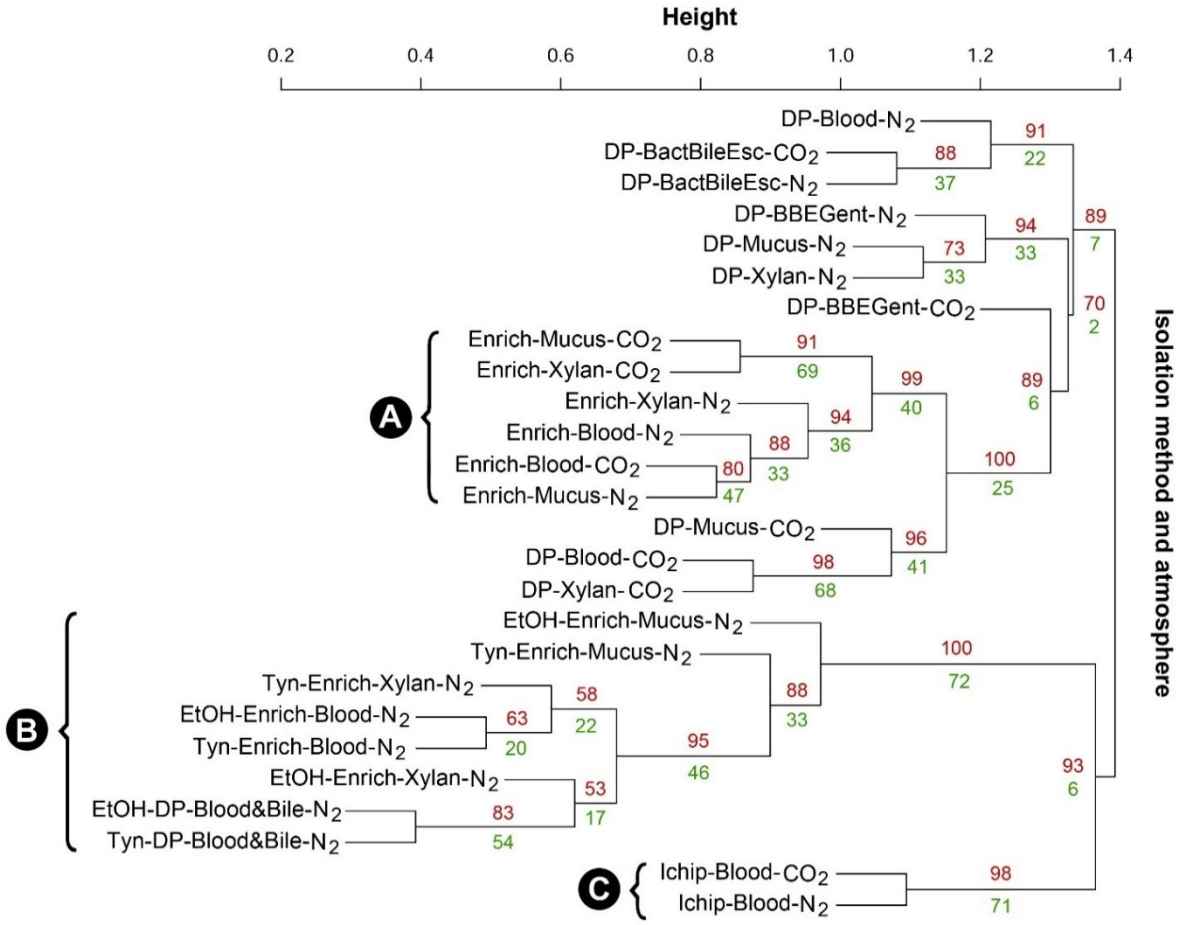

Figure 3.6 Dendrograms of Euclidean distances generated through the UPGMA function of the *vegan* package of R (Oksanen et al. 2007). The *pvclust* package in R was used to generate values of approximately unbiased (AU; red) P-values (in percent) and bootstrap probability (BP; green) values (nboot = 1,000) as measures of certainty for clusters. Encircled letters indicate collections of bacteria where AU ≥ 95% indicating these clusters are strongly supported by data. Bacterial communities were generated from bacterial isolated from within N_2 or CO_2 anaerobic environments. Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3-h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's blood (Blood); modified Dehority's media with 5% porcine mucus III (Mucus); modified Dehority's medium with 5% xylan (Xylan); or Columbia agar supplemented with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The parenthesis denoted with A shows clustering of bacterial taxa isolated by enrichment, the parenthesis denoted with B shows clustering of bacteria isolated using methods that select for endospore-forming taxa, and the parenthesis denoted with C shows clustering of bacterial taxa isolated using the Ichip. Data are combined across intestinal locations.

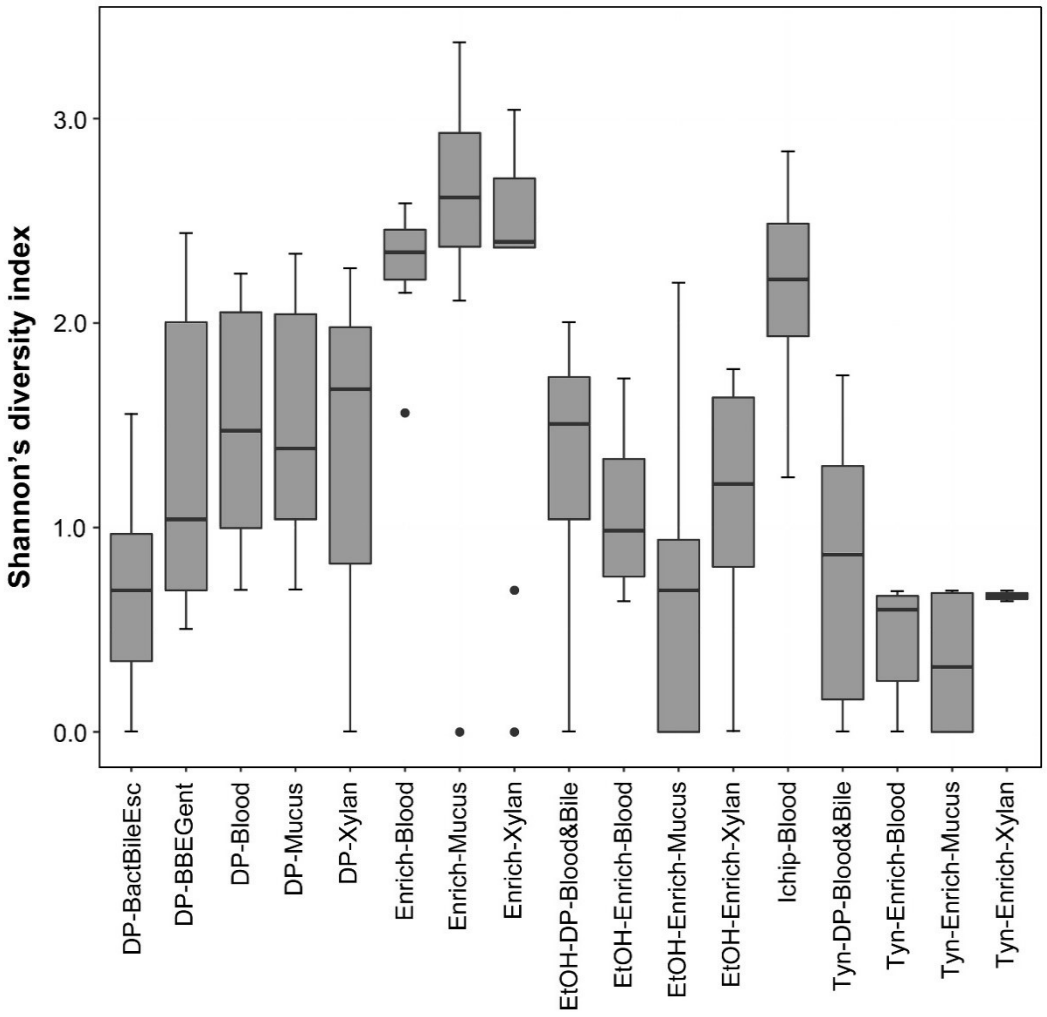

Figure 3.7 Distributions of the Shannon's diversity index of bacteria isolated from pigs using different isolation methods. Bacteria were isolated in this study using direct plating (DP) methods, enrichments (Enrich), a modified Ichip (Ichip) method, after a 3-h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's blood (Blood); modified Dehority's agar with 5% porcine mucus III (Mucus); modified Dehority's medium with 5% xylan (Xylan); or Columbia agar with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The center lines in the box plot represent the median value, the size of the box plot represents the distribution within a confidence of 95%, and the vertical lines and dots associated with the box plot represent the total distribution of the data. The boxplots were generated using the *geom_boxplot* function of the *ggplot* package in R, and Shannon's diversity was determine using the *vegan* package of R (Oksanen et al. 2007). Data are combined across intestinal location.

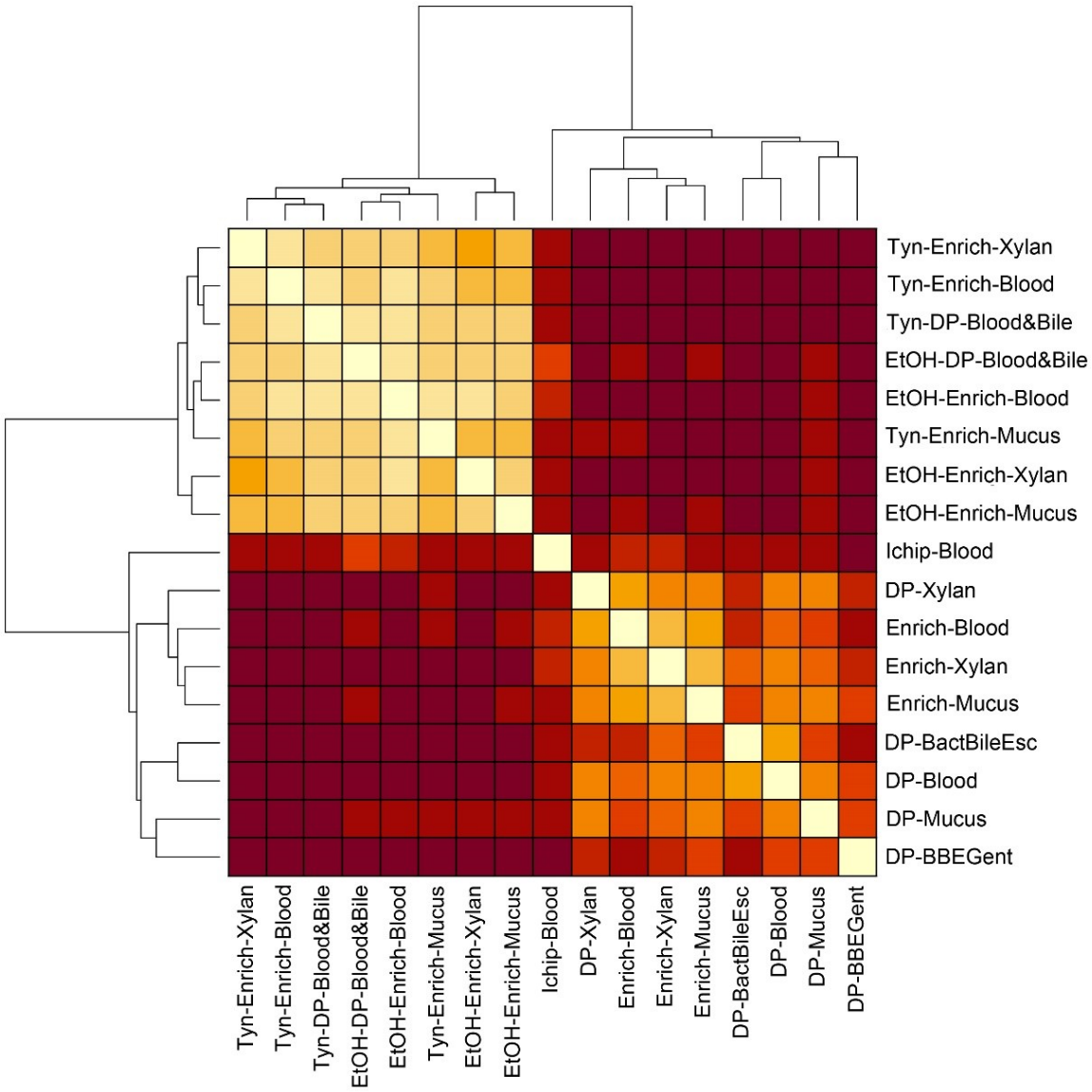

Figure 3.8 Cluster heatmap generated with the Euclidean distances calculated among isolation methods and media. Isolation methods included direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3-h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's blood (Blood); modified Dehority's medium with 5% porcine mucus III (Mucus); modified Dehority's medium with 5% xylan (Xylan); or Columbia agar with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The heatmap and dendrogram was generated using the *heatmap* and *Hclust* (*vegan* package) functions in within R (Oksanen et al. 2007). Data are combined across intestinal location.

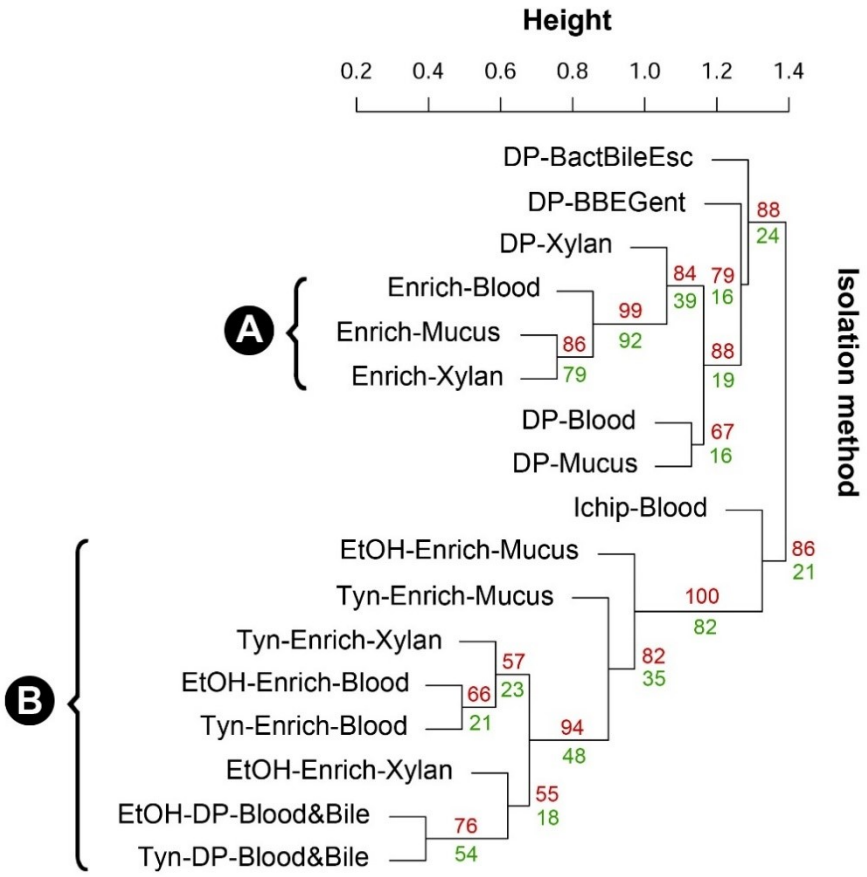

Figure 3.9 Dendrograms of Euclidean distances generated through the UPGMA function of the *vegan* package of R (Oksanen et al. 2007). The *pvclust* package in R was used to generate values of approximately unbiased (AU; red) P-values (in percent) and bootstrap probability (BP; green) values (nboot = 1,000) as measures of certainty for clusters. Encircled letters indicate collections of bacteria where AU≥95% indicating these clusters are strongly supported by data. Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3-h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's blood (Blood); modified Dehority's medium with 5% porcine mucus III (Mucus); modified Dehority's medium with 5% xylan (Xylan); or Columbia agar with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The parenthesis denoted with A shows clustering of bacterial taxa isolated by enrichment, and the parenthesis denoted with B shows clustering of bacteria isolated using methods that select for endospore-forming taxa. Data are combined across intestinal location.

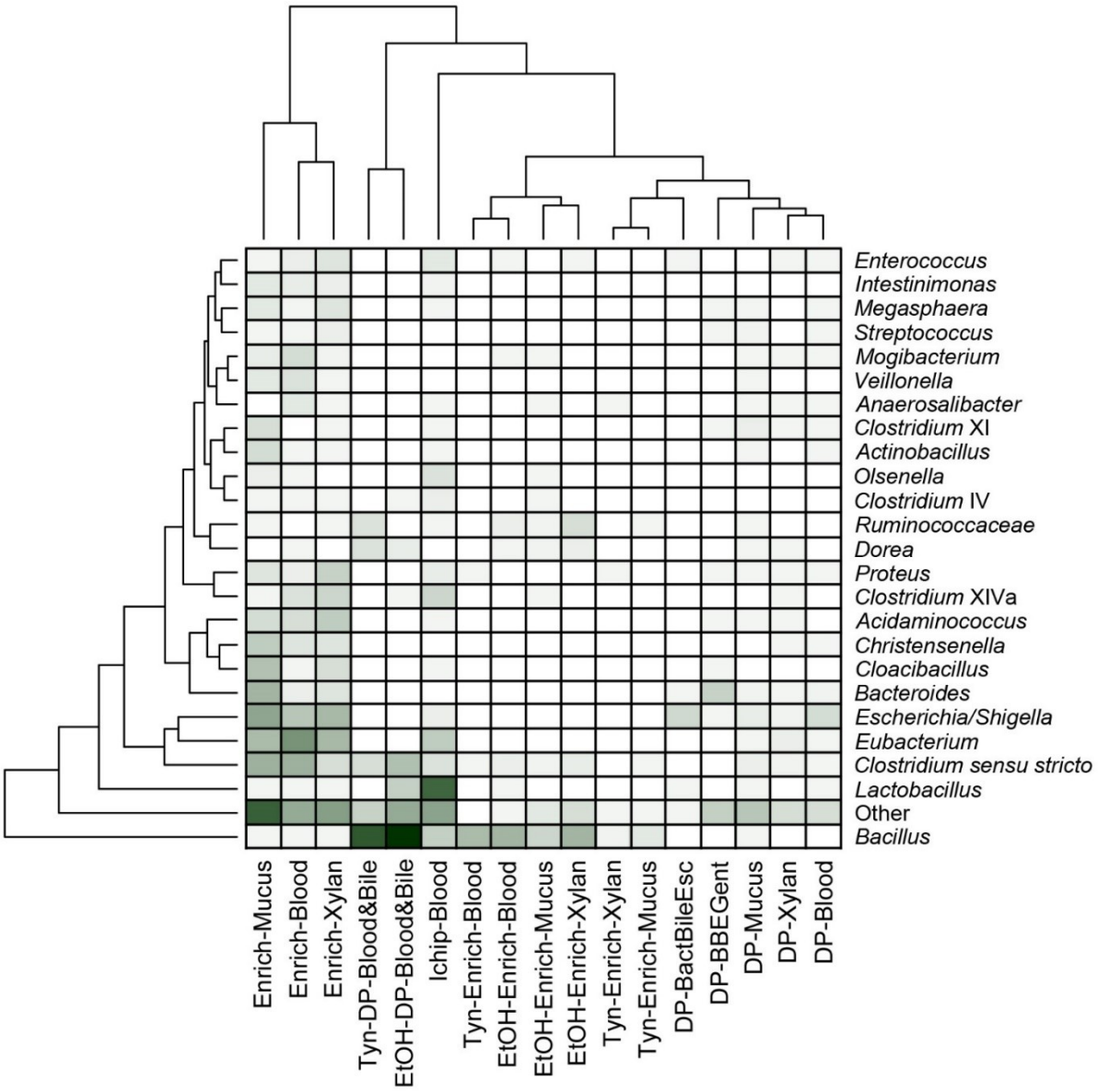

Figure 3.10 Heatmap of Euclidean distances and relative abundance of the 25 most abundantly isolated bacterial genera as well as "other" bacteria. Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3-h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used during were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine Agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's (Blood); modified Dehority's medium with 5% porcine mucus III (Mucus); modified Dehority's medium with 5% xylan (Xylan); or Columbia agar supplemented with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The heatmap and dendrogram were generated using the *heatmap* and *Hclust* functions in the *vegan* package of R (Oksanen et al. 2007). Bacteria were isolated from the ileum, cecum, ascending colon, and spiral colon. Data are combined across intestinal locations.

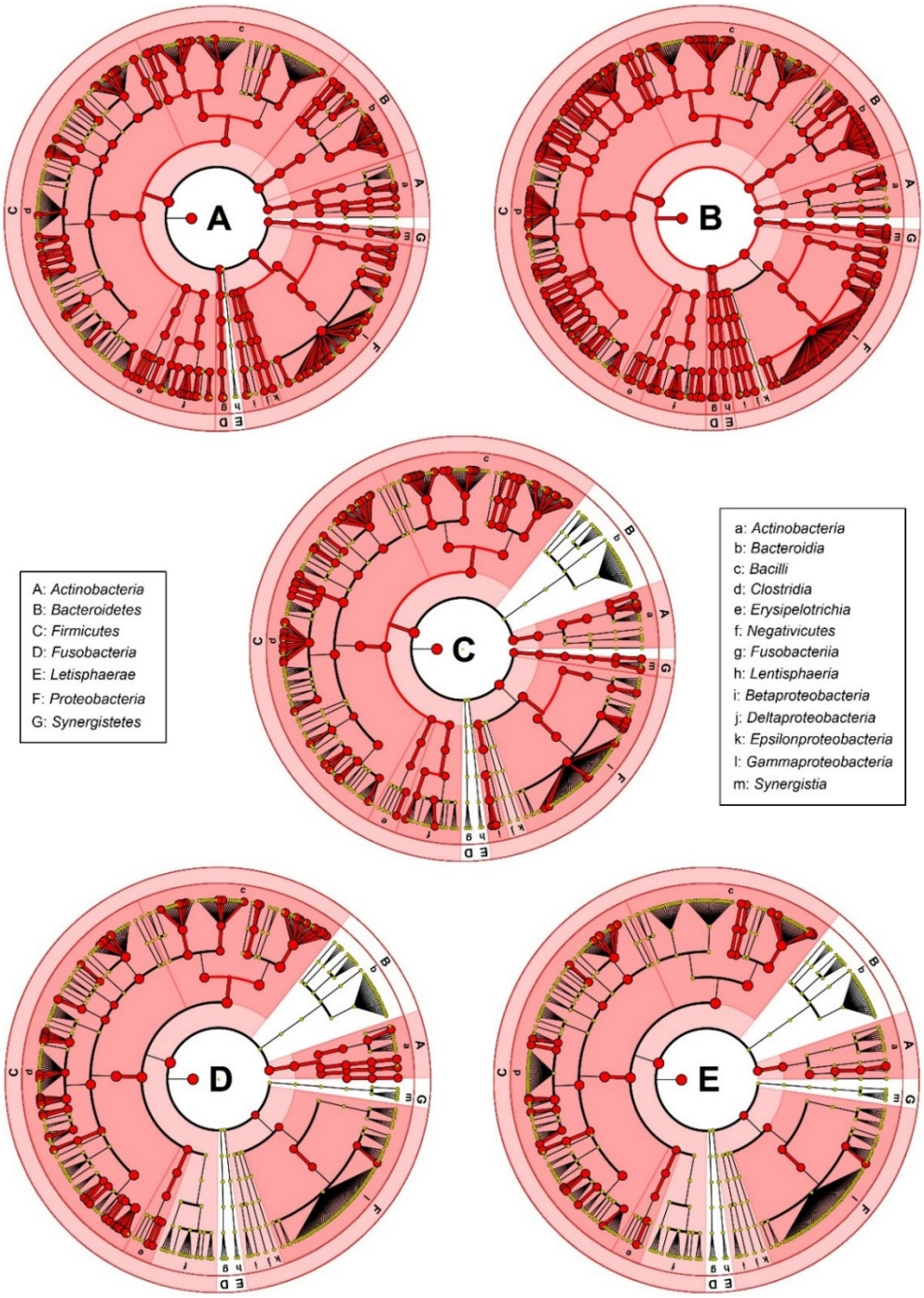

Figure 3.11 Cladograms showing the distribution of bacterial taxa isolated by an individual isolation method (red circles) relative to bacteria isolated using other methods (gold circles). Bacteria were isolated using: (A) direct plating; (B) enrichment; (C) a modified Ichip method; (D) after a 3-h exposure to 70% ethanol; and (E) after a 30 min exposure to 100°C. Taxonomic levels in the cladograms range from superkingdom (in the center) to species (at the perimeter). Cladograms were generated using the online *LEfSe* tool available on the Huttonhower Lab Galaxy instance (available from https://huttenhower.sph.harvard.edu/galaxy).

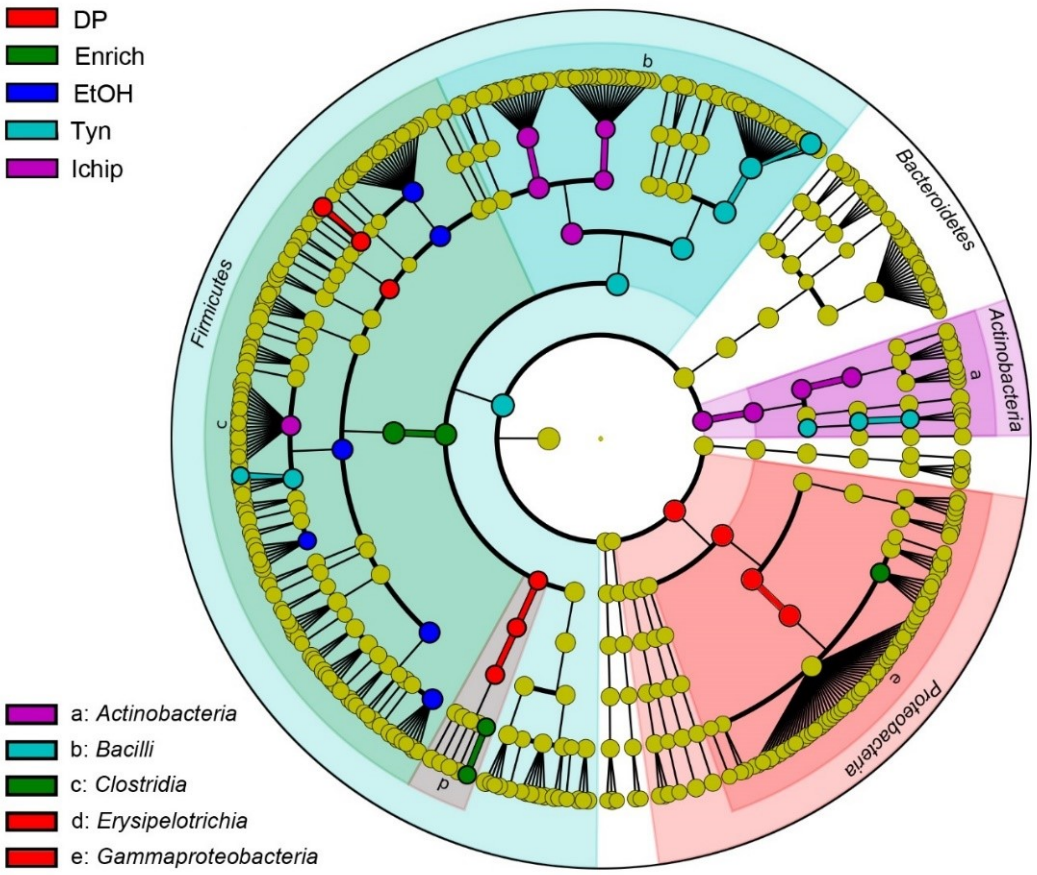

Figure 3.12 Cladogram showing isolation methods with differential abundance of the bacteria taxa isolated were observed. Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3-h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Taxonomic levels in the cladogram range from superkingdom (in the center) to species (at the perimeter). The color of the taxonomic markers and cladogram background illustrate the methods that yielded differences in bacterial abundance as indicated by LEfSe (linear discriminant analysis effect size) analysis; taxa that were not affected by the isolation method are shown as yellow markers. Data are combined across intestinal location.

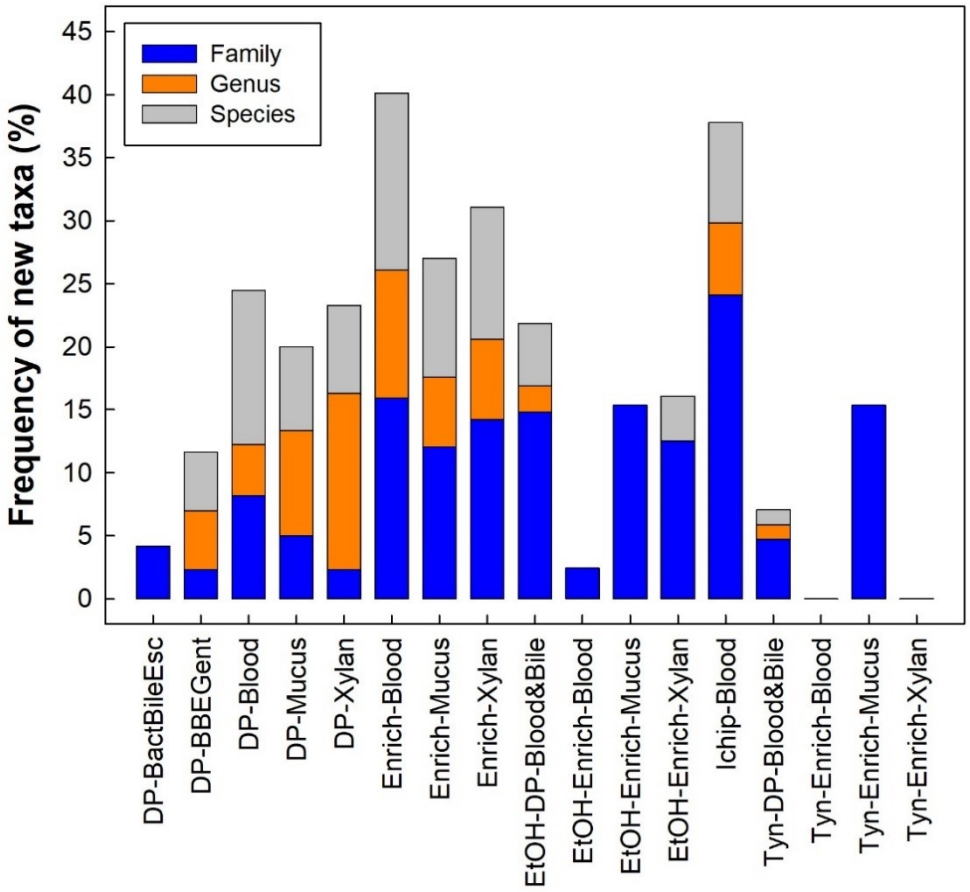

Figure 3.13 Frequency of novel bacterial taxa (%) recovered using the evaluated isolation methods and media. Novel taxa were delineated at a >95 and ≤97% similarity (species), >92 and ≤95% similarity (genus), and ≤92% similarity (family and higher) relative to 16S rRNA gene sequences within the Ribosomal Database Project database (Cole et al. 2014). Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3-h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's blood (Blood); modified Dehority's medium with 5% porcine mucus III (Mucus); modified Dehority's medium with 5% xylan (Xylan); or Columbia agar with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). Bacteria were isolated from the ileum, cecum, ascending colon, and spiral colon. Data are combined across intestinal locations.

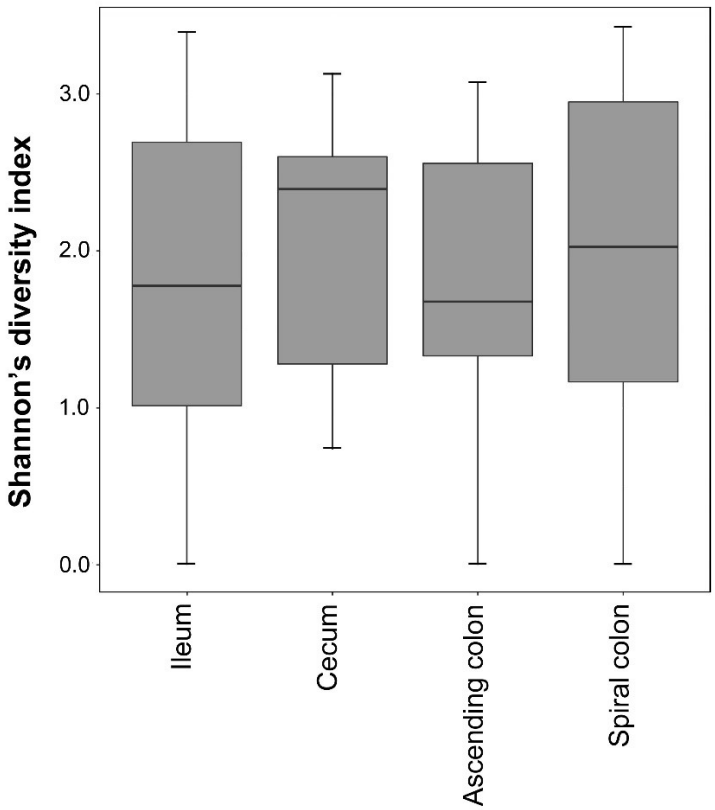

Figure 3.14 Distributions of the Shannon's diversity index of isolated bacteria by intestinal site (i.e. ileum, cecum, ascending colon, or spiral colon). Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3 h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's blood (Blood); modified Dehority's medium with 5% porcine mucus (Mucus); modified Dehority's medium with 5% xylan; or Columbia agar with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The center lines in the box plot represent the median value, the size of the box plot represents the distribution within a confidence of 95%, and the vertical lines and dots associated with the box plot represent the total spread of data. The boxplots were generated using the *geom_boxplot* function of the *ggplot* package in R, and Shannon's diversity was determine using the *vegan* package of R (Oksanen et al. 2007). Data are combined across isolation method and media.

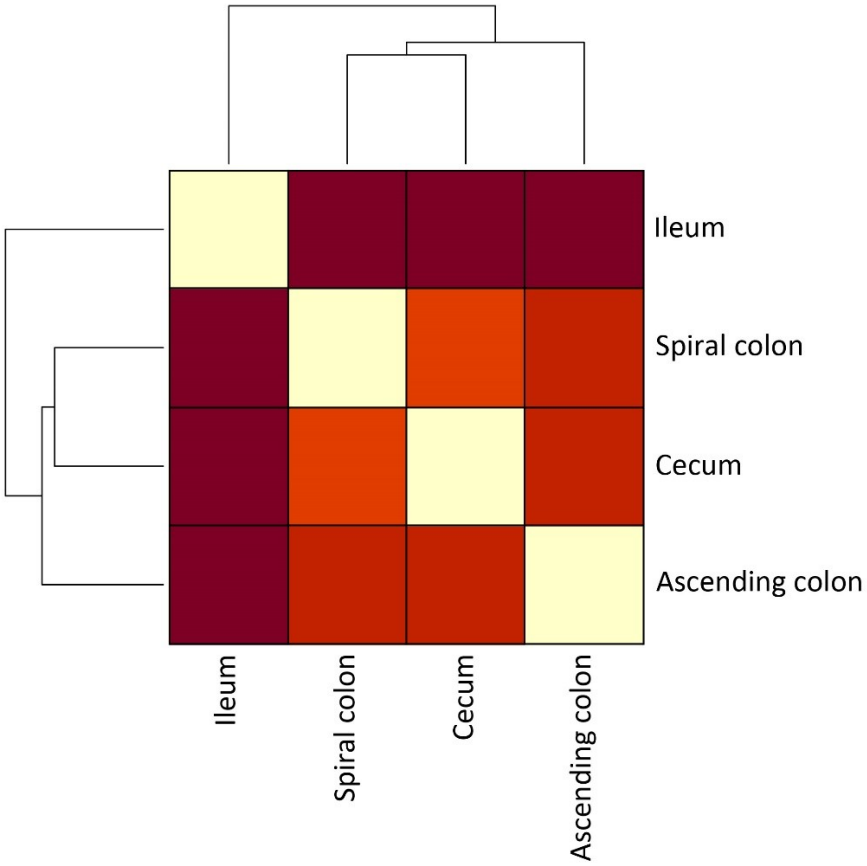

Figure 3.15 Cluster heatmap generated with the Euclidean distances calculated among isolated bacteria from the ileum, cecum, ascending colon, and spiral colons of pigs. Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3 h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate (BBEGent); Columbia medium with 10% sheep's blood (Blood); modified Dehority's medium with 5% porcine mucus (Mucus); modified Dehority's medium with 5% xylan; or Columbia agar with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The heatmap and dendrogram were generated using the *heatmap* and *Hclust* functions in the *vegan* package of R (Oksanen et al. 2007). Data are combined across isolation method and media.

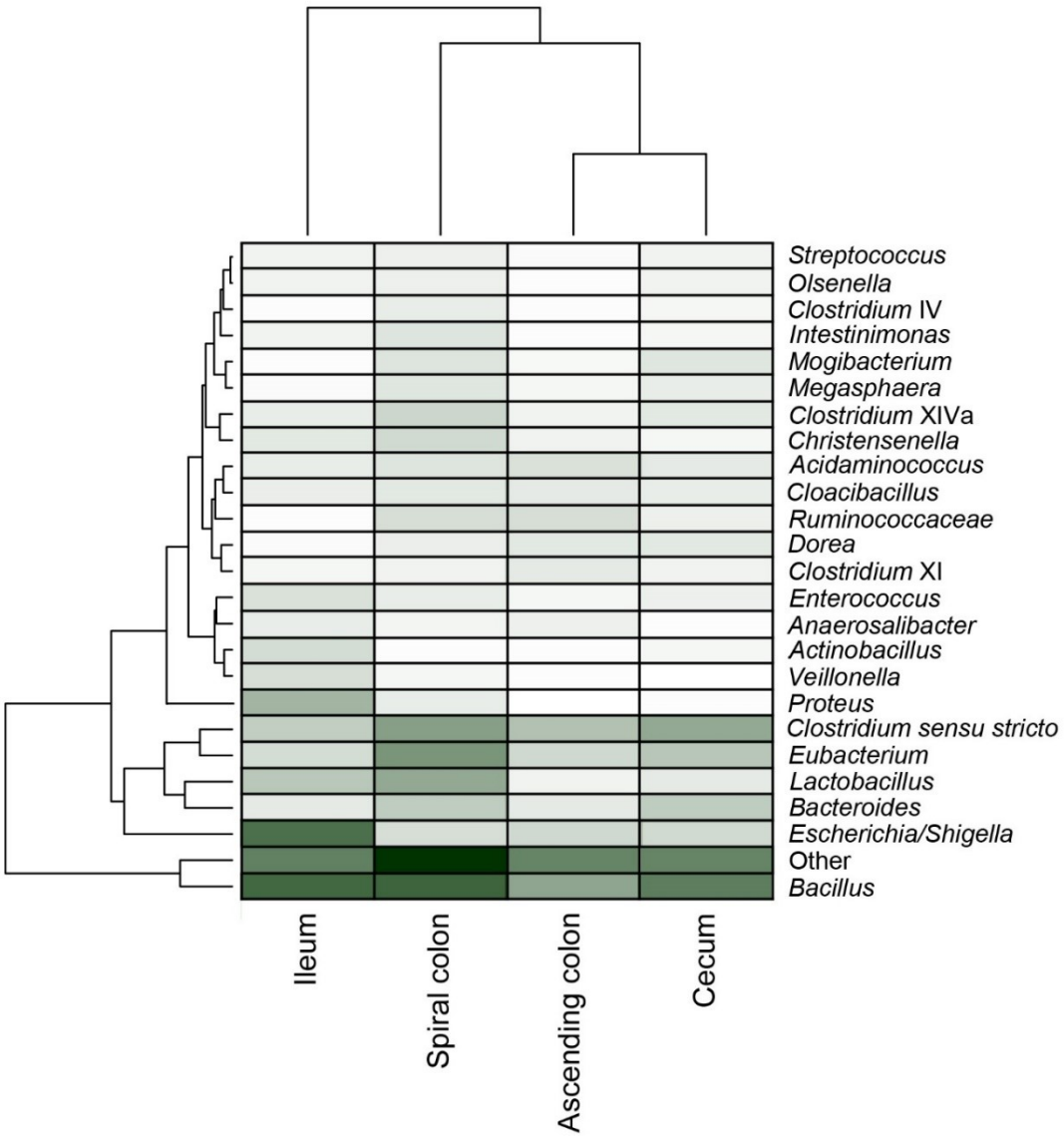

Figure 3.16 Heatmap of Euclidean distances and relative abundance of the 25 most abundantly isolated bacterial genera as well as "other" bacteria from the ileum, cecum, ascending colon, and spiral colon of pigs. Bacteria were isolated using direct plating (DP), enrichment (Enrich), a modified Ichip (Ichip) method, after a 3 h exposure to 70% ethanol (EtOH), or after a 30 min exposure to 100°C temperatures (Tyn). Media used were: *Bacteroides* bile esculine agar (BactBileEsc); *Bacteroides* bile esculine agar with 100 µg ml^{-1} gentamicin sulfate; Columbia medium with 10% blood (Blood); modified Dehority's medium with 5% porcine mucus (Mucus); modified Dehority's medium with 5% xylan (Xylan); or Columbia agar with 10% sheep's blood and 0.1% sodium taurocholate (Blood&Bile). The heatmap and dendrogram were generated using the *heatmap* and *Hclust* (*vegan* package) functions in R (Oksanen et al. 2007). Data are combined across isolation method and media.

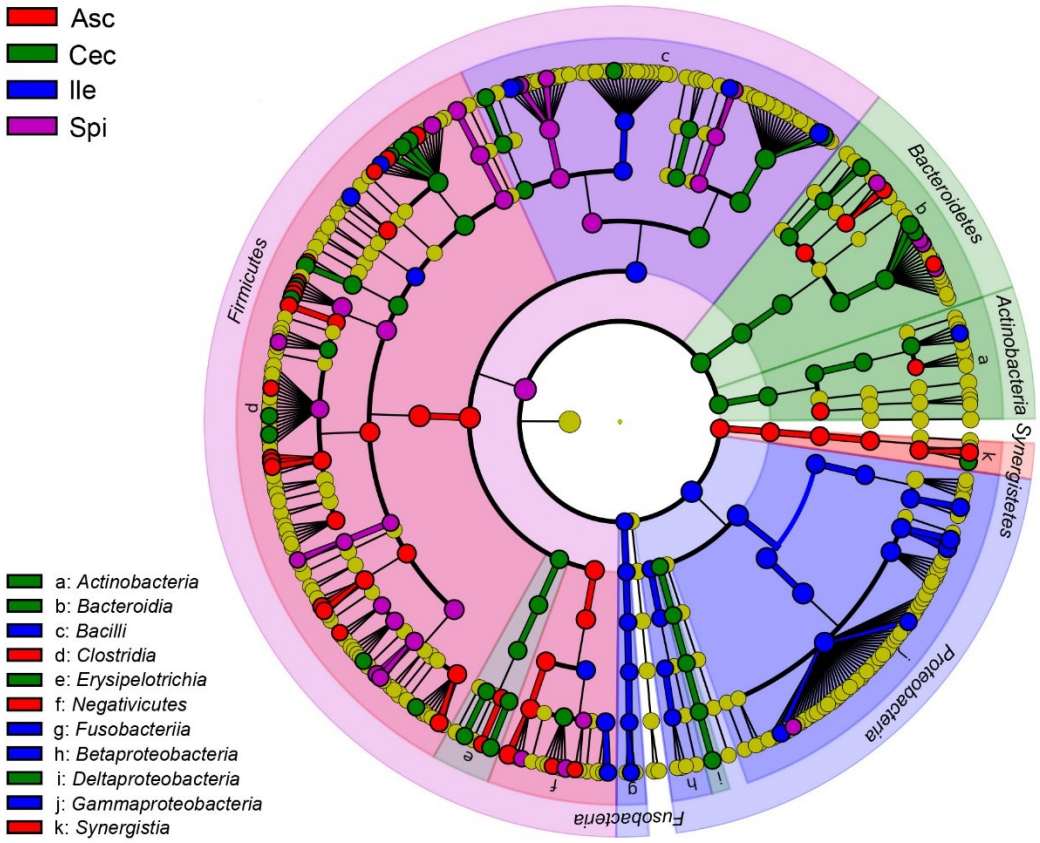

Figure 3.17 Cladogram showing intestinal sites for which changes in the abundance of the bacteria taxa isolated were observed. Sites were the ileum (Ile), cecum (Cec), ascending colon (Asc), and spiral colon (Spi). Taxonomic levels in the cladogram range from superkingdom (in the center) to species (at the perimeter). The color of the taxonomic markers and cladogram background illustrate the methods that yielded differences in bacterial abundance as indicated by LEfSe (linear discriminant analysis effect size) analysis; taxa that were not affected by the isolation method are shown as yellow markers. The cladogram was generated using the online version of Galaxy (available from https://huttenhower.sph.harvard.edu/galaxy). Data are combined across isolation method and media.

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Chapter 4 *Salmonella* enterica serovar Typhimurium temporally modulates the enteric microbiota and host responses to overcome colonization resistance in swine [3]

4.1 Introduction

The gastrointestinal tract (GIT) of mammals is inhabited by commensal and mutualistic microorganisms referred to as the normal microbiota. Bacterial densities in the GIT system vary within sites ranging from 10^3 cells per milliliter in the stomach to 10^{11} cells per gram in the colon (Xu and Gordon 2003). These bacterial communities have been shown to play an essential role in nutrient acquisition, development of gut-associated lymphoid tissue, defense against pathogens, and maturation of the intestine (Hooper et al. 2002; Sansonetti 2004). Changes in numbers and diversity of bacteria that comprise the normal microbiota can be triggered by diverse factors potentially leading to health consequences for the host. Dramatic changes in the intestinal microbiota of swine have been observed after enteropathogenic infections (Drumo et al. 2016), as a result of nutritional and dietary additives (Liu et al. 2012), and due to the administration of antibiotics (Allen et al. 2011). Furthermore, the presence of an inflammatory response triggered by infections with enteric pathogens such as *Salmonella enterica*, *Citrobacter rodentium*, and *Campylobacter jejuni* have been associated with alterations to the composition of the intestinal microbiota (Lupp et al. 2007; Drumo et al. 2016).

Salmonella enterica serovar Typhimurium is an important zoonotic pathogen that successfully infects the intestinal tract of swine. A number of studies to elucidate mechanisms of pathogenesis (Boyen et al. 2008; Agbor and McCormick 2011), host immune responses (Wang et al. 2007; Collado-Romero et al. 2010), and the epidemiology and control of salmonellosis have been conducted in pigs (Boyen et al. 2008). High throughput sequencing methods have been used to profile the enteric microbiota of swine under the influence of different feed efficiencies (Quan et al. 2018) and different levels of fatness (Yang et al. 2016). A similar culture-independent approach was employed to investigate changes in the ileal microbiota of swine subjected to an early *S.* Typhimurium infection (Argüello et al. 2018). However, the majority of studies conducted to date have focused on the evaluation of differences in the fecal microbiota of pigs. For example, evaluation of fecal microbiota during weaning transition (Pajarillo et al. 2014), after antibiotic administration (Looft et al. 2012), and a comparison between high or low *Salmonella* shedders (Bearson et al. 2013). Importantly, evidence indicates that the fecal microbiota is not representative of the intestinal microbiota (e.g. mucosa-associated communities) (Zhao et al. 2015). Differences in protocols among laboratories, specifically in DNA extraction, variation

[3] A version of this chapter has been accepted for publication in Applied and Environmental Microbiology pending minor revisions

of sequencing coverage to detect minority populations (Lynch and Neufeld 2015), differences within methods for bioinformatics analyses, the incapacity to differentiate live from dead bacteria, and relatively poor taxonomic resolution are some of the salient limitations of the culture-independent strategies. Moreover, bacteria are not recovered for additional analysis (e.g. for empirical function determinations). Thus, comprehensive analyses that include culturomics together with sequence-based microbiome analysis approaches are necessary to properly characterize the intestinal microbiota.

Arguello et al. (2018) and Bearson et al. (2013) examined changes in the composition of the enteric microbiota of pigs infected by *S.* Typhimurium (Bearson et al. 2013; Argüello et al. 2018). However, they did not characterize the composition and structure of the microbiota as a function of inflammation intensity; in pigs, salmonellosis follows a temporal progression developing from acute infection characterized by severe diarrhea, vomiting and fever to subclinical infection represented by intermittent shedding of the pathogen (Scherer et al. 2008). Additionally, they exclusively used high throughput sequencing techniques to characterize the microbiota, and they did not characterize communities associated with the mucosa. It has been observed that the composition of bacterial communities in association with mucosa can vary with those in digesta within the intestinal lumen (Zhang et al. 2018). Thus, a primary objective of the current study was to comprehensively characterize the microbiota in the ileum, cecum, and colon of pigs at different stages of salmonellosis experimentally incited by *S.* Typhimurium DT104 (i.e. acute, subacute, and recovery phases) using both culture-based and culture-independent methods. Moreover, we collated temporal changes to the microbiota with a variety of host metrics to glean information on how the pathogen overcomes colonization resistance. Our study provides a more comprehensive and extensive understanding of temporal interactions between the host and the intestinal microbiota, and the impact that enteric inflammation incited by *S.* Typhimurium has in this interplay.

4.2 Materials and methods

4.2.1 Ethics

The project was approved by the LeRDC Animal Care Committee (Animal Use Protocol Review 1512), and LeRDC Biosafety and Biosecurity Committee before commencement. As infection by *S.* Typhimurium in pigs is reportable in Alberta, approval to conduct *Salmonella* inoculations in piglets was also obtained from the Head Provincial Veterinarian, Dr. Gerald Hauer, Alberta Agriculture and Forestry, Edmonton, Alberta.

4.2.2 Experimental design.

The experiment was arranged as a completely randomized design with three levels of sample time (2, 6, and 10 dpi) and two levels of immunological stress (± *S.* Typhimurium) (Figure 4.1). The goal was to obtain samples that corresponded temporally to acute, subacute, and recovery phases of salmonellosis in swine. Each replicate included six piglets. To ensure humane animal care, piglets were housed in pairs within individual animal rooms. The limited number of animal rooms available in the Livestock Containment Unit [LCU] at LeRDC necessitated that the experiment was conducted on separate occasions (i.e. two replicates at time one, and two replicates at time two); the separate times were treated as a random effect in the statistical model.

4.2.3 Animal maintenance.

Castrated male piglets at 6-weeks of age were used in the experiment. Piglets were vaccinated for circovirus, ileitis, and erysipelothrix. Neither the sow (during pregnancy or post-partum) nor piglets were administered antibiotics. Piglets were group housed for 3 d in the LCU under a 14:10 h dark:light cycle. After the adaptation period, arbitrarily-selected animals were assigned to individual pens, with two animals per room. Piglets were provided a mini-pellet ration diet that was free of antibiotics (Proform Pig Starter 2; Hi-Pro Feeds, Okotoks, AB). Feed was provided daily, and piglets were permitted to eat and drink *ad libitum*. Straw was used for bedding, and toys were provided for environmental enrichment. Animals were monitored daily for activity level, and behavioral signs of pain and stress (i.e. diarrhea) were recorded. Bedding, and residual food and water were replaced daily. Initial body weights were recorded upon assignment of animals to individual cages, and every other day thereafter. Food consumption was determined daily.

4.2.4 Inoculation.

Piglets were orally inoculated with *S. enterica* Typhimurium DT104 (strain SA970934) (Yin et al. 2014). The bacterium was grown aerobically on MacConkey's agar (MA) (Difco BD, Mississauga, ON) at 37°C for 16-24 hours. Biomass was removed from the surface of the agar and transferred into Columbia Broth (CB) (Difco BD, Mississauga, ON). Cultures were maintained for 180 to 210 min at 37°C, shaking at 150 rpm, until an optical density (600 nm) of greater than 1.2 was obtained. Cultures were centrifuged at 4,000 x g for 15 min, supernatants were removed to a volume of 20 ml, and the density adjusted to a target of 3.0×10^9 cells/ml. To confirm densities of viable cells, inoculum was diluted in a 10-fold dilution series, 100 µl of each dilution was spread in duplicate onto MA, cultures were incubated at 37°C, and the number of *S*. Typhimurium colonies were counted at the dilution yielding 30 to 300 CFU after 24 hours. Individual piglets were gavaged on 2 consecutive days with *S.* Typhimurium cells in CB (1.0 ml,

ST+) or with CB alone (1.0 ml; ST-). Each piglet was administered the two treatments in 1 ml aliquots using sterile 3.0 ml syringes. The animals were individually restrained, the syringe was placed in the rear of the mouth and the liquid slowly dispensed taking care to avoid aspiration.

4.2.5 Body temperature, feed consumption, weight gain, and feces collection.

Rectal temperature and food consumption were measured daily. In addition, body weights were measured at 2-day intervals using a Model 75 scale (Reliable Scale Corporation, Calgary, AB). Fresh feces were collected from the pen floor immediately before inoculation and at 2-day intervals thereafter, including just before humane euthanization. Fecal samples were transported to the laboratory within 30 min of collection for analysis. Samples of fresh feces were processed for the presence of *S.* Typhimurium via dilution plating. In addition, aliquots of the feces were weighed and placed at -80°C for qPCR.

4.2.6 Intestinal tissue collection.

At 2, 6, and 10 dpi, randomly designated animals were anaesthetized for sample collection from live animals. Piglets were sedated with a cocktail of ketamine (Ketaset, Pfizer, Kirkland, QC) and xylazine (Xylamax, Bimeda, Cambridge, ON) at a dose of 22 mg/kg and 2.2 mg/kg respectively. An intramuscular injection was administered. Animals were placed in dorsal recumbency on a v-trough surgical table, intubated, and general anesthesia established with isoflurane (2-3% isoflourane at 15000mg/ml O_2; Abbott Laboratories, Chicago, IL). The abdomen was sanitized with Stanhexidine (Omega Laboratories Ltd., Montreal, QC), 70% ethanol, and Prepodyne (West Penetone Inc., Ville D'Anjou, QC).

From animals under general anesthesia, segments of intestine (≈10 cm-long) were collected from the duodenum, proximal jejunum, mid-jejunum, distal jejunum, ileum, cecum (two segments located at the free end and adjacent to the ileal-cecal junction), ascending colon, spiral colon (two segments located at the central flexure and ≈100 cm distal from the flexure), and descending colon. To ensure the integrity of the intestinal segment and to minimize release of ingesta, double ligatures were established at the two ends of the segment, and the segment was excised from the intestine by cutting between the two ligatures. Care was taken to ligate mesentery blood vessels immediately prior to intestinal segment removal, and to ensure the maintenance of blood flow to adjacent intestinal tissue. All intestinal samples were processed within ca. 5-10 min of their removal from live animals. To prevent introduction of air, additional ligatures were established on segments from which anaerobic bacteria were to be isolated (i.e. ileum, cecum, and spiral colon). Ligated sub-segments for culturomics were removed and immediately placed in an anaerobic jar; the ambient atmosphere was removed by vacuum, replaced with nitrogen (N_2), and the segments transported to the laboratory. With the exception of intestinal segments used to isolate anaerobic bacteria, segments were longitudinally incised in ambient

atmosphere, and luminal contents (i.e. digesta) were aseptically collected and weighed for DNA extraction (e.g. for quantitation of *S.* Typhimurium and select commensal bacterial taxa, and characterization of bacterial communities). Sections of the intestine were collected for RNA extraction (i.e. gene expression), and DNA extraction (e.g. quantitation of mucosa-associated *S.* Typhimurium and select commensal bacterial taxa, histology, and characterization of bacterial communities). Samples for characterization of mucosa-associated *S.* Typhimurium and microbial communities were gently flooded with sterile phosphate buffered saline (≈3 ml) to remove residual ingesta with minimal disruption to the adhered mucus. Samples for RNA extraction (three subsamples per segment) were placed in RNAprotect® (Qiagen Inc., Toronto, ON) and maintained at -80°C until processed. For histopathological scoring, intestinal segments were placed in 10% neutral buffered formalin (Surgipath Canada, Inc., Winnipeg, MB) for a minimum of 24 hours.

4.2.7 Blood collection and animal euthanization.

Blood (≈20 ml) was collected from the portal vein (draining the intestine) and from the heart (systemic blood) using an 18 gauge needle. Blood was collected in BD Vacutainer® with K_2EDTA (BD, Franklin Lake, NJ) for complete blood count (CBC) analysis and detection of *S.* Typhimurium. Immediately after blood removal, the animal was euthanized under general anesthesia.

4.2.8 Accessory tissue collection.

Within 5-10 min of death, ileal-cecal lymph nodes, jejunal lymph nodes (one to two per animal), the liver, and spleen were removed, and samples excised from these tissues were placed in RNAprotect® (Qiagen Inc.) and maintained at -80°C until processed. In addition, samples from the above tissues, and from the frontal cortex, midbrain, and brain stem were collected for isolation of *Salmonella*.

4.2.9 Histopathology.

Tissues for hematoxylin and eosin (H&E) were prepared by our standard procedure (Jiminez et al. 2017). Tissues from duodenum, proximal jejunum, medium jejunum, distal jejunum, ileum, cecum, spiral colon, ascending colon and distal colon in formalin were dehydrated using a tissue processor (Leica TP 1020, Leica Biosystems), and then embedded in paraffin (Fisherfinest™ Histoplast PE, Thermo Fisher Scientific, Edmonton, AB) using a Shandon Histocentre 3 (Thermo Fisher Scientific). Five µm sections were transferred to positively charged slides (Fisherbrand Superfrost™ Plus Gold; Thermo Fisher Scientific) and allowed to dry prior to being deparaffinized with xylene. Slides were then rehydrated in ethanol and stained with H&E using a standard protocol. Histopathologic changes were assessed by a veterinary pathologist, (Dr. Richard R.E. Uwiera), blinded to the treatment protocol. The scoring system used was a modification of previous scoring protocols developed by Boyer et al. (2015), and Garner et al.

(2009). The tissues were scored for villus blunting, villus fusion, lymphoid depletion, neutrophil infiltration, epithelial injury, and fibrosis. Villus blunting (i.e. crypt to villus ratio), 0 = normal with no changes in villus height, 1 = <25% reduction in villus height, 2 = 26-50% reduction in villus height, 3 = 51-75% reduction in villus height, and 4 = complete villus loss. Villus fusion, 0 = normal with no increases in the presence of villus fusion, 1 = mild with small increases in the numbers fused villi, 2 = moderate with prominent increases in the numbers of fused villi, and 3 = severe with substantive increases in the numbers of fused villi. Lymphoid depletion, 0 = none, 1 = mild with a small reduction in lymphoid cells, 2 = moderate with a prominent reduction in lymphoid cells and 3 = severe with a marked reduction in lymphoid cells. Neutrophil infiltration was examined in both lamina propria (D1), as well as muscularis and serosa (D3). Neutrophil infiltration, 0 = none, 1 = rarely observed in tissue, 2 = few scattered neutrophils within the tissue, 3 = many foci with collections of few numbers of neutrophils, and 4 = large numbers of neutrophils present within the tissue. Epithelial injury, 0 = none, 1 = rare (< 10 surface epithelial cells shedding), 2 = mild (focal epithelial erosions; 11-50 surface epithelial cells shedding), 3 = moderate (multi focal surface epithelium erosions; <50 of surface epithelial cells shedding), and 4 = severe (multi focal to coalescing area of surface epithelium erosions; >50 of surface epithelial cells shedding). Fibrosis (desmoplasia), 0 = normal, 1 = rare with small foci of collections of reactive fibroblast or small areas with increased amounts of mature collagens, 2 = small focal to multifocal areas of collections of reactive fibroblast or small focal to multifocal areas increased amounts of mature collagens, and 3 = large areas collections of reactive fibroblast or large areas increased amounts of mature collagens. The total histopathologic score was sum of all individual tissue measurements (maximum score of 21).

4.2.10 Blood analysis.

Complete blood counts were performed on a Hematrue blood analyser (Heska, Des Moines, IA) within 45 min of collection. Blood chemistry was analyzed on a Vettest blood analyzer (Idexx Laboratories, Westbrook, ME) using a pre-anesthetic blood panel. The pre-anesthetic blood panel included tests for alanine aminotransferase, alkaline phosphatase, creatinine, glucose, total protein, and blood urea nitrogen.

4.2.11 Isolation and genotyping of *Salmonella*.

Salmonella was isolated and identified from feces, systemic blood, ileal-cecal lymph nodes, jejunal lymph nodes, liver, spleen, frontal cortex, midbrain, and brain stem as previously described (Molla et al. 2010). Briefly, for feces, ≈1 g of fecal matter, was weighed, diluted 1:10 in buffered peptone water (Oxoid Inc., Nepean, ON), and suspended in the liquid by vortexing. For blood, 1 ml of systemic blood

was added to 9 ml of buffered peptone water, and vortexed. For tissues, any bacteria on the outside of the samples were killed by immersion into boiling water for 10 s; this method does not kill *Salmonella* internalized within the tissues (Vieira-Pinto et al. 2005). As with feces, ≈1 g of tissue was weighed and diluted 1:10 in buffered peptone water. Samples were homogenized using a Tissue Tearor (Model 985370; Biospec Products Inc., Bartlesville, OK). All suspensions were incubated at 37°C for 16-24 hours, and 50 µl of the suspension was transferred to 5 ml of Rappaport-Vassiliadis enrichment broth (Oxoid Inc.), and incubated at 42°C for 16-24 hours. A 10 µl inoculation loop was used to transfer a subsample of the Rappaport-Vassiliadis enrichment broth to both Brilliant Green Agar (BGA; BD Difco, Mississauga, ON) and Modified Lysine Iron Agar (MLIA; Oxoid Inc.). The BGA and MLIA cultures were incubated for 48 h at 37°C to allow time for H_2S production to manifest. Representative red colonies on BGA were transferred to Triple Sugar Iron agar (TSIA; BD Difco) slants. Two representative black colonies from MLIA were transferred to TSIA slants. The TSIA slant cultures were incubated at 37°C for 16-24 hours. Representative colonies from *Salmonella* positive TSIA slants were transferred to MacConkey Agar (BD Difco) and incubated at 37°C for 16-24 hours. Colorless colonies on MacConkey Agar are considered *Salmonella* positive and were stored in 30% glycerol in broth at -80°C.

To identify presumptive *Salmonella* positive colonies, genomic DNA was extracted using an automated DNA extraction robot (Model Autogen 740; Autogen, Inc., Holliston, MA) according to the manufacturer's recommendations. Taxon-specific PCR was conducted using the primers F-(Sal) and R-(Sal), which target the *invA* gene of *S. enterica* (Kumar et al. 2010). The reactions were run on Eppendorf Mastercycler Pro S thermocycler (VWR international, Mississauga, ON) at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 64°C for 1 min, 72°C for 1 min, and one cycle at 72°C for 10 min. DNA obtained from *S.* Typhimurium SA970934 was used as a positive control, and Optima water (Fisher Scientific, Edmonton, AB) was used as a template negative control. To genotype *Salmonella* isolates, the PFGE protocol used by PulseNet (PNL05) was applied to representative *Salmonella* isolates recovered from piglets relative to *S.* Typhimurium SA970934.

4.2.12 Meat characteristics.

Exsanguinated animals were hung in a 4°C cooler, and the pH of the right *longissimus dorsi* muscle (≈2-3 cm posterior to the last rib) was determined at 45 min and 24 hours post-mortem using a portable meat pH meter (HI99163; Hanna Instruments, Laval, QC). At 24 hours post-mortem, the right and left longissimus dorsi muscles were removed, and moisture drip loss test was conducted. Briefly, the *longissimus dorsi* muscle from each side was treated as an observation (n = 2). The individual muscles

were trimmed to equal dimensions, cut in two, weighed, placed in a porous nylon bag, suspended within a liquid impervious bag for 48 h at 4°C, and re-weighed.

4.2.13 RNA extraction.

To quantify mRNA of targets of interest, RNA was extracted from samples (≈0.5 x 0.5 cm) from the intestines (duodenum, jejunum, ileum, cecum, ascending colon, spiral colon, and descending colon) and spleen stored in RNAprotect® (Qiagen Inc.) using an RNeasy Mini kit (Qiagen Inc.) with a DNase step added to eliminate residual genomic DNA (Qiagen Inc.). RNA quantity and quality was determined using a Bioanalyzer 2100 (Agilent Technologies Canada Inc., Mississauga, ON), and 1000 ng of RNA was transcribed to cDNA following the manufacturer's protocol (Qiagen Inc). Reactions were run on a 384-well plate, and each reaction contained 5.0 µl QuantiTect SYBR Green Master Mix (Qiagen Inc.), 0.5 µl of each primer (10 µM) (Table 4.1), 3.0 µl of RNase-free water, and 1.0 µl of cDNA. Quantitative PCR was performed using ABI7900HT thermocycler (Applied Biosystems, Carlsbad, CA) with the following cycle conditions: 95°C for 15 min; 40 cycles of 95°C for 15 sec, at the primer annealing temperature for 30 sec, and 72°C for 30 sec; and melt curve analysis from 55-95°C. Some primer sequences specific to gene targets were generated using NCBI Primer-BLAST to generate an amplicon between 75 and 200 base pairs. Reactions were run in triplicate and average Ct values were used to calculate expression relative to Peptidylprolyl isomerase A (*PPIA*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), and beta-glucuronidase (*GUSβ*) reference genes. These genes were selected using the geNorm algorithm in qbase+ (Biogazelle, Zwijnaarde, Belgium), which identifies stability of expression among samples (Vandesompele et al. 2002).

4.2.14 Bacterial genomic DNA extraction from digesta and tissue samples.

For quantification of *S.* Typhimurium and select commensal bacteria associated with mucosa by qPCR, DNA was extracted from the ileal, cecal, and spiral colonic samples using the Qiagen Blood and Tissue kit (Qiagen Inc.) gram positive protocol. For characterization of mucosa-associated bacteria by NGS analysis, DNA extracted from intestinal tissue of the spiral colon was enriched using the NEBNext® Microbiome DNA Enrichment Kit (New England Biolabs, Ipswich, MA). For quantification of *S.* Typhimurium and select commensal bacterial taxa, and characterization of bacterial communities in feces and digesta from the ileum, cecum, and spiral colon, DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen Inc.). A bead homogenization step using 5.0 mm-diam stainless steel beads following the Qiagen protocol for isolation of bacterial DNA using a TissueLyser LT (Qiagen Inc.) at 30 Hz was included to ensure comprehensive extraction of genomic DNA. The bead homogenization step (30 s) was conducted three times per sample.

4.2.15 Quantification of *Salmonella.*

To enumerate *S.* Typhimurium by qPCR, duplicate reactions (20 µl) were prepared as follows: 10 µl of QuantiTect SYBR® Green Mastermix (Qiagen Inc.), 0.5 µM of each primer (IDT, San Diego, CA), 2 µl BSA (0.1 µg/µl) (Promega, Madison, WI), 2 µl DNA, and 4 µl DNase free water (Qiagen Inc.). The primers used were F-(Sal) and R-(Sal) (Kumar et al. 2010). Data was collected using an Mx3005p Real Time PCR instrument (Agilent Technologies Canada Inc. Mississauga, ON). Cycle conditions used were 95°C for 5 min, followed by 40 cycles of 94°C for 15 s, 64°C for 30 s, and 72°C for 30 s. Serial dilutions of genomic DNA containing 1.5 x 10^6 copies/µl were used to prepare a standard curve, concentrations of the *Salmonella* DNA in the sample were determined based on standard curve Ct values, and copies per cm^2 were calculated. A dissociation curve was included with each run to verify amplicon specificity.

4.2.16 Characterization of bacterial communities by culturomics.

4.2.16.1 Bacteriological media.

The basic protocol used was recently published elsewhere (Moote et al. 2020). All media (Table 4.2-Table 4.3) were reduced before use. Media were prepared without the addition of cysteine and autoclaved for 5 min. Once autoclaved, warmed media was immediately transferred into chamber containing a nitrogen (N_2)-predominant atmosphere consisting of 85:10:5% N_2:CO_2:H_2 or a carbon dioxide (CO_2)-predominant atmosphere consisting of 90:10% CO_2:H_2, and vigorously agitated to displace oxygen from the media. For media used in the CO_2 atmosphere, 40 ml of 8% sodium carbonate (Sigma-Aldrich, Ottawa, ON) was added to prevent acidification. When cooled, media were decanted into separate bottles containing the desired L-cysteine monohydrate content to remove any residual oxygen, the bottles were sealed with a screw cap containing a rubber stopper, removed from the chamber, and autoclaved for 30 min at 121°C and 15 kPa. The pH of media was tested using pH paper (Micro Essential Laboratory, Brooklyn, NY).

Agar (1.5% agar; BD Difco) and resazurin as an oxygen indicator (25 µg/ml; Temecula, CA) were added to media before autoclaving for 30 min. Media was dispensed into Petri dishes, and maintained in the N_2 and CO_2 atmosphere chambers for 24 hours before use. For enrichments, resazurin sodium salt (25 µg/ml) was added, media (10 ml) were dispensed into 15-ml glass Hungate tubes (Kimble-Chase, Vineland, NJ), tubes were sealed with a screw cap fitted with a black butyl rubber stopper (Bellco Glass Inc., Vineland, NJ), autoclaved for 30 min, and then transferred to the N_2 and CO_2 atmosphere chambers.

4.2.16.2 Liberation of bacteria from digesta and the mucosal surface.

Ligated intestinal samples were transferred into an anaerobic chamber (Forma Scientific, Inc., Marietta, OH) containing the N_2 atmosphere. The anoxic status of anaerobic chambers was routinely monitored using a resazurin anaerobic indicator (Oxoid Inc.). Once in the chamber, ligations were aseptically removed, and the intestinal segment was incised to expose the mucosal surface and digesta. A 1 cm^2 sample of the intestinal wall with digesta was removed, and transferred into 5 ml of reduced CB (HiMedia Laboratories LLC, West Chester, PA) in a 50-ml Falcon tube, where it was gently mixed by rocking side to side for 30 s. To isolate mucosa-associated bacteria, individual washed tissue segments were transferred to a new tube containing 5 ml of CB. The remaining suspension in the initial tube was used to isolate digesta-associated bacteria. Tubes were then vortexed vigorously (high setting for 0.5 min), and the resultant suspensions were used to isolate bacteria. Half of the tubes were retained within the N_2 atmosphere chamber, and half of the tubes were transferred to a CO_2 atmosphere chamber.

4.2.16.3 Direct plating.

Bacteria suspended in CB (10 µl) were streaked onto each agar medium within the N_2 and CO_2 atmosphere chambers, and cultures were maintained for 7 days at 37°C. Where possible, cells from a minimum of five morphologically distinct colonies per culture were transferred to fresh reduced Columbia agar supplemented with 10% whole sheep blood (CBA), and streaked for purity as warranted.

4.2.16.4 Enrichment.

Bacterial suspended in CB (10 µl) were added to enrichment media, and cultures were maintained at 37°C for 12 weeks in the N_2 and CO_2 atmosphere chambers as done previously (Moote et al. 2020). After the incubation period, bacteria were isolated by streaking the culture onto CBA as described above for direct plating.

4.2.16.5 Ichip.

A modified version of the original Ichip method (Berdy et al. 2017) was used. The primary modification was the pipette tip holder used to construct the Ichip was modified to contain a 0.02 µm pore membrane (Sterlitech Corporation, Kent, WA) attached using Silicone II sealant (General Electric Company, Fairfield, CT). In addition, the Ichip enrichment medium was modified to be rumen fluid. Specifically, the assembled Ichip was created and sterilized by autoclaving and maintained in the N_2 and CO_2 atmosphere chambers for 24 hours before use. Warm (37°C) reduced phosphate buffered saline (PBS; 0.1 M, pH 7.2) containing 0.5% agarose was used to dilute bacteria liberated from digesta and mucosal surfaces in CB. Bacterial cells in suspension (20 µl) were stained with Trypan Blue (20 µl) (Sigma-Aldrich), and enumerated using a Petroff-Hausser chamber at 100X magnification. Bacterial suspensions were diluted in the reduced PBS-agarose medium to a target density of one cell in 200 µl,

and 200 µl aliquots were dispensed into individual cells within the Ichip apparatus. The top of the Ichip was sealed with a non-porous adhesive membrane (BioRad Laboratories Inc., Hercules, CA). Another salient modification from previous reported applications of the Ichip was the continuous submergence of the apparatus in freshly collected rumen fluid, which provided limiting nutrients including vitamins and co-factors; rumen fluid was collected from fistulated beef cattle (LeRDC Animal Use Protocol Review 1614). Ichips were maintained for 12 weeks, and rumen fluid was replaced with fresh fluid at 2-week intervals. After the incubation period, the Ichips were removed, and left on sterile paper towel within anaerobic chambers to dry. The Ichip top seal was then carefully removed taking care to prevent cross contamination among wells, and a 10 µl aliquot was removed from each well, and streaked onto reduced CBA as above.

4.2.16.6 Endospore-forming taxa.

Two strategies were applied to kill vegetative bacteria, and to facilitate the isolation of endospore-forming taxa. These were the basic ethanol-killing method described by Browne et al (2016), and Tyndallization. For the former, an equal volume of ethanol (70%) was added to 750 µl of the bacterial cells suspended in CB in 2 ml sample vials, rigorously vortexed, and maintained at 37°C for 4 h. Subsamples of the CB-ethanol mixture (10 µl) were streaked onto CBA containing 0.1% taurocholic acid (Millipore, Burlington, MA). In addition, 10 µl of the suspensions were transferred into 10 ml Hungate tubes containing reduced Dehority's medium amended with 0.5% xylan (Sigma-Aldrich) or 0.5% porcine mucin (Sigma-Aldrich), as well as CB with 5% sheep blood for enrichment. Enrichments were maintained for 12 weeks at 37°C, and 10 µl aliquots were streaked onto CBA as above.

For Tyndallization, ≈500 µl of the bacterial suspension was transferred to a sterile 1.5 ml glass tube, and then sealed within the anaerobic chambers. The samples were transferred to an oven, and maintained at 100°C for 30 min to kill vegetative cells and to stimulate the germination of endospores. After the heat treatment, the tubes were returned to the anaerobic chambers, and processed as for the ethanol-killing method.

4.2.16.7 Recovery and storage of bacteria.

Based on colony morphology, five representative colonies were selected per culture (1,523 isolates in total), and cells from the colonies were streaked for purity on CBA. After ≥7 days, bacterial biomass was suspended in 1.5 ml of CB containing 40% glycerol, the tubes were sealed, snap frozen on dry ice, and transferred to -80°C for medium-term storage.

4.2.16.8 Bacterial identification.

Bacteria were rejuvenated from glycerol stocks on CBA at 37°C in the atmospheres from which they were originally isolated until sufficient biomass was produced. Biomass was scraped from the agar surface, placed in CB, and sedimented by centrifugation (13,200 x *g* for 10 min). Genomic DNA was extracted using an automated DNA extraction robot (Autogen, Inc.) according to the manufacturer's recommendations. Amplicons of the 16S rRNA gene were generated using the primers 27F and 1492R (Costa et al. 2009). Amplicons were purified using a QIAquick PCR Purification kit (Qiagen N.V., Hilden, Germany) and sequenced by Eurofins genomics (Toronto, ON) using the 27F primer. Sequence chromatograms were visualized, assessed for quality, and trimmed using Geneious (Biomatters, Inc., San Diego, CA).

4.2.16.9 Analysis of isolated bacteria.

Bacterial taxonomy was assigned using the Seqmatch tool of the Ribosomal Database Project (RDP) with the following settings selected: strain = both (Type and Non-Type); source = Isolates; size = both (>1200 and <1200; quality = good; taxonomy = nomenclature; and KNN matches = 1 (Cole et al. 2014). Diversity metrics were made using functions of the *vegan* package in R (Version 3.4.3) and phylogenetic relationships were analyzed using the LEfSe tool on the Galaxy Instance of the Huttenhower lab (http://huttenhower.sph.harvard.edu/galaxy/). Phylogenetic trees were generated and evolutionary analyses were conducted from the 16S rRNA gene sequences using the MEGA-X software package (Kumar et al. 2018). Briefly, a multiple sequence alignment was made using MUSCLE software and a tree was generated using the unweighted pair group method with arithmetic mean (UPGMA) method (Edgar 2004). A bootstrap test for phylogeny was used with the number of replications set to 1,000. A tree figure was generated with FigTree version 1.4.4 using the resulting Newick file (Rambaut 2012; Pohlert 2018). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option); there were a total of 1,258 positions in the final dataset (Kumar et al. 2018).

4.2.17 Bacterial community characterization by NGS analysis.

DNA extracted from digesta and mucosa was processed with an Illumina protocol for creating 16S rRNA sequencing libraries. Extracted genomic DNA was amplified with Illumina indexed adaptor primers (V4 Schloss primers (Kozich et al. 2013)). The PCR reaction contained 5 µl of PCR buffer, 1 µl of 10 mM dNTPs, 1 µl of 25 mM of $MgCl_2$, 2.5 of µl of each primer, 0.25 µl of Hot Start Taq (Qiagen Inc.), 32.8 µl of molecular grade water, and 5 µl of bacterial community DNA. Amplicons were purified with QIAquick PCR Purification kit (Qiagen Inc.) following the manufacturer's recommendations. The effectiveness of

the clean-up was checked by agarose gel electrophoresis, followed by quantification with a Qubit (Fisher Scientific, Ottawa, ON). Indexed DNA libraries were normalized to 1.5 ng/µl and pooled together. A PhiX control (10%) was run with the normalized DNA library, and both were denatured and diluted to 8 pM prior to loading into the MiSeq Reagent Kit v2 500-cycle (Illumina, San Diego, CA). The raw sequencing reads were submitted to the Sequencing Read Archive of NCBI under BioProject accession PRJNA612572. An average of 5,673 16S rRNA gene amplicon reads were obtained per sample with 94% of the reads passing the Q30 score. QIIME2 (Bolyen et al. 2019) was used to classify bacterial reads for digesta and mucosa-associated communities. This analysis was done using the tourmaline Snakemake reproducible workflow to automate QIIME2 (version 2019.7) analyses (https://github.com/ropolomx/tourmaline). Briefly, raw reads were denoised with DADA 2 (Callahan et al. 2016), and representative ASVs were generated. A phylogenetic tree of ASV sequences was generated, and the taxonomy of each ASV was identified by using a Naïve Bayes classifier pre-trained with the reference SILVA 132 database (silva-132-99-515-806-nb-classifier.qza). Alpha diversity metrics including number of taxa observed, the Shannon's index of diversity, and the Inverse Simpson index were calculated. The phyloseq package (version 1.28.0) of R version 3.6.1 was used to evaluate beta-diversity with a Principal Coordinate Analysis (PCoA) of the calculated Unweighted Unifrac distances, generating an ordination plot. Detection of differential abundance between tissues was done with the analysis of composition of microbiomes (ANCOM) in QIIME2 (Mandal et al. 2015).

4.2.18 Biochemical pathway inference.

Analysis of biochemical pathways inferred from the 16S rRNA gene phylogenies of the mucosa datasets was done with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2 (Douglas et al. 2020)). The following outputs were generated with PICRUST2: biochemical pathways; KEGG numbers; and EC numbers. Differential abundance of each of the PICRUST outputs in ST+ versus ST- treatment samples was completed for individual tissues for each of the dpi. Differential abundance of biochemical pathways, KEGG numbers, and EC numbers were tested with the ANOVA-Like Differential Gene Expression Analysis method (ALDEx2 package version 1.20.0; R version 4.0.0 (Fernandes et al. 2013, 2014)).

4.2.19 Network analysis.

Interaction networks were generated based on Bray-Curtis distances of the taxonomically classified ASVs using the phyloseq package (version 1.32.0 (McMurdie and Holmes 2013)) in R (version 4.0.0).

4.2.20 Quantification of commensal bacterial taxa by quantitative PCR.

To enumerate commensal taxa of interest (e.g. *B. uniformis* and *S. gallolyticus*) by qPCR, duplicate reactions (20 µl) consisting of 10 µl of QuantiTect SYBR® Green Mastermix (Qiagen Inc.), 0.5 µM of each primer (Table 4.4), 2 µl of community DNA, and 6 µl of DNase free water (Qiagen Inc.) were prepared. Data was collected using an Mx3005p Real Time PCR instrument (Agilent Technologies Canada Inc.). Cycle conditions were 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, at the individual annealing temperatures (Table 4.4) for 30 s, and at 72°C for 30 s. Serial dilutions of genomic DNA containing 1.0 x 10^6 copies/µl were used to prepare a standard curve. Concentrations of the bacterial DNA in the sample were determined based on standard curve Ct values, and copies per mg or g were calculated. A dissociation curve was included with each run to verify amplicon specificity.

4.2.21 Statistical analyses.

The experiment was arranged as a two (*Salmonella* ±) by three (2, 6, and 10 dpi) factorial experiment with four replicates. These two factors and the interaction between them were treated as fixed effects. Given the experiment was run on two separate occasions (i.e. runs), run was treated as a random effect in the statistical model. Analyses for gene expression, histopathologic measurements, *Salmonella* quantification, bacteria quantification, physiopathology changes, and meat pH were performed using Statistical Analysis Software (SAS; SAS Institute Inc. Cary, NC). Normality was checked and analyzed in continuous data using the MIXED procedure of SAS. In the event of a main treatment event effect ($P \leq 0.050$), the least squares means test was used to compare treatments within factors. Histopathologic measurement data was analyzed using the pairwise Fisher's exact test in SAS. Quantification data for commensal bacteria was not normally distributed, and the data was analyzed using the Kruskal-Wallis test in SAS. Data are represented by mean ± standard error of the mean.

4.3 Results

4.3.1 Infection by *Salmonella* Typhimurium induced temporal changes in health status.

All piglets inoculated with *S.* Typhimurium (ST+) showed behavioral evidence of disease in a temporal manner. Inoculated piglets rapidly developed transient watery diarrhea (some with mucus discharge). This was particularly conspicuous within 2-3 days post-inoculation (dpi). Moreover, all inoculated piglets were modesty depressed and lacked appetite. By 6 dpi, diarrhea had largely abated, and by 10 dpi all piglets showed distinct evidence of recovery (e.g. semi-solid stools) and restoration of normal food consumption.

4.3.2 Infection by *Salmonella* Typhimurium affected body temperature, feed consumption, and weight gain.

ST+ animals exhibited an increase (P = 0.028) in body temperature over time (P = 0.007) (Figure 4.2A); the body temperature of inoculated piglets was elevated (P ≤ 0.050) 1, 2, and 3 dpi. The consumption of feed was suppressed (P ≤ 0.001) in infected animals 2 to 5 dpi (Figure 4.2B). ST+ piglets lost weight for 4 days, and weighed less (P = 0.003) than control piglets (ST-) at all experimental end points (Figure 4.2C).

4.3.3 Infection by *Salmonella* Typhimurium induced gross pathologic changes.

At 2 and 6 dpi, all ST+ piglets showed gross evidence of enteritis in the cecum, ascending colon, and spiral colon, and to a lesser extent in the ileum. This included evidence of hyperemia and excessive gas and liquefied digesta accumulation. Fibrinous mucosal necrosis with casts were frequently observed in the spiral and ascending colon of ST+ piglets. All ST+ piglets displayed enlarged lymph nodes, and some inoculated animals exhibited splenomegaly. In no instance were conspicuous gross pathologic changes observed in the duodenum or jejunum.

4.3.4 Infection by *Salmonella* Typhimurium induced temporal histopathologic alterations in the distal small intestine and large intestine.

Substantive histopathologic changes to the intestine were observed in ST+ relative to ST- piglets. No differences were observed in histologic changes within infected animals between the two cecal locations (P = 0.408) or the two spiral colon locations (P = 1.000) examined; therefore, information from the two locations within the cecum and spiral colon were grouped. Higher (P ≤ 0.041) total histopathologic scores (i.e. epithelial injury, neutrophil infiltration, fibrosis, villus fusion) were observed in ST+ piglets in the distal small intestine (distal jejunum and ileum), and throughout the large intestine at 2, 6, and 10 dpi (Figure 4.3). The degree of neutrophil infiltration (mucosa to submucosa) between ST+ and ST- piglets in the distal small intestine and large intestine was the highest (P ≤ 0.001) at 2 dpi, and progressively subsided by 10 dpi (Figure 4.4). Changes were particularly evident in these intestinal sites, as there is marked transmural neutrophilic inflammation with multifocal to coalescing areas of epithelial erosion (Figure. 4.5). In contrast, fibrosis in the large intestine of ST+ pigs was more prominent (P ≤ 0.001) at 10 dpi (Figure. 4.6).

4.3.5 Infection by *Salmonella* Typhimurium affected total white cells densities in blood.

There was no difference (P = 0.627) in the numbers or types of immune cells collected from the portal vein or systemic venous blood. Densities of total white blood cells, and the percentage of granulocytes in blood of ST+ piglets were elevated (P ≤ 0.001) only at 10 dpi (Figure 4.7-A,D). Moreover,

the percentage of lymphocytes circulating in the blood of ST+ piglets was lower ($P \leq 0.005$) at 10 dpi (Figure 4.7-B). The percentage of monocytes in blood were lower ($P \leq 0.020$) at 10 dpi (Figure 4.7-C). Inoculation of pigs with *S*. Typhimurium had no effect ($P > 0.500$) on blood chemistry, including alanine aminotransferase, alkaline phosphatase, creatinine, glucose, total protein, and blood urea nitrogen (*data not presented*).

4.3.6 Higher densities of Salmonella Typhimurium were observed in infected piglets at 2 days post-inoculation.

Salmonella Typhimurium was not isolated from the feces of any of the piglets upon arrival at the Lethbridge Research and Development Centre (LeRDC), nor was bacterium isolated from ST- treatment animals during the experimental period. In contrast, *S.* Typhimurium was isolated from the feces of ST+ at high densities throughout the study period; densities of the bacterium shed in feces peaked at day 2 and 4 dpi (Figure 4.8). High densities of *S.* Typhimurium DNA were also observed in digesta and associated with mucosa throughout the intestinal tract (Figure 4.9). Densities of the pathogen associated with mucosa, and to a lesser extent in digesta, were highest ($P \leq 0.039$) in the distal small intestine and large intestine, and decreased ($P \leq 0.017$) over time. *Salmonella* Typhimurium was frequently isolated from the jejunal and ileal-cecal lymph nodes, and from the liver and spleen. The bacterium was isolated from the brain (frontal cortex and mid-brain) of one piglet 2 dpi, and from systemic blood of an additional animal 10 dpi. Genotyping of arbitrarily selected strains of *S.* Typhimurium isolated from piglets showed that recovered isolates possessed the same pulsed field gel electrophoresis (PFGE) fingerprint as the *S.* Typhimurium SA970934 (*data not presented*).

4.3.7 The pH of meat was affected in piglets infected with *Salmonella* Typhimurium.

The pH of *longissimus dorsi* muscle from ST+ piglets was lower ($P < 0.001$) than ST- piglets (Figure 4.10). Moreover, the pH of *longissimus dorsi* muscle was lower ($P < 0.001$) in pigs at 6 and 10 dpi regardless of the *Salmonella* treatment. There was no effect ($P = 0.824$) of *S.* Typhimurium infection on drip moisture loss from the *longissimus dorsi* muscle harvested from piglets (*data not presented*).

4.3.8 Infection by *Salmonella* Typhimurium temporally modulated immune responses.

Expression of genes encoding immune response proteins were evaluated to temporally characterize responses incited by *S.* Typhimurium throughout the intestinal tract. Differential expression of mRNA for immune genes was not observed in the duodenum or jejunum (*data not presented*). In contrast, the differential regulation of a variety of immune genes was observed in the ileum, cecum, and spiral colon (Figure 4.11). In the ileum, *βD2* ($P = 0.011$), *MUC4* ($P = 0.038$), and *REGIIIγ* ($P = 0.011$) were upregulated at 6 dpi and/or at 2 dpi. In the spiral colon, *βD2* ($P = 0.029$) and *MUC1* ($P = 0.024$) were downregulated

in ST+ at 2 dpi and/or 6 dpi. At 2 dpi, *TNFα* ($P < 0.001$), *IFNγ* ($P = 0.006$), *IL17* ($P = 0.014$), *IL10* ($P = 0.006$), *PR39* ($P = 0.002$), *IL1β* ($P < 0.001$), *IL8* ($P = 0.016$), *iNOS* ($P = 0.006$), *TLR4* ($P = 0.033$), and *TGFβ* ($P = 0.030$) were upregulated in the cecum of ST+ piglets as compared with ST- animals (Figure 4.12). At 6 dpi, *TNFα* ($P = 0.043$), *IL8* ($P = 0.008$), *IL17* ($P = 0.016$), and *IL1β* ($P = 0.045$) were upregulated in cecum of ST+ piglets compared with ST- animals. Additionally, at 6 dpi, *MUC1* ($P = 0.028$) was downregulated in the cecum of ST+ piglets compared with ST- animals (Figure 4.11).

4.3.9 Bacterial communities characterized by next-generation sequence analysis differed in the small and large intestine.

The digesta microbiota within the ileum mainly contained bacteria within the *Firmicutes* (75.0 ± 18.6%) and *Proteobacteria* (24.9 ± 18.8%) (Figure 4.13). In the cecum and spiral colon, the digesta microbiota was primarily dominated by bacteria within the *Firmicutes* (39.0 ± 13.6% and 33.9 ± 21.9%, respectively) and *Bacteroidetes* (58.7 ± 13.9% and 64.0 ± 21.3%, respectively). At a family level of resolution, greater numbers of bacteria within *Clostridiaceae*-1, *Peptostreptococcaceae*, and *Pasteurellaceae* were observed in the ileum than in the large intestine. The large intestine was mainly dominated by bacteria within *Prevotellaceae, Lachnospiraceae*, *Ruminococcaceae,* and *Veillonellaceae*. At a genus level of resolution, an increase in the relative abundance of *Clostridium sensu stricto, Streptococcus*, and *Romboutsia* was observed in the ileum, and *Prevotellaceae*-9 abundance increased in the cecum and spiral colon.

Number of amplicon sequence variants (ASVs), Shannon index, Pielou's evenness, and Faith's phylogenetic diversity were evaluated to characterize the alpha diversity of luminal samples of each intestinal site. In all instances, bacterial communities in the ileum differed from the cecum and spiral colon, which were similar to each other. For example, ASV counts were lower in ileum as compared with cecum ($P < 0.001$) and spiral colon ($P < 0.001$). Moreover, Shannon's index indicated lower diversity in the ileum as compared to the cecum ($P < 0.001$) and spiral colon ($P < 0.001$), and no difference ($P = 0.589$) was observed between the cecum and spiral colon. There was no difference ($P = 0.290$) in species evenness between the cecum and spiral colon, but evenness was reduced ($P < 0.001$) in the ileum relative to the other two sites. A clear separation in beta diversity of bacterial communities (using unweighted principal coordinate analysis) was observed between digesta in the ileum relative to the cecum and spiral colon (Figure 4.14). There was no difference in beta diversity of bacteria between the cecum and spiral colon.

To determine if bacterial communities observed in digesta corresponded with those associated with mucosa, we examined the composition of the microbiota associated with the mucosa of the spiral

colon. Bacterial communities associated with mucosa were similar in composition to those within digesta, and no significant differences were observed at the phyla, family, or genus levels (Figure 4.15).

4.3.10 Next-generation sequence analysis showed taxon- and location-specific changes in bacterial communities in piglets infected with *Salmonella* Typhimurium.

ASV counts were lower (P = 0.008) in ST+ animals. Piglets infected with *S*. Typhimurium showed an increase in the relative abundance of *Prevotellaceae* in digesta within the cecum at 2 and 6 dpi (71-79% and 50-56%) (Figure 4.13). A higher abundance of *Enterobacteriaceae* was observed in infected animals at day 6, and to a lesser extent at day 2 in the ileum. Although some genera, such as *Ruminococcaceae* and *Veillonellaceae*, tended to decrease in the cecum and spiral colon of ST+ piglets, no significant differences in the relative abundance were observed in ST+ piglets.

A lower (P = 0.048) alpha diversity of bacteria was observed in the ceca of ST+ relative to ST- piglets at 2 dpi, but not at 6 and 10 dpi (Figure 4.16). In contrast, alpha diversity was equivalent within the ileum and spiral colon of ST+ and ST- piglets (Figure 4.16).

4.3.11 The culturable bacteria differed in piglets infected with *Salmonella* Typhimurium.

To increase the taxonomic resolution and ascertain information on function, comprehensive isolation methods were applied to recover bacteria from the ileum, cecum, and spiral colon of ST+ and ST- piglets. A total of 1,526 bacterial isolates were isolated and identified; of these, 419, 331 and 484 were collected from the ileum, cecum and spiral colon, respectively. The composition of the culturable and culture-independent bacteria communities differed (Figure 4.17). For example, no *Bacteroides* or *Parabacteroides* were detected by next-generation sequence (NGS) analysis, whereas, twelve species of these two genera were isolated (*Bacteroides caccae*, *B. denticanum*, *B. eggerthii*, *B. fragilis*, *B. pyogenes*, *B. stercoris*, *B. uniformis*, *B. vulgatus*, *B. xylanisolvens*, *B. heparinolyticus*, *P. distasosis*, and *P. merdae*) (Figure 4.18). Moreover, *Streptococcus* species were not detected in cecum and spiral colon by NGS analysis, whereas *Streptococcus gallolyticus* was isolated from the ileum, cecum, and spiral colon (Figure 4.19). Analysis of taxa revealed that the abundance of taxa isolated differed between infected and control pigs (Figure 4.20). For example, *S. gallolyticus* was isolated only from the intestines of pigs infected with ST+ but not from ST- piglets. Additionally, higher abundances of *Gammaproteobacteria* (*Escherichia/Shigella, Proteus*, and *Salmonella*) were observed in ST+ piglets at 6 dpi (Figure 4.20). Bacteria that were more common in ST- animals included unclassified members of the *Ruminococcaceae* family (Figure 4.19). These bacteria included putative members of the *Intestinimonas* spp., as well as some bacteria that are most closely related to *Soleaferrea massiliensis*, among other previously undescribed bacteria. At 10 dpi, the composition of the culturable microbiota from ST+ animals did not

differ from control piglets with the exception of *Gammaproteobacteria* whose abundance remained higher in ST+ animals (Figure 4.20). In the ileum, members of the *Actinobacteria* order (e.g. *Bifidobacterium pseudolongum*) were more commonly isolated from ST- piglets (Figure 4.19). In contrast, *Gammaproteobacteria* (*Escherichia/Shigella* and *S. enterica*) and *Fusobacterium* (*F. varium* and *F. gastrosuis*) were commonly isolated from ileum of ST+ animals. In the cecum and spiral colon of ST+ piglets, members of *Bacteroidia* (*B. uniformis*, *B. fragilis* and *B. heparinolyticus*) were more abundant. Bacteria within the *Firmicutes* phylum (i.e. *Bacilli*, *Clostridia*, *Erysiphelotrichia*, and *Negativicutes*) were recovered from all locations, and from both ST+ and ST- piglets. However, members of the *Bacilli* (i.e. *Lactobacillales and Bacilliales*) were more prevalent in ST- piglets. Although *Clostridia* bacteria were more commonly associated with samples collected from the spiral colon of ST+ piglets, a higher abundance of the *Clostridiaceae* family was observed in cecum of ST- piglets.

4.3.12 Quantitative PCR confirmed that densities of bacterial genera differed by intestinal location and in piglets infected by *Salmonella* Typhimurium.

Lower quantities of DNA for *B. uniformis*, *S. gallolyticus, Intestinimonas* spp., *Prevotella* spp., and *Ruminococcus* spp. were observed in the ileum compared to the cecum ($P < 0.001$) and spiral colon ($P < 0.001$) (Figures 4.21; Figure 4.22). Although the concentration of *Clostridium* cluster I spp. DNA was higher in ileum in comparison to the other genera examined, concentrations were still lower ($P < 0.007$) in the ileum relative to the cecum (Figure 4.22-C). *Bacteroides uniformis* and *S. gallolyticus* DNA was detected in the digesta and in association with mucosa (*data not presented*) of the ileum, cecum, and spiral colon, albeit at low densities. Consistent with the frequency of isolation, densities of *B. uniformis* were higher ($P \leq 0.058$) in the digesta of infected animals in cecum at 10 dpi and spiral colon at 6 dpi (Figure 4.21). Similarly, a trend for higher ($P \leq 0.124$) densities of *S. gallolyticus* in the digesta of infected animals was observed in the cecum and spiral colon at both 6 and 10 dpi.

4.3.13 Biochemical pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) numbers and Enzyme Commission (EC) numbers did not differ between *Salmonella* Typhimurium infected and control animals.

Biochemical pathway inference was carried out with 16S phylogenies obtained from mucosa samples of the spiral colon. No biochemical pathways, KEGG numbers and EC numbers were detected outside of the effect size ($P \geq 0.873$) between ST- and ST+ treatment animals at day 2, 6 and 10 dpi.

4.3.14 Interaction networks showed lower ASVs associations in *Salmonella* Typhimurium infected animals.

Interaction networks were generated based on Bray-Curtis distances of the taxonomically classified ASVs. Modestly lower ASVs interactions were observed in ST+ as compared to ST- treatment animals (Figure 4.23). Network associations tended to decrease between the *Campylobacter* and *Prevotella* genera in ST+ animals.

4.4 Discussion

The composition of the intestinal microbiota of piglets has been studied by a number of research groups (Looft et al. 2012; Liu et al. 2012; Pajarillo et al. 2014; Kelly et al. 2017; Dou et al. 2017). However, the impact of infection and ensuing inflammation on the enteric microbiota is poorly understood at present. In our study, we temporally (2, 6 and 10 dpi) and spatially (small to the large intestine) characterized host responses due to *S.* Typhimurium infection in piglets. In this regard, we concomitantly measured immune responses, histopathologic alterations, and changes to the structure of the enteric microbiota at the acute, subacute, and chronic stages of salmonellosis. The current study is the first to comprehensively characterize the microbiota of piglets infected with *S.* Typhimurium using both culture-dependent and culture-independent methods. The highest degree of inflammation was observed in the cecum. NGS analysis showed that the primary changes to the microbiota happened in the cecum at the earliest stages of disease (i.e. 2 dpi), which correlated with the highest level of inflammation. A substantially lower Shannon diversity, an increase in the relative abundance of *Proteobacteria,* and a decrease in the relative abundance of *Clostridiaceae* and *Lachnospiraceae* were observed in the cecum of infected animals. Additionally, culture-based methods allowed us to examine bacteria at a higher resolution than NGS analysis, and demonstrated higher association of some species with inflamed tissues (e.g. *S. gallolyticus* and *B. uniformis*). Our study provides novel information on the coordinated influence that S. Typhimurium has on the enteric microbiota and host in infected piglets.

Salmonellosis is a concern to the swine sector for two primary reasons. Firstly, the disease causes significant economic losses, and secondly, *S*. Typhimurium is an important foodborne pathogen, responsible for significant morbidity of people (Scherer et al. 2008), as well as additional losses to the swine sector (e.g. meat recalls). Swine infected with *S.* Typhimurium exhibit a disease progression, but the temporal characteristics of acute salmonellosis in pigs are not well defined. We incited salmonellosis by inoculating piglets with a highly virulent strain of *S.* Typhiumurium DT104 (SA970934) (Yin et al. 2014), and we temporally measured a variety of metrics in infected and uninfected animals. Similarly to Scherer et al. (2008) we observed evidence of enteritis (Balaji et al. 2000; Johnson et al. 2005) in piglets

1 to 2 dpi, including elevated body temperature, inappetence, body weight loss and diarrhea. To temporally characterize the impacts of salmonellosis on the host, we humanely euthanized piglets, and intestinal (i.e. duodenum to the descending colon) and extra-intestinal (e.g. portal vein and systemic blood, mesenteric lymph nodes, spleen, liver, brain, and muscle) samples were recovered and analyzed at 2, 6, and 10 dpi. Substantive histopathologic changes were observed throughout the intestine in infected piglets at all three time points. In this regard, pathogen densities in digesta and mucosa samples, and total histopathologic scores, including epithelial injury and neutrophil infiltration, were higher at 2 dpi in piglets infected with *S*. Typhimurium. We observed that densities of *S.* Typhimurium within digesta and associated with mucosa decreased over time. There was also an attenuation of intestinal injury, which had returned near normal levels by 10 dpi. However, levels of fibrosis were elevated at 10 dpi in the cecum, ascending colon, spiral colon and descending colon of piglets infected with *S*. Typhimurium; an expected observation of tissue repair following injury. Spatially, histopathologic changes were most pronounced in the distal small intestine, and within the cecum and spiral colon, which is consistent with previous studies (Wilcock et al. 1976; Chirullo et al. 2015). It is suggested that the location of tissue injury may be attributed to the lower concentration of bile salts and beta-defensins present at these sites (Prouty and Gunn 2000). The three sample times at which piglets were evaluated in the current study (i.e. 2, 6, and 10 dpi), represented, acute, subacute, and recovery stages of salmonellosis, respectively.

Examination of immune marker modulation is essential to more fully understand the progression of salmonellosis in swine. Immune responses triggered after infection with *S.* Typhimurium in swine have been previously described (Uthe et al. 2007; Meurens et al. 2009; Collado-Romero et al. 2010). However, evaluation of the immune response within the entire GIT has not been previously examined. In the current study we measured different T helper cell responses that occurred after *Salmonella* infection, and the temporal progression of these responses. More specifically, we evaluated Th1 (*IFNγ*, *TNFα*), Th2 (*IL4*), Th17 (*IL17*) and T regulatory (*IL10* and *TGFβ*) responses. We also examined innate mechanisms of defense such as, host defense peptides (HDPs), including defensins (*βD1* and *βD2*), C-type lectins (*REGIIIγ*), and cathelicins (*PR39*). As well, we evaluated the expression of pattern recognition receptors (*TLR4*). Importantly, these metrics were comparatively investigated both temporally and spatially along the intestinal tract. We observed that *S.* Typhimurium did not affect the expression of the target genes in the duodenum, jejunum, and proximal ileum. In contrast, targeted genes were upregulated in *S*. Typhimurium infected piglets in the distal ileum, cecum, and colon, which corresponded to the histopathologic changes observed. In these locations, differential regulation of

gene expression primarily occurred at 2 dpi (acute stage), with modest changes at 6 dpi, and no difference between infected and non-infected piglets at 10 dpi (recovery). The cecum was the most affected intestinal site. In the cecum, we observed elevated expression of *IL17* and *IFNγ*, which corresponds to the findings of other studies (Godinez et al. 2008; O'Donnell et al. 2014). Secretion of IL17 and contact between the pathogen and enterocytes have been shown to enhance the secretion of IL8 attracting neutrophils from the microvasculature of the intestinal mucosa (McCormick et al. 1995). In the current study, a greater neutrophilic infiltration was observed in cecum at 2 dpi, which corresponded with the elevated expression of *IL8* that was observed at this time point. We observed elevated expression of inducible nitric oxide synthase (*iNOS*) in the cecum at 2 dpi. Since its secretion has been associated with elimination of *S*. Typhimurium and also with epithelial injury (Cherayil and Antos 2001), this is consistent with the mucosal damage that we observed in the cecum at 2 dpi. Induction of a pro-inflammatory response by pathogens is often accompanied by an anti-inflammatory response triggered by the host (Singh et al. 2001). We observed that *S*. Typhimurium infected piglets which exhibited a higher pro-inflammatory response in the cecum at 2 dpi, concurrently presented a higher expression of *IL10* and *TGFβ*. Both of these anti-inflammatory cytokines have previously been shown to incite a regulatory response to attenuate inflammation within the intestine (Singh et al. 2001); therefore, the elevated expression of these cytokines is likely directed to control the inflammatory response triggered by *S.* Typhimurium.

One of the main mechanisms of innate defense that the host utilizes to eliminate pathogens, and also to shape the microbiota, is the secretion of HDPs (Salzman et al. 2007). In the current study, we examined the expression of β-defensins (e.g. *βD2*), C-type lectins (e.g. *REGIIIγ*), and cathelicidins (e.g. *PR39*). We observed that these three types of HDPs were upregulated after *Salmonella* infection. The secretion of β-defensins is induced after detection of pathogens due to their bactericidal activity (Wang 2014). In piglets, the secretion of βD2 has previously been shown to inhibit the growth of *S.* Typhimurium (Veldhuizen et al. 2008). Elevated expression of REGIIIγ has also been observed following infection with enterotoxigenic *E. coli* in pigs, which was implicated with its bactericidal action by pore-forming activity (Soler et al. 2015). A primary group of HDPs are the cathelicidins (Holani et al. 2016). In pigs, *PR39*, a homologue of *LL37* and *mCRAMP* in human and mice, respectively, has been studied due to its broad antimicrobial spectrum, but not extensively in pigs with salmonellosis (Veldhuizen et al. 2014; Holani et al. 2016). The release of cathelicidins by neutrophils and their expression in the mucosa surfaces have been associated with local host defense. Their antimicrobial activity is not only limited to potent, rapid and broad spectrum bactericidal function, but also to their capacity to bind and neutralize

endotoxins (Mookherjee et al. 2007). PR39 has been observed to inhibit bacterial DNA and protein synthesis (Veldhuizen et al. 2014). Its immunomodulatory effects include leukocyte chemotaxis, modulation of cytokine production, as well as stimulation of wound healing (Gallo et al. 1994) and angiogenesis (Li et al. 2000 p. 39). In the current study, HDPs were the only genes upregulated not only in the cecum of *S*. Typhimurium infected piglets, but also in the ileum of infected animals. In this regard, two- and three-fold increases in expression of *βD2* and *REGIIIγ*, respectively, were observed in the distal ileum of infected piglets at 2 dpi, while *PR39* showed a three-fold increase in the cecum of infected animals at 2 dpi. The differential regulation of *βD2* and *REGIIIγ* in the ileum compared with the cecum was expected, since the higher expression of both of these immune markers in the ileum of infected pigs has previously been reported (Veldhuizen et al. 2008; Soler et al. 2015). Since *PR39* is mainly secreted by neutrophils, the higher expression in the cecum, that we observed in the current study, could likely be related to the level of neutrophil infiltration observed at this intestinal site. Although the roles that cathelicidins perform in modulating the enteric microbiota and salmonellosis are enigmatic at present, the changes in expression that we observed could be intimately related to their bactericidal activity directed against *S.* Typhimurium (Mukherjee and Hooper 2015).

A primary goal of the current study was to describe how the commensal microbiota is temporally modified during the induction and progression of intestinal disease. We comparatively characterized bacterial communities in the ileum, cecum, and spiral colon of *S*. Typhimurium infected and control piglets using a combination of culture-independent and culture-dependent methods. Results from NGS analysis showed a clear separation between the ileal and large intestinal communities. In the small intestine, and primarily in the ileum, the microbiota was predominated by facultative anaerobic bacteria that are adapted to acidic pH, high concentrations of bile acids, antimicrobial peptides, and higher levels of oxygen, which allows them to outcompete many obligate anaerobic bacteria for simple carbohydrates (e.g. oligosaccharides) (Zhang et al. 2018). It has been proposed that the microbiota of the ileum originates from the cecum, a hypothesis that is primarily based on the presence of similar taxa at both sites (Brooks et al. 2011). In the ileum, we observed a higher abundance of *Enterobacteriaceae* in the luminal digesta relative to the large intestine. This may be attributed to the higher concentration of oxygen found in the small intestine that would differentially benefit facultative anaerobic taxa in this family (Donaldson et al. 2016). In contrast, the neutral pH, lower concentration of antimicrobials, slower transit time, and greater access to complex carbohydrates in the large intestine facilitate the colonization and proliferation of a substantially higher diversity of autochthonous bacteria, with cell

densities in the range of 10^{11} to 10^{12} cells/g in the large intestine compared with 10^{4} to 10^{8} cells/g in the small intestine (Rastall 2004; Donaldson et al. 2016).

In the cecal and spiral colonic digesta, we observed that bacteria within the *Bacteroidetes* and *Firmicutes* were most prevalent, which has been observed previously (Zhang et al. 2018). Overall, we observed taxon-specific changes in the composition of bacterial communities within digesta between *S*. Typhimurium infected and control piglets. The most pronounced changes between the two treatment groups occurred at 2 dpi, which corresponded with the highest degree of intestinal damage (i.e. acute salmonellosis). Communities in cecal digesta were more affected by inflammation than in the spiral colon. In this regard, a decrease in alpha diversity was observed in the cecal digesta of *S*. Typhimurium infected piglets at 2 dpi, which corresponds to a similar observation in chickens infected with *S.* Typhimurium (Mon et al. 2015). A higher abundance of *Proteobacteria* (e.g. *Enterobacteriaceae*) was observed in both the cecum and spiral colon of *S*. Typhimurium infected piglets, and this was most pronounced in the cecum at 2 dpi as has been observed previously (Litvak et al. 2017). An increase in bacterial proportion within this phylum is a hallmark of dysbiosis and epithelial injury (Mon et al. 2015; Litvak et al. 2017), and since the *Proteobacteria* phylum contains many human and porcine pathogens, including *Salmonella*, the increase in abundance of pathogenic organisms within inflamed tissues is expected. Correspondingly, obligate anaerobes such as *Clostridiaceae*-1 and *Lachnospiraceae* decreased in abundance within the cecum and spiral colon of *S*. Typhimurium infected animals at 2 dpi, but recovered to normal levels by 10 dpi. Changes in the relative abundance of *Enterobacteriaceae*, *Clostridiaceae*, and *Lachnospiraceae* are influenced by levels of oxygen in the lumen of infected piglets (Rigottier-Gois 2013; Litvak et al. 2017). When inflammation is triggered within the GIT, higher levels of oxygen are released into the lumen (Rigottier-Gois 2013). The increase in luminal oxygen could be due to higher irritation of the GIT in chronic inflammation with an extravasation of hemoglobin carrying oxygen into the lumen (Rigottier-Gois 2013). A change in oxygen level could also be the result of inflammation itself, as an oxidative burst triggered by neutrophils releases reactive oxygen species into the lumen benefiting facultative anaerobes (Winter et al. 2010). Moreover, a negative correlation between *Enterobacteriaceae* and *Prevotellaceae*, *Ruminococcaceae,* and *Lachnospiraceae* has previously been described in other hosts infected with *Salmonella* (Mon et al. 2015).

The composition of the microbiota varies in the cross-sectional axis of the intestine. Unique niches for enteric bacteria (Kelly et al. 2017) are provided by higher concentrations of oxygen, the proximity to the mucosa, and the abundance of mucin glycoproteins and other nutrient sources. We observed higher densities of *S.* Typhimurium in association with the mucosa as compared with the luminal digesta in the

cecum and spiral colon. This is consistent with the lowered densities of *Prevotellaceae*, *Lachnospiraceae,* and *Ruminococcaceae* previously observed in association with cecal mucosa (Kelly et al. 2017). We observed that communities associated with the mucosa were not significantly different in composition than those in the digesta of the spiral colon (i.e. via Illumina sequencing of the community). Although we observed a trend for a higher abundance of bacteria within the *Enterobacteriaceae* and *Campylobactereaceae* in association with mucosa (i.e. families that contain species known to associate with mucosa) these differences were not statistically different. Studying mucosa-associated bacteria is challenging. For example, removal of residual digesta from mucosa surfaces without disrupting the integrity of the loosely adherent mucus is exceptionally difficult. Furthermore, the predominance of host DNA can lead to preferential amplification of host DNA; to circumvent this possibility we used a DNA enrichment method that allowed capture and elimination of methylated host DNA while conserving and enriching bacterial DNA. Although some studies have reported a unique bacterial community associated with mucosa (Zhang et al. 2018), conclusions regarding differential composition of mucosa-associated and luminal communities without supporting evidence (e.g. fluorescent *in situ* microscopy) should be interpreted carefully. Network interaction analyses carried out in the mucosa samples did not show significant effects; however, an increased in the number of microbial associations in controls compared to *Salmonella* infected animals was observed. The decreased number of network interactions observed between *Campylobacter* and *Prevotella* genus in infected animals could be strictly related to the lower diversity observed under infection. The lower ASVs from genus *Prevotella* associated with the mucosa of infected animals could be the result of the higher level of inflammation and damage detected in the spiral colon of ST+ which is in line with previous studies (Kelly et al. 2017).

Despite the current emphasis on NGS technologies to characterize the intestinal microbiota of mammals, there are inherent limitations of sequence-based methods (e.g. poor taxonomic resolution, taxa overrepresentation, poor sensitivity, and incapacity to differentiate live from dead bacteria) (Inglis et al. 2012). Thus, we also employed culture-based methods to characterize enteric communities in the intestines of *S*. Typhimurium infected and control piglets. To isolate bacteria, we applied a variety of media and strict anaerobic methods (e.g. differential killing of vegetative cells, induction of endospore germination, direct plating, long-term enrichments, and an Ichip method modified to isolate enteric bacteria) (Lagier et al. 2012; Browne et al. 2016; Berdy et al. 2017). We observed that no single isolation method was comprehensive, and that a combination of methods was required. In particular, the use of long-term enrichments and Ichip recovered the highest diversity of bacteria. Similarly to Lagier et al. (2016) we observed that Columbia agar supplemented with sheep's blood (CBA) provided good recovery

of bacteria by direct plating. Of the isolation methods utilized in the current study, the use of heat treatment was found to noticeably change the diversity of isolated bacteria, which is in agreement with recent studies (Fenske et al. 2020). However, the diversity of the bacteria isolated via Tyndallization as well as via ethanol exposure was reduced relative to the long-term enrichment and the Ichip methods. This decrease in diversity is in disagreement with that of Browne et al. (2016), who suggested that endospore germination methods such as the ethanol exposure would increase the diversity and isolation of novel taxa. Using a single method, previous studies have isolated and characterized bacteria from the intestines of healthy pigs. They recovered 46 presumptive taxa that were identified using physiological characters. In contrast, we recovered a minimum of 173 different bacterial species, representing seven phyla using a combination of methods in concert. Although our findings of prominent taxa broadly correspond with that of Russell et al. (1979) in that *Eubacterium*, *Lactobacillus*, and Gram positive cocci were frequently recovered, that we targeted bacteria associated with inflamed tissues coupled with the use of a combination of culturomic methods likely contributed to the much higher diversity of bacteria that we recovered from piglets (Russell 1979).

Our culturomic and NGS analyses of enteric bacteria in piglets with salmonellosis both showed an elevated abundance of *Gammaproteobacteria* (*Escherichia/Shigella, Proteus* and *Salmonella*), which has been previously described (Zeng et al. 2017). However, in other instances the composition of the bacterial communities as determined by culturomics and NGS analysis differed conspicuously. In this regard, analysis of culturomics data showed that a number of taxa were differentially associated with inflamed tissues. For instance, *S. gallolyticus* was solely isolated from *S*. Typhimurium infected piglets, and *Bacteroides fragilis*, *Bacteroides heparinolyticus, B. uniformis*, and *Acidominococcus fermentants* were more commonly isolated from pigs with salmonellosis; these three genera were not detected using NGS analysis. That we isolated *S. gallolyticus* at a conspicuously higher frequency from inflamed tissues of piglets infected with *S*. Typhimurium suggests that *S. gallolyticus* is favored by this condition. An association between *S. gallolyticus* and colon cancer tissues in human beings has been recently reported (Boleij and Tjalsma 2013). Therefore, the presence of this bacterium in *S*. Typhimurium infected piglets may be a result of a propensity of this bacterium to colonize inflamed tissue sites (Fyderek et al. 2009; Al-Jashamy et al. 2010). A characteristic of *S*. *gallolyticus* that may favor the colonization in animals with inflammation is its ability to form biofilms to exposed collagen in inflamed tissues (Boleij and Tjalsma 2013). Another possibility is that *S. gallolyticus* is able to evade the immune response mounted by the host (e.g. HDPs) as has been observed for other taxa effective at overcoming colonization resistance in the intestine. Of the ten species of *Bacteroides* that we isolated, only *B. fragilis*, *B. uniformis*, and *B.*

heparinolyticus were more commonly isolated from piglets with salmonellosis. An association of enterotoxigenic *B. fragilis* with inflamed tissues in piglets and human beings has been described previously (Sears et al. 2008; Duimstra et al. 2016). This bacterium is known to colonize crypts (Lee et al. 2013). Moreover, the formation of capsules provides resistance to the complement system, and to phagocytic uptake and killing (Wexler 2007). The utilization of a comprehensive isolation strategy revealed a number of bacteria that are differentially associated with infected piglets, and demonstrated the value of using culture-dependent methods in concert with NGS analysis of communities to characterize bacteria potentially associated with salmonellosis. Ancillary examination of cell densities by taxon-specific quantitative PCR (qPCR) showed a tendency of *S. gallolyticus* and *B. uniformis* to increase in cecum and spiral colon of *S*. Typhimurium infected piglets. However, the low level of detection of these taxa associated with mucosa and the limitation of real-time qPCR to differentiate between nucleic acids from live and dead cells emphasizes the requirement of conducting *in vivo* analyses to confirm the propensity of these taxa to colonize inflamed tissue.

In conclusion, we evaluated the progression of salmonellosis in pigs by characterizing histopathologic changes in the GIT, host immune responses, and alterations to the enteric microbiota. To characterize the microbiota, we applied a combination of culture-dependent and culture-independent methods, and found that a reliance on NGS analysis was insufficient to detect taxon-specific changes associated with inflammation. Furthermore, we recovered a number of taxa that were differentially abundant in pigs with salmonellosis (e.g. *S. gallolyticus*), and the acquisition of these bacteria will facilitate functional assessments. A detailed evaluation of the host immune responses pointed to upregulation of HDPs as an important mechanism modulating disease. It is currently believed that these peptides execute their bactericidal function in a prescribed manner; however, the mechanisms by which HDPs trigger an immune response and also modify the microbiota are not well understood. The utilization of knock-out mice lacking HDP genes could be used to elucidate the role that these peptides play in salmonellosis. Moreover, delivery of HDPs to sites of enteric inflammation may represent a novel and non-antibiotic strategy to mitigate enteritis

4.5 Tables and figures

Table 4.1 Sequences and annealing temperatures (Ta) for primers used for gene expression.

Target	Primer	Sequence (5' to 3')	Ta (°C)	Source
Ppia	Ppia-F	GACAGCAGAAAACTTCCGTG	58	This study
	Ppia-R	ACCACCCTGGCACATAAATC		
HPRT	pHPRT-F	AGGCTATGCCCTTGACTACA	58	This study
	pHPRT-R	GGCTTTGTATTTTGCCTTTCCA		
Gusβ	pGusB-F	CATGAGGCCTACCAGAAACC	58	This study
	pGusB-R	GAGGTGGATCCTCGTGAAAC		
βD2	pBD2-F	AGCTGGCTGCAGGTATTAAC	58	This study
	pBD2-R	TCAATCCTGTTGAAGAGCGG		
Muc4	pMuc4-R	GTCCCCTGGGTGTTTCTGAG	58	This study
	pMuc4-R	CATAGTGTTTCCACCCAGGAC		
RegIIIγ	REG3g-F	AGCCTGTCAAGAAACACAGGATA	58	This study
	REG3g-R	TCCAATCTCATCTAGCCCTTG		
Muc1	pMuc1-F	ACCCCTATGAGCAGGTTTCT	58	This study
	pMuc1-R	CCCCTACAAGTTGGCAGAAG		
TNFα	pTnf-a-F	CCACGTTGTAGCCAATGTCA	58	This study
	pTnf-a-R	GTTGTCTTTCAGCTTCACGC		
IFNγ	pIFN-g-F	AGAATTGGAAAGAGGAGAGTGAC	58	This study
	pIFN-g-R	TCAGTTTCCCAGAGCTACCA		
IL17	IL17a-F	CCAGACGGCCCTCAGATTAC	65	(Meurens, Berri, *et al.* 2009)
	IL17a-R	CACTTGGCCTCCCAGATCAC		
IL10	pIL-10-F	CTGGAAGACGTAATGCCGAA	58	This study
	pIL-10-R	CAGAAATTGATGACAGCGCC		
PR39	PR39-F	TAATCTCTACCGCCTCCTGG	62	(Meurens, Berri, *et al.* 2009)
	PR39-R	CCCGTTCTCCTTGAAGTCAC		
IL1β	pIL1b-F	CCCATCATCCTTGAAACGTG	58	This study
	pIL1b-R	CTCATGCAGAACACCACTTC		
IL8	IL8-F	TCCTGCTTTCTGCAGCTCTC	62	(Meurens, Berri, *et al.* 2009)
	IL8-R	GGGTGGAAAGGTGTGGAATG		
INOS	iNOS-F	GAGAGGCAGAGGCTTGAGAC	62	This study
	iNOS-R	TGGAGGAGCTGATGGAGTAG		
TLR4	pTLR4-F	CAGCCATGGCCTTTCTCTC	58	This study
	pTLR4-R	ATGTTAGGAACCACCTGCAC		
TGFβ	pTGF-B1-F	CCGGAACCTGTATTGCTCTC	58	This study
	pTGF-B1-R	TGACATCAAAGGACAGCCAC		

Table 4.2 Dehority's agar.

Ingredient	Amount
Carbohydrate	5.0 g/L
Tripticase	4.5 g/L
Yeast extract	0.5 g/L
Mineral 1[a]	40 ml/L
Mineral 2[b]	40 ml/L
Hemin (0.01%)[c]	10 ml/L
VFA solution[d]	10 ml/L
Resazurin solution (25 mg/ml)	1 ml/L
L-Cysteine HCl solution	10 ml/L
Clarified rumen fluid[e]	50 ml/L
Agar	15 g/L

[a]Mineral 1 = K_2HPO_4.(6 g/L)
[b]Mineral 2 = KH_2PO_4 (6 g/L), $(NH_4)_2SO_4$ (6 g/L), NaCl (12 g/L), $MgSO_4$ (2.45 g/L), $CaCl_2 \cdot 2H_2O$ (1.69 g/L).
[c]Hemin was dissolved in 1 M NaOH and then diluted to appropriate concentration.
[d] VFA solultion = acetic acid (6.85 ml/L), propionic acid (3 ml/L), butyric acid (1.85 ml/L), isobutyric acid (0.5 ml/L), 2-methyl butyric acid (0.55 ml/L), N-valeric acid (0.55 ml/L), and isovaleric acid (0.55 ml/L).
[e]Clarified rumen fluid was prepared by centrifuging to rumen fluid to remove debris and gently decanting in a fresh vial for storage at -20°C.

Table 4.3 Columbia blood agar.

Ingredient	Amount
Columbia broth	35 g/L
Cysteine	1 g/L
Agar	15 g/L
Sheep blood[a]	100 ml/L

[a]Add blood to cooled sterile medium (≈50°C). The anticoagulant CPDA-1 was added to blood bags in advance of blood collection from sheep to prevent clotting. CPDA-1 is composed of 26.30 g/L trisodium citrate, 3.27 g/L citric acid, 3.22 g/L sodium dihydrogen phosphate, 3.18 g/L dextrose, and 0.275 g/L adenine dissolved in water at a pH of 5.6 to 5.8.

Table 4.4 Sequences and annealing temperatures (Ta) for primers used to quantify bacteria.

Target organism	Primer	Sequence (5' to 3')	Annealing Tmp (°C)	Reference
Prevotella spp.	PrevF	CACCAAGGCGACGATCA	58	(Larsen *et al.* 2010)
	PrevR	GGATAACGCCYGGACCT		
Ruminococcus	Rflbr730F	GGCGGCYTRCTGGGCTTT	58	(Ramirez-Farias *et al.* 2009)
	Clep866mR	ACCTTCCTCCGTTTTGTCAAC		
Clostridium cluster I	CI-F1	TACCHRAGGAGGAAGCCAC	55	(Song *et al.* 2004)
	CI-R2	GTTCTTCCTAATCTCTACGCAT		
Intestinimonas AF211	PFF590F	AAAACTATGGGCTCAACCCA	58	(Bui *et al.* 2015)
	PFF702R	GTCAGTTAATGTCCAGCAGG		
Total bacteria	F-tot	GCAGGCCTAACACATGCAAGTC	56	(Castillo *et al.* 2006)
	R-tot	TGCTGCCTCCCGTAGGAGT		
Bacteroides uniformis	BaUNI-F	TACCCGATGGCATAGTTCTT	55	(Rashidan *et al.* 2018)
	BaUNI-R	GGACCGTGTCTCAGTTCCAA		
Streptococcus gallolyticus	Sg-F	TGACGTACGATTGATATCATCAAC	60	(Kumar *et al.* 2017)
	Sg-R	CGCTTAACACATTTTTAGCTAATACG		

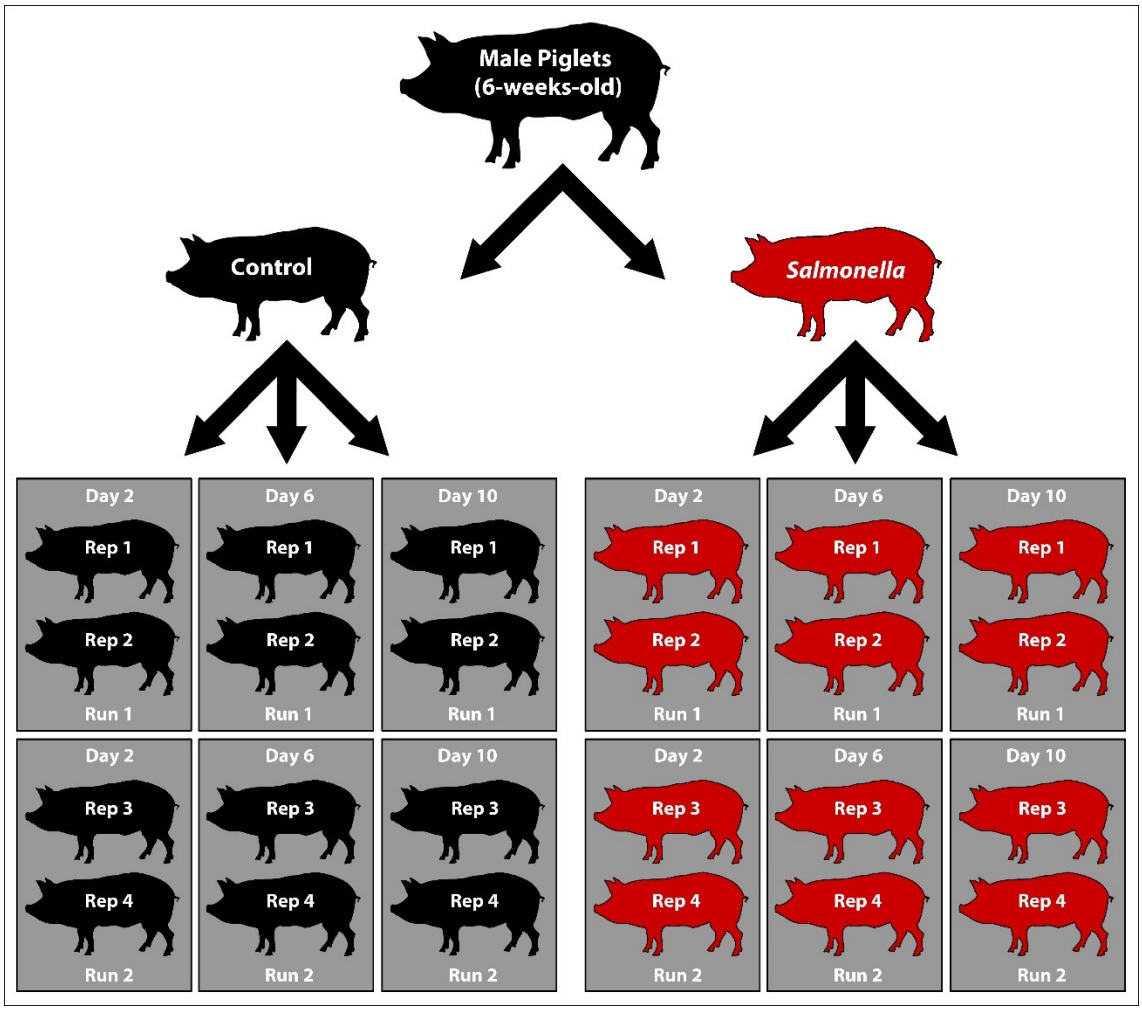

Figure 4.1 Schematic representation of the experimental design. The experiment was arranged as a two (Salmonella ±) by three (2, 6, and 10 days post-inoculation) factorial with four replicate piglets per treatment. The experiment was run on two separate occasions (i.e. run 1 and run 2).

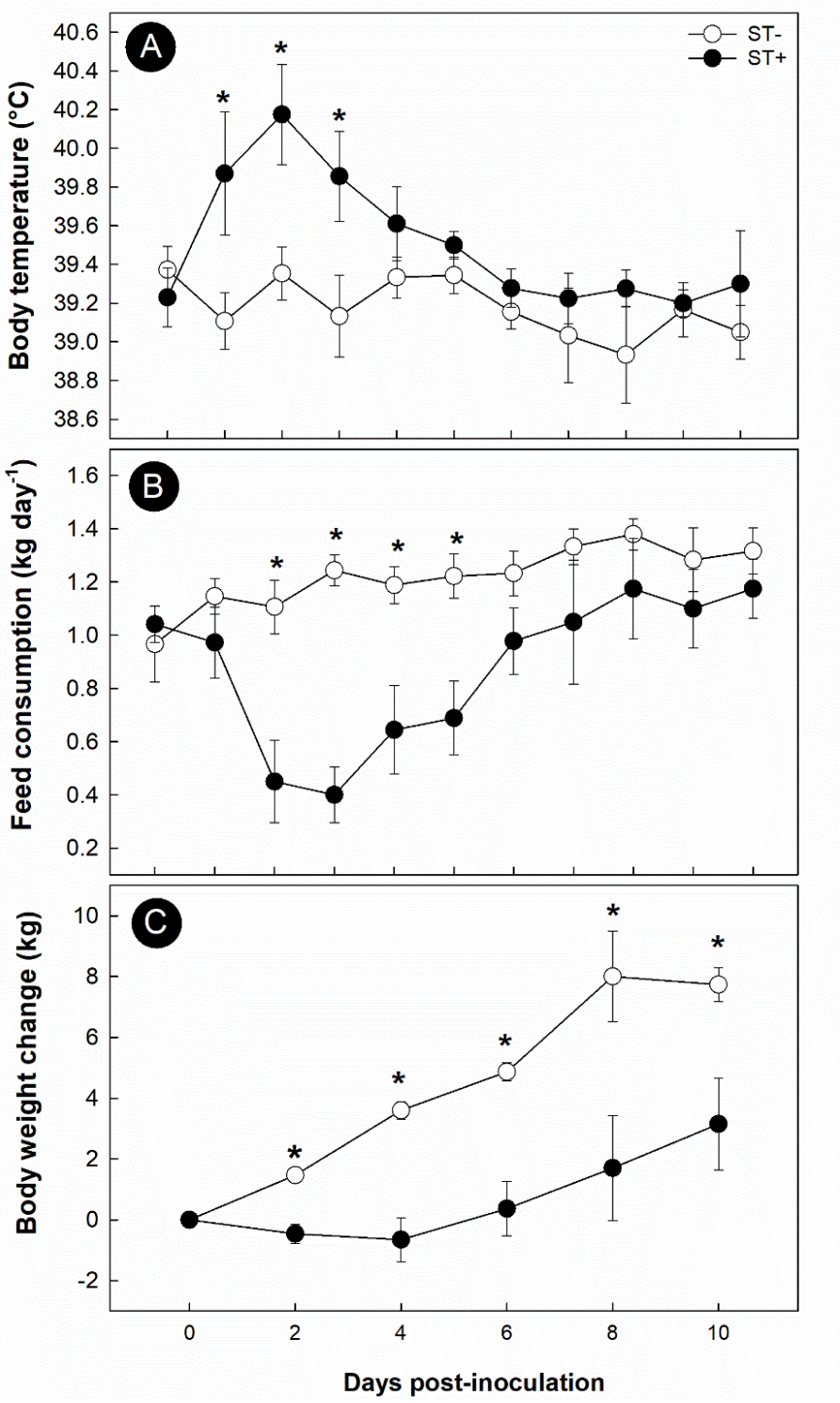

Figure 4.2 Temporal changes in host parameters of piglets orally inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). (A) Body weight; (B) rectal temperature; (C) daily feed consumption. Vertical lines associated with markers are standard errors of the mean. Markers with an asterisk indicate a difference ($P < 0.050$) between treatments at the corresponding time point.

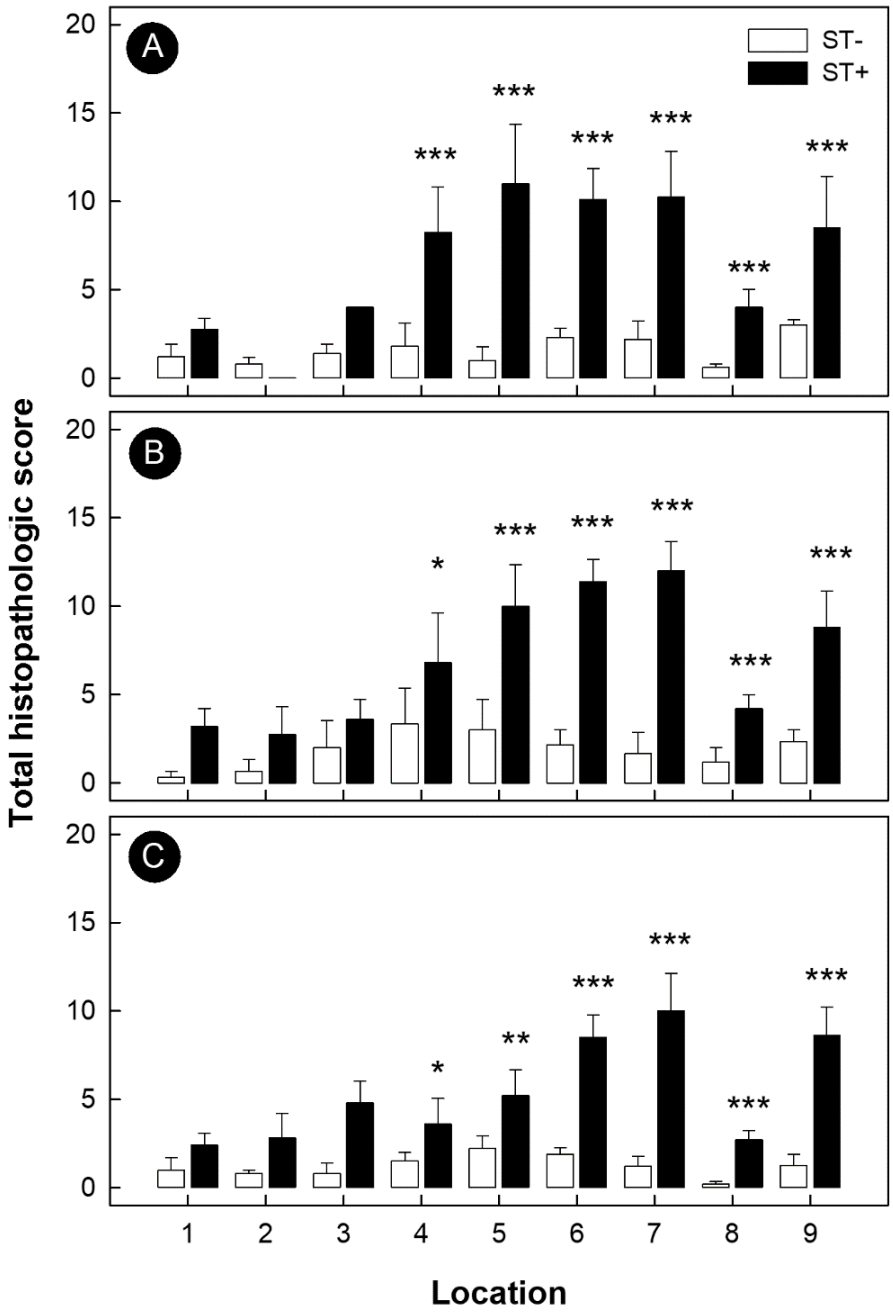

Figure 4.3 Total histopathologic scores in piglets orally inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Intestinal locations are: (1) duodenum; (2) proximal jejunum; (3) mid-jejunum; (4) distal jejunum; (5) ileum; (6) cecum ; (7) ascending colon; (8) spiral colon; and (9) descending colon. (A) Two days post-inoculation (dpi); (B) 6 dpi; (C) 10 dpi. Vertical lines associated with markers are standard errors of the mean. Histogram bars with asterisks indicate differences (*P < 0.050, **P < 0.010, ***P < 0.001) between treatments at the corresponding intestinal location.

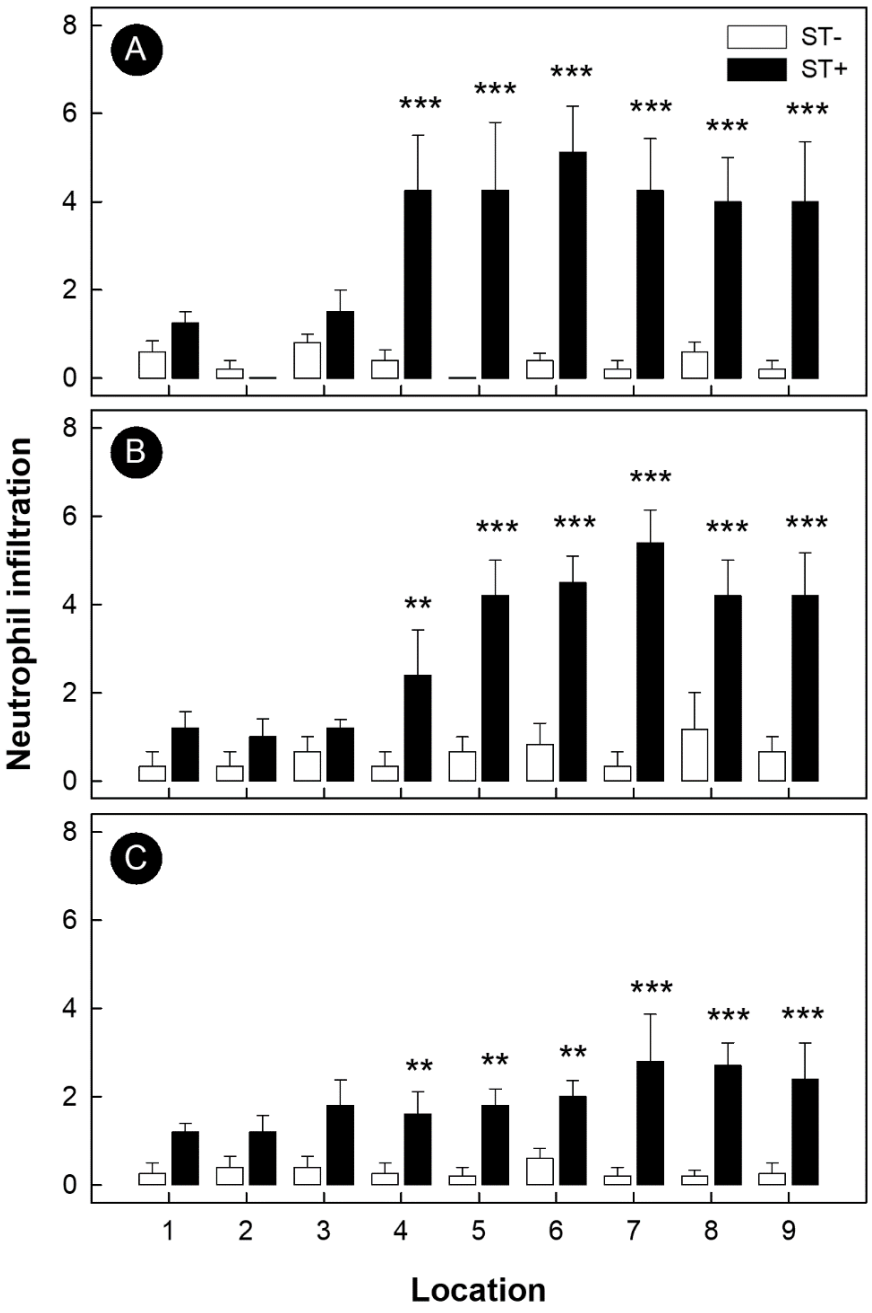

Figure 4.4 Neutrophil infiltration in piglets orally inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Intestinal locations are: (1) duodenum; (2) proximal jejunum; (3) mid- jejunum; (4) distal jejunum; (5) ileum; (6) cecum ; (7) ascending colon; (8) spiral colon; (9) descending colon. (A) Day 2 post-inoculation (p.i.); (B) Day 6 p.i.; (C) Day 10 p.i.. Vertical lines associated with markers are standard errors of the mean. Histogram bars indicated with an asterisk indicate significant difference (*P < 0.050, **P < 0.010, ***P < 0.001) between the two treatments at the corresponding intestinal location.

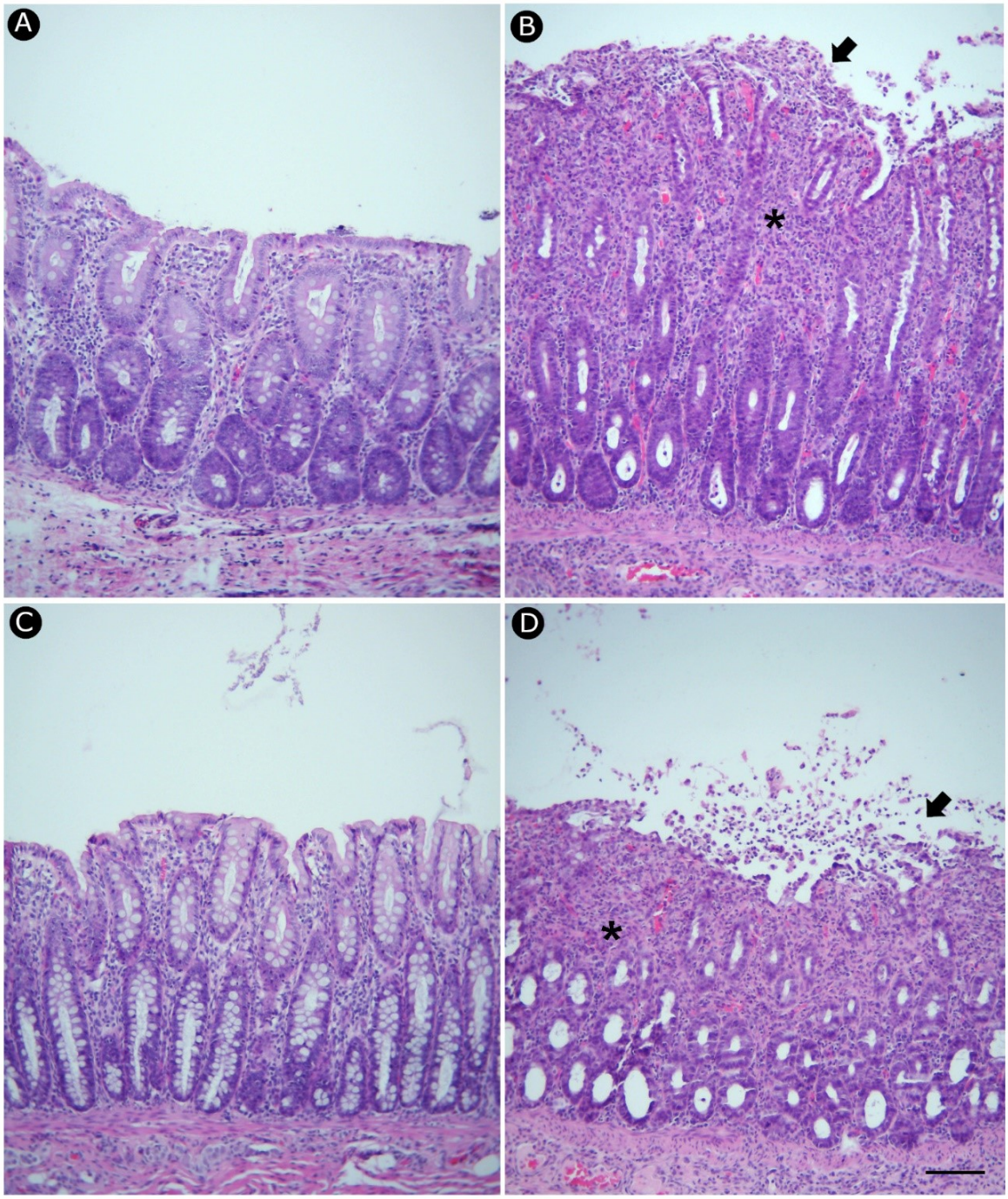

Figure 4.5 Histological representation of intestinal tissue harvested on day 2 post-inoculation from piglets orally inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Arrows indicate epithelial injury. Asterisks indicate leukocytes infiltration. (A) Cecum ST-; (B) Cecum ST+; (C) Spiral Colon ST-; (D) Spiral Colon ST+. Bar = 100 µm.

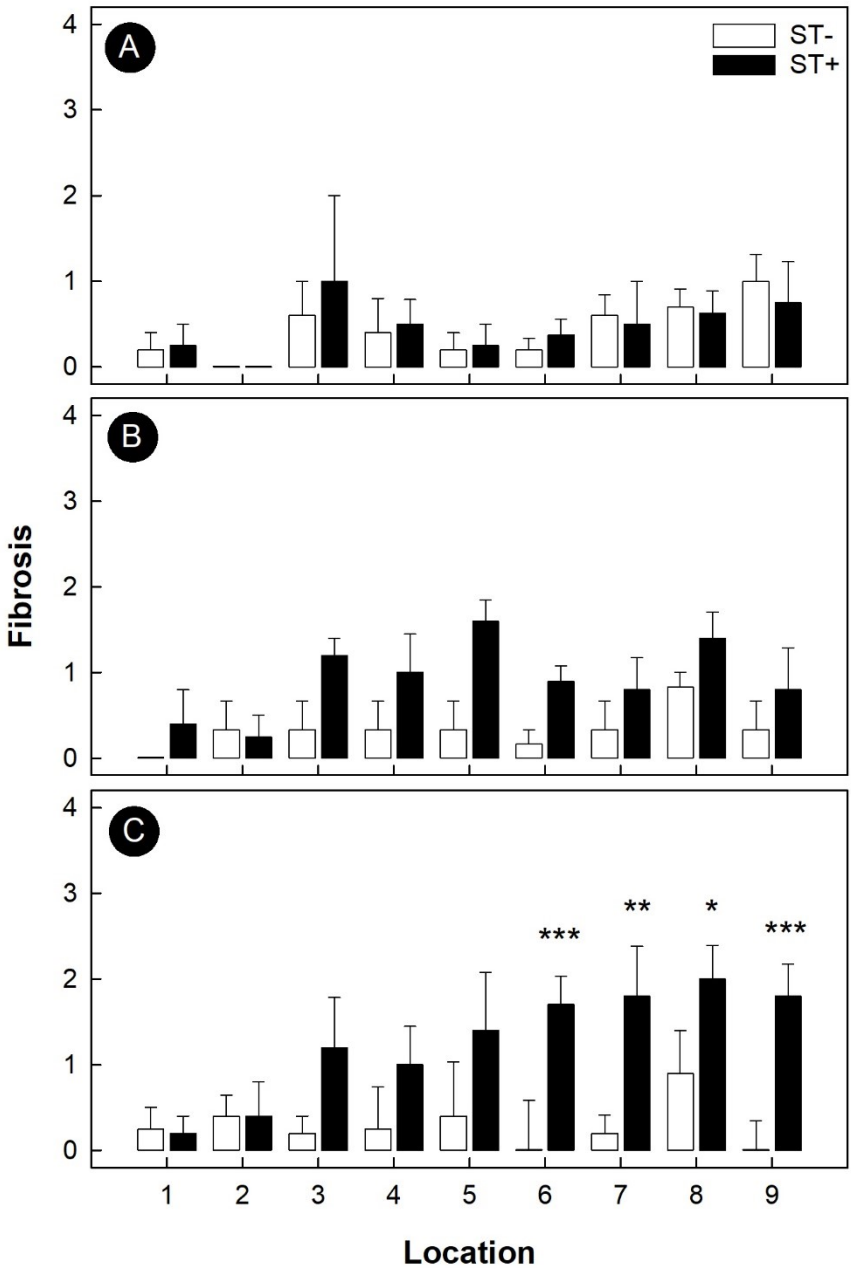

Figure 4.6 Fibrosis in piglets orally inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Intestinal locations are: (1) duodenum; (2) proximal jejunum; (3) mid-jejunum; (4) distal jejunum; (5) ileum; (6) cecum; (7) ascending colon; (8) spiral colon; (9) descending colon. (A) Day 2 post-inoculation (p.i.); (B) Day 6 p.i.; (C) Day 10 p.i.. Vertical lines associated with markers are standard errors of the mean. Histogram bars indicated with an asterisk indicate significant difference (*P < 0.050, **P < 0.010, ***P < 0.001) between the two treatments at the corresponding intestinal location.

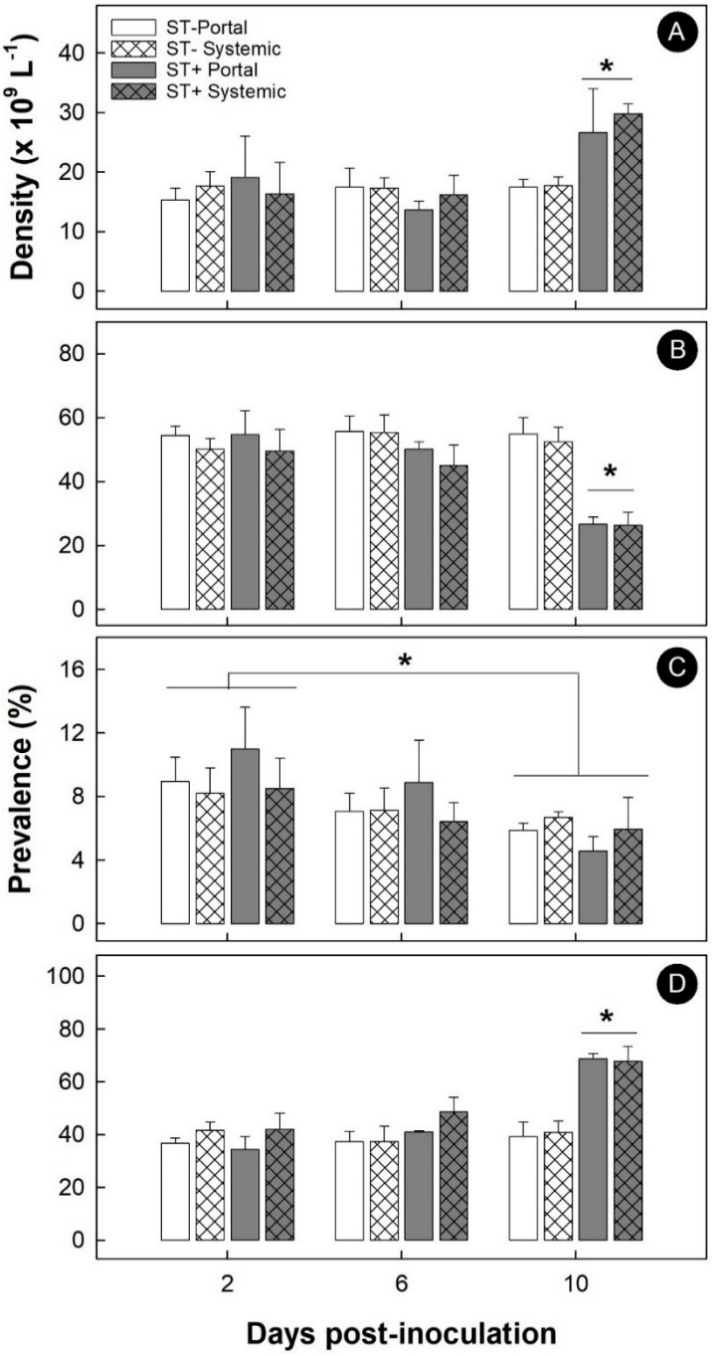

Figure 4.7 Densities of total white blood cells (x 109 L-1), and percentage of lymphocytes, monocytes, and granulocytes in portal vein and cardiac (i.e. systemic) blood of piglets at 2, 6, and 10 days post-inoculation with *Salmonella enterica* Typhimurium (ST+) or medium alone (i.e. ST-). (A) White blood cells; (B) lymphocytes; (C) monocytes; (D) granulocytes. Vertical lines associated with histogram bars represent standard error of the means. Asterisks indicate a significant effect (*P < 0.050).

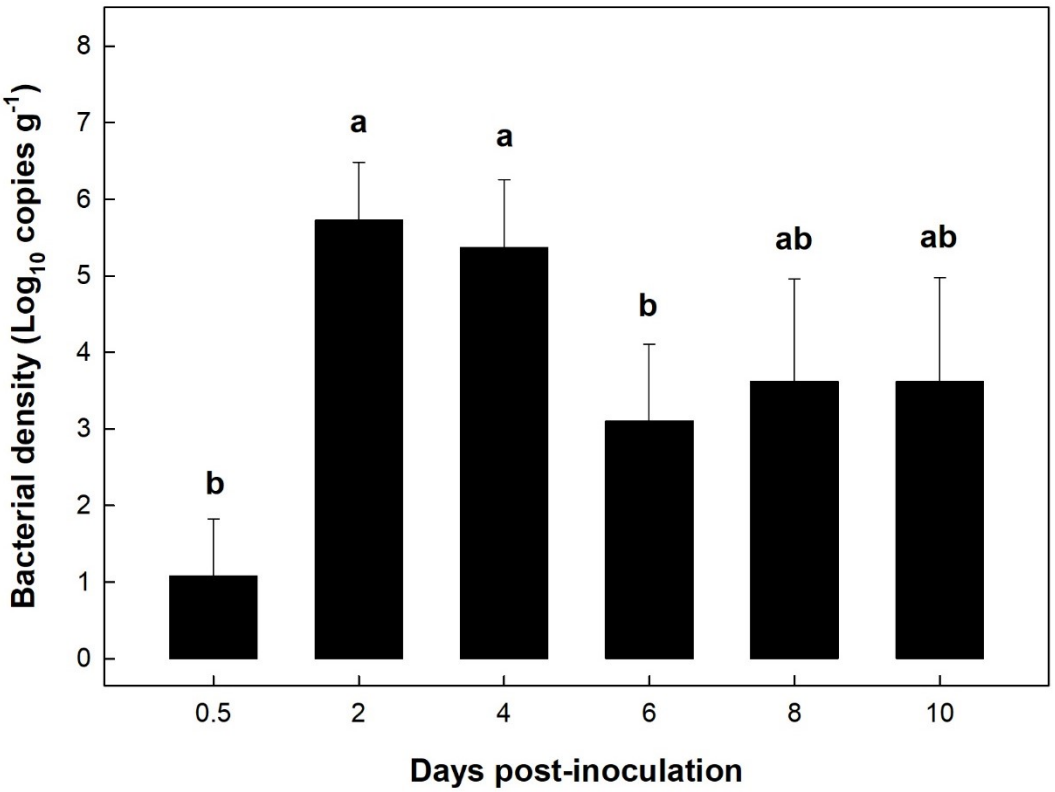

Figure 4.8 Temporal shedding of *Salmonella enterica* Typhimurium in feces from piglets orally inoculated with the pathogen. Vertical lines associated with histogram bars represent standard errors of the mean. Histogram bars indicated by different letters differ ($P < 0.050$). No *Salmonella* was detected in feces from piglets orally administered medium alone (i.e. control).

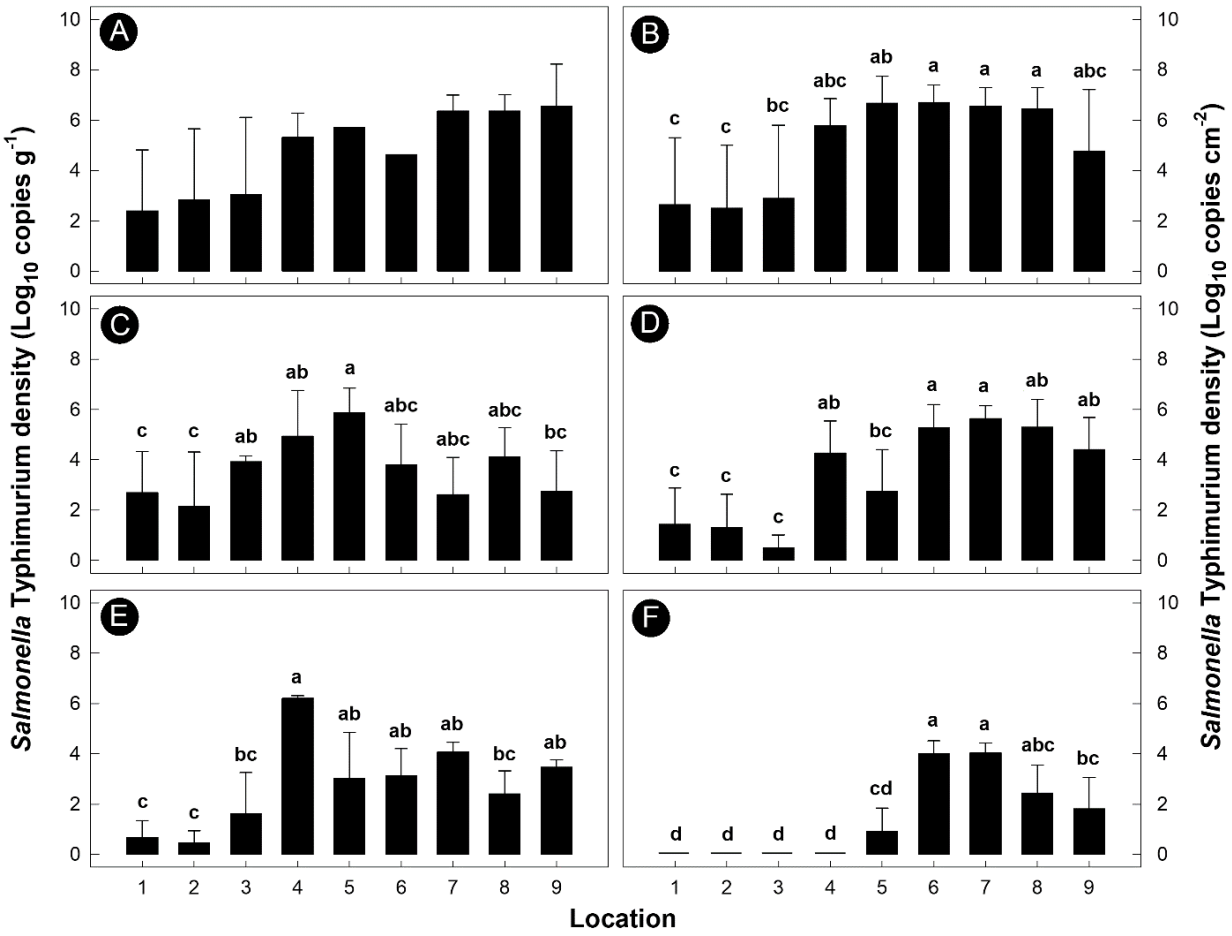

Figure 4.9 *Salmonella* densities from piglets at 2, 6, and 10 days post-inoculation (dpi) with *Salmonella enterica* Typhimurium. Locations are: (1) duodenum; (2) proximal jejunum; (3) mid-jejunum; (4) distal jejunum; (5) ileum; (6) cecum; (7) ascending colon; (8) spiral colon; and (9) descending colon. Vertical lines associated with histogram bars represent standard error of the means. Histogram bars indicated by different letters at each time post-inoculation differ ($P < 0.050$). (A) Digesta at 2 dpi; (B) mucosa-associated at 2 dpi; (C) digesta at 6 dpi; (D) mucosa-associated at 6 dpi; (E) digesta at 10 dpi; (F) mucosa-associated at 10 dpi. No *Salmonella* was detected in digesta or associated with mucosa from piglets orally administered medium alone.

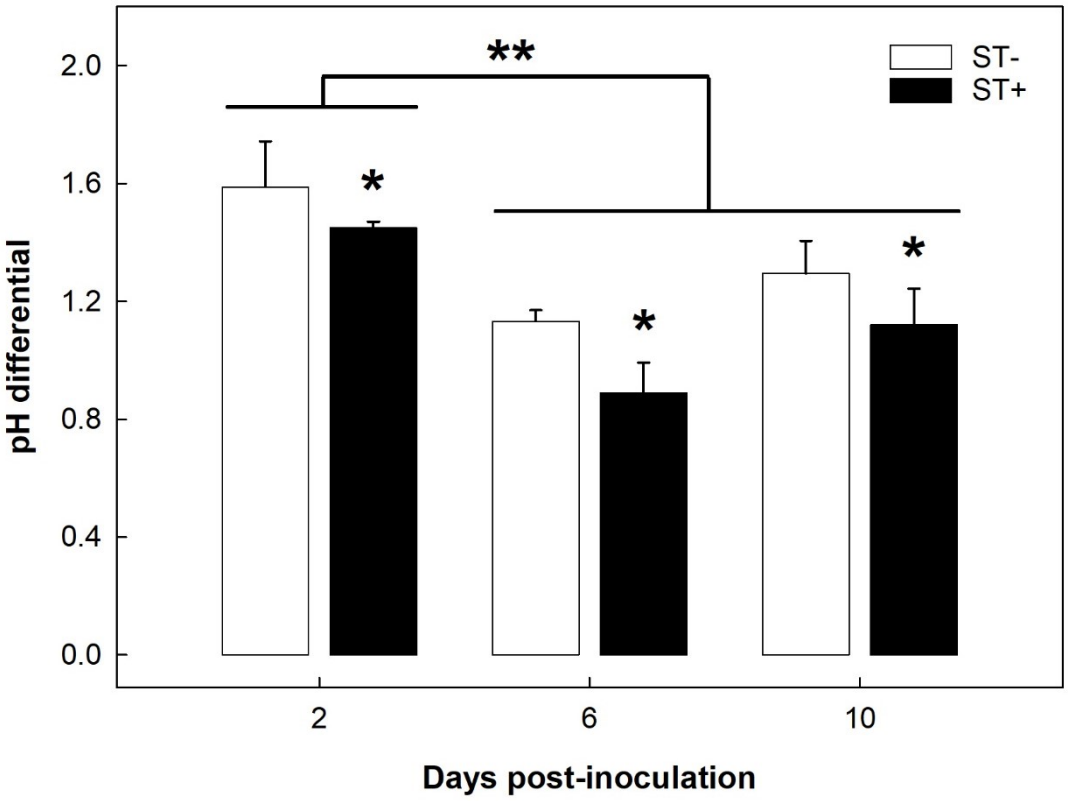

Figure 4.10 pH differential between initial measurement (45 minutes) and final measurement (24 hours post-mortem) in animals inoculated with *Salmonella* (ST+) or with medium alone (ST-). Vertical lines associated with histogram bars represent standard error of the mean. Asterisks indicate a significant effect (*P < 0.050, **P < 0.010).

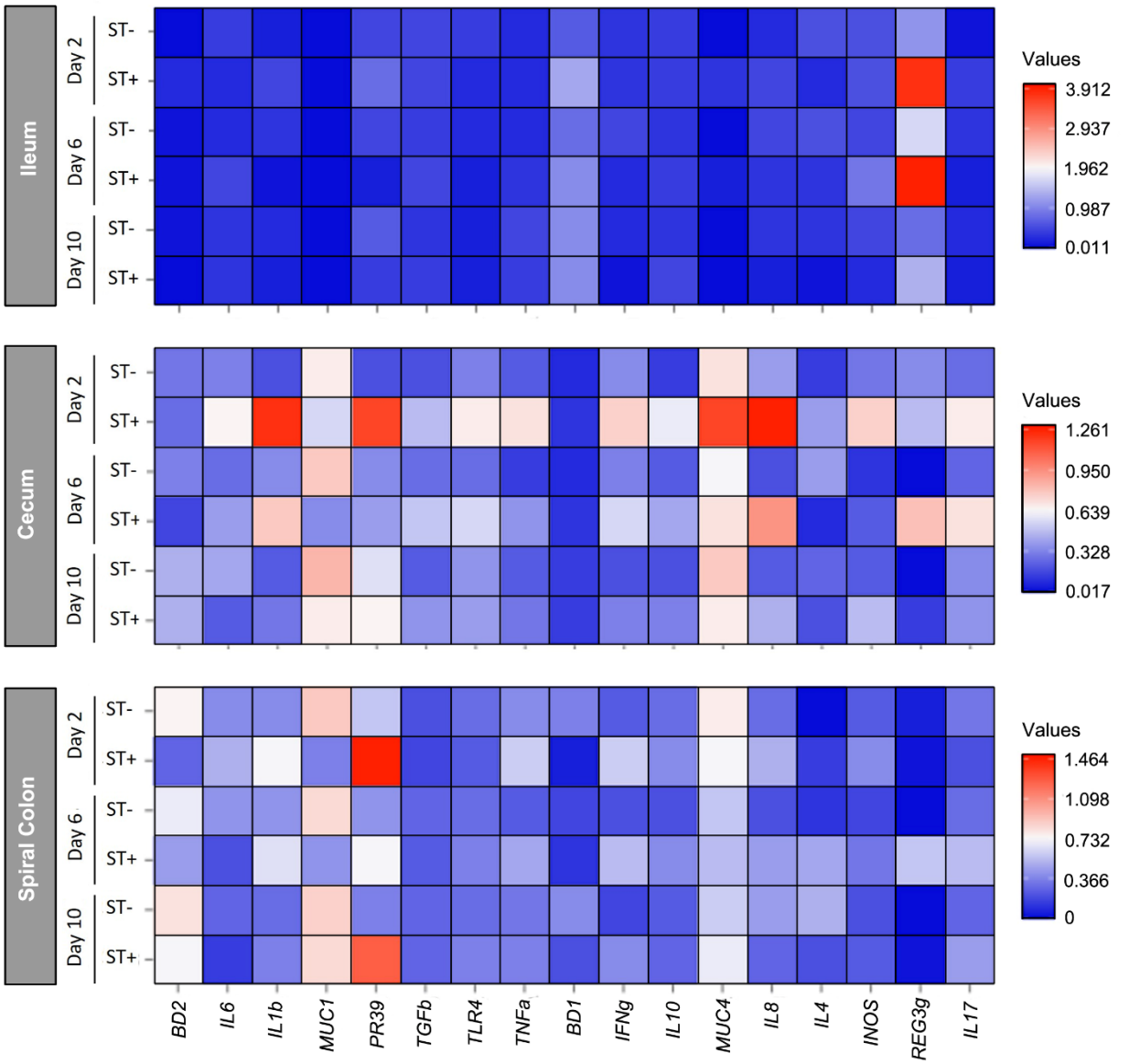

Figure 4.11 Relative gene expression of ileal, cecal, and spiral colonic tissue from animals inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Tissues were sampled at 2, 6, and 10 days post-inoculation. Low to high expression are represented by a change of colours from blue to red, respectively.

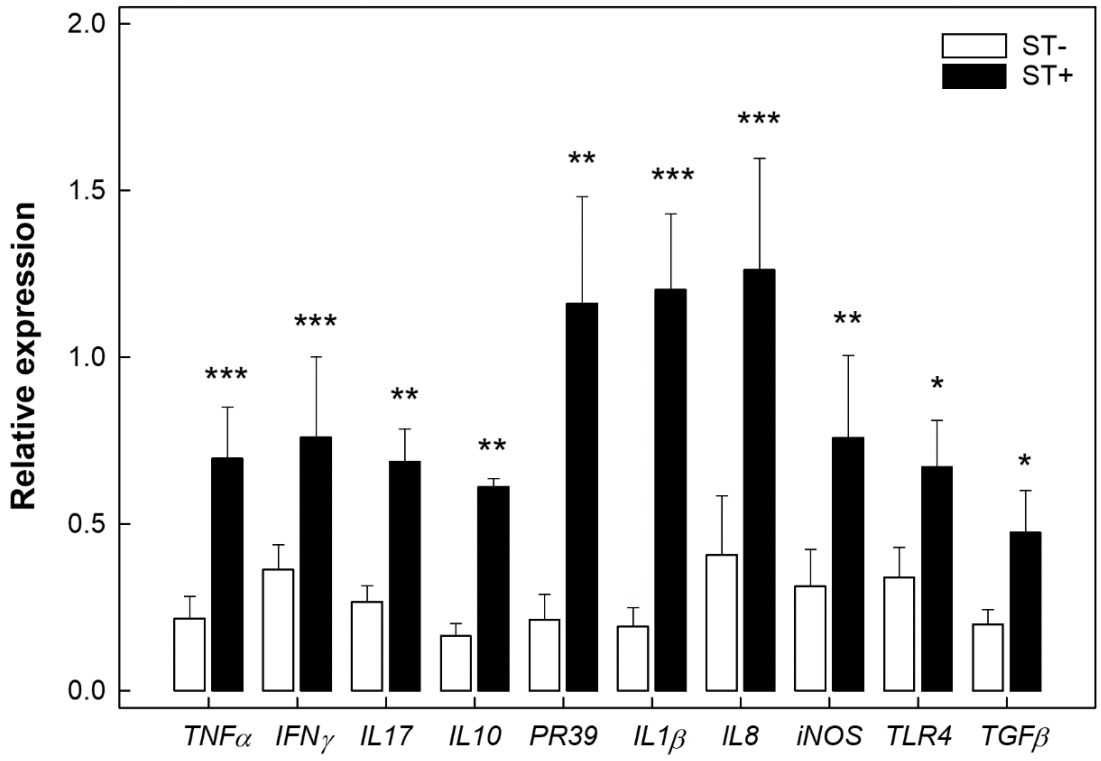

Figure 4.12 Relative gene expression in cecal tissue of piglets orally inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-) at 2 days post-inoculation. Vertical lines associated with markers are standard errors of the mean. Histogram bars with asterisks indicate differences (*P < 0.050, **P < 0.010, ***P < 0.001) between the two treatments.

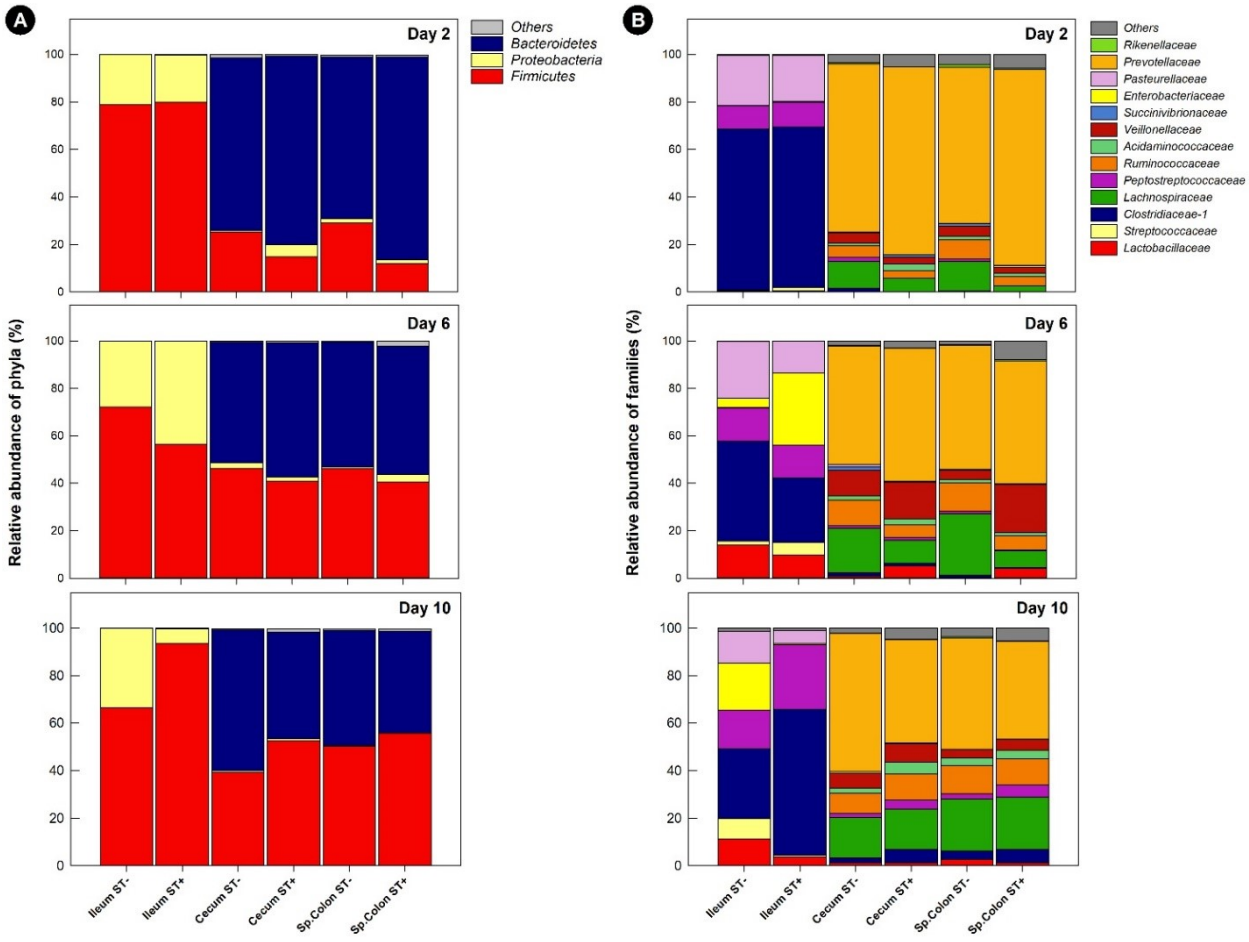

Figure 4.13 Spatial characterization of the main taxa in digesta from the ileum, cecum, and spiral colon of piglets inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Samples were obtained from piglets 2, 6, and 10 days post-inoculation. Relative abundances (%) are represented at different taxonomic levels. (A) Phyla; (B) families.

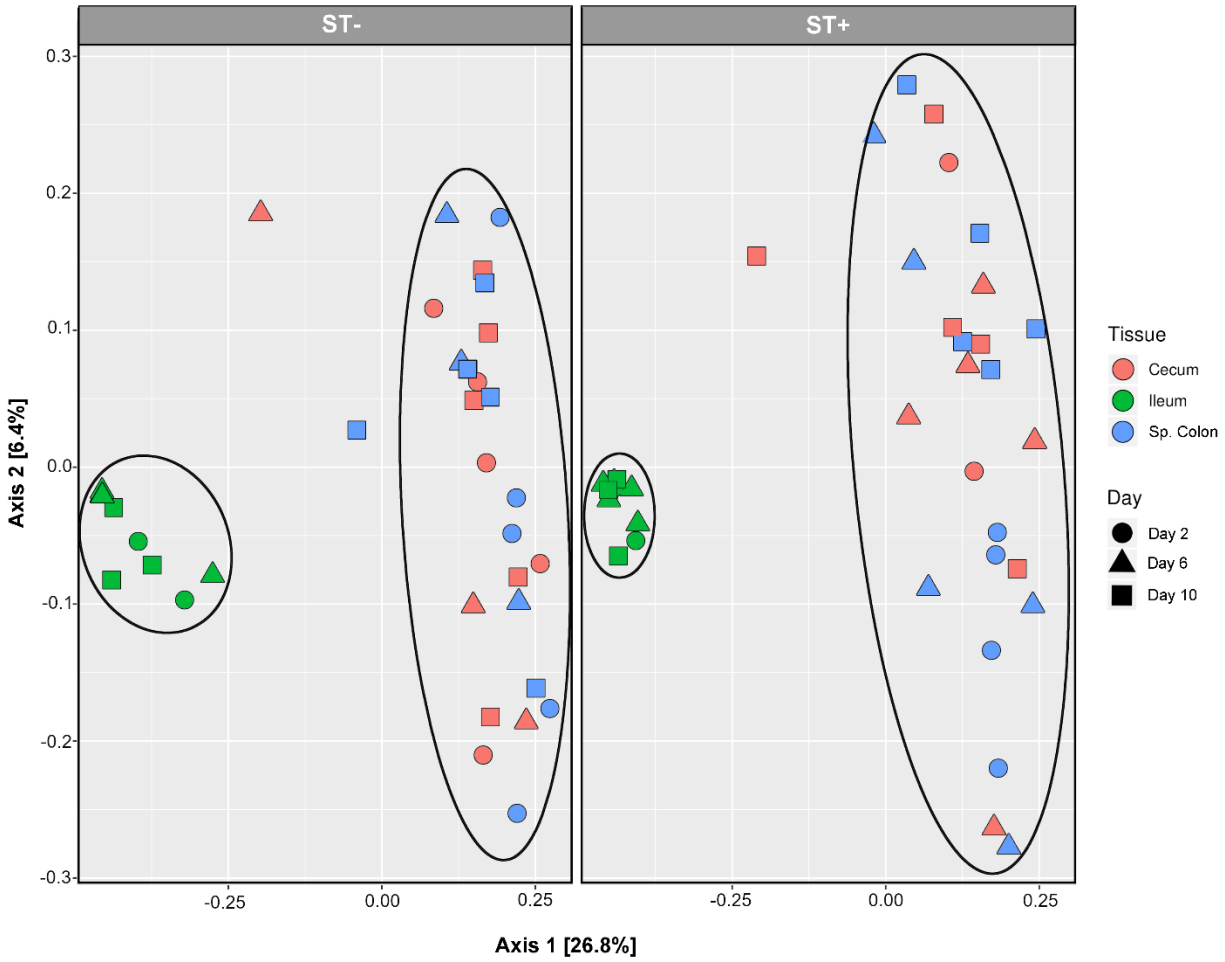

Figure 4.14 PCoA plot based on unweighted UniFrac distances of bacterial communities in digesta from the ileum, cecum and spiral colon of animals inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Samples were obtained from piglets at three time points post-inoculation.

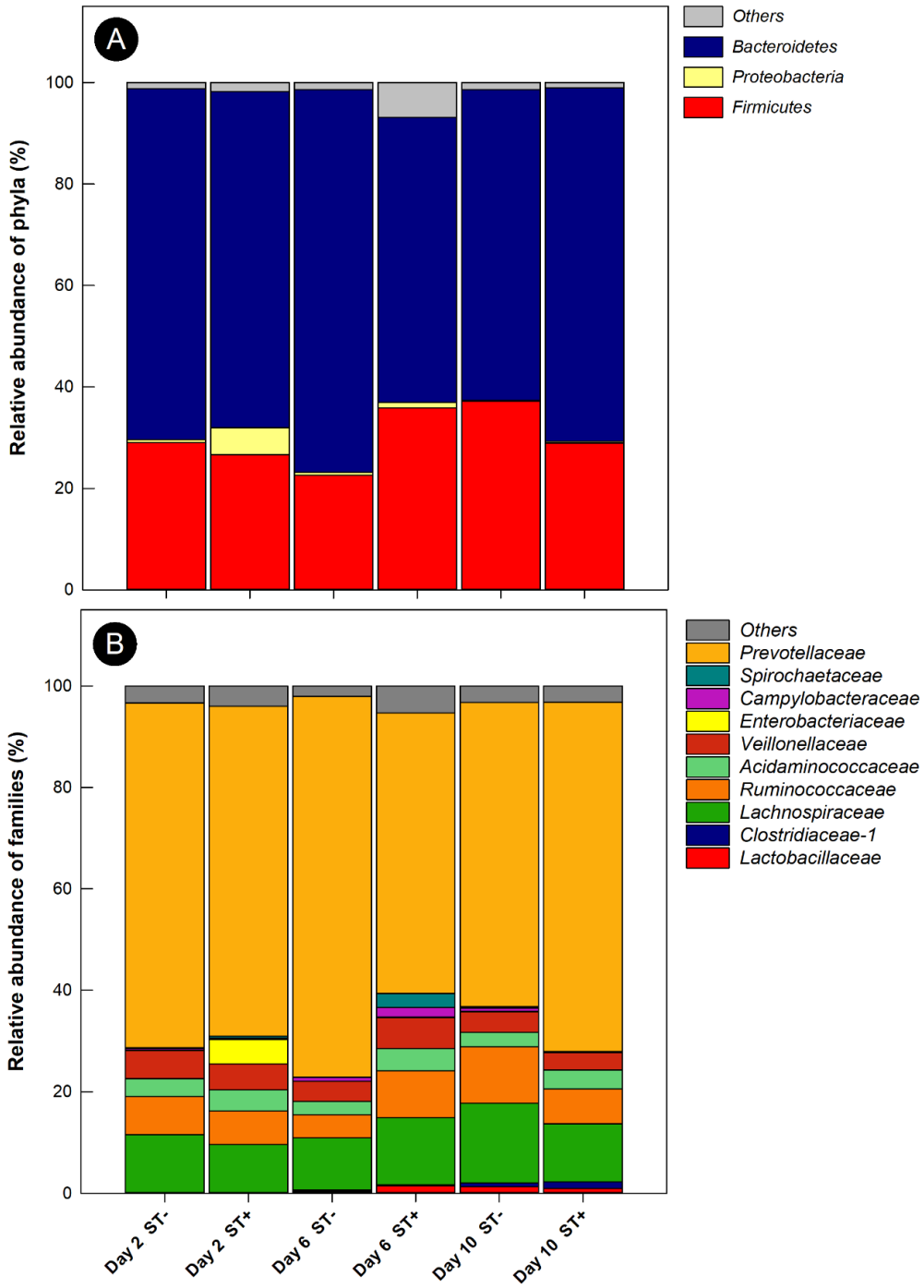

Figure 4.15 Relative abundance (%) of bacterial associated with spiral colonic mucosa of animals inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Samples were obtained at 2, 6 and 10 days post-inoculation. Communities were characterized by Illumina sequencing. (A) Phyla; (B) families.

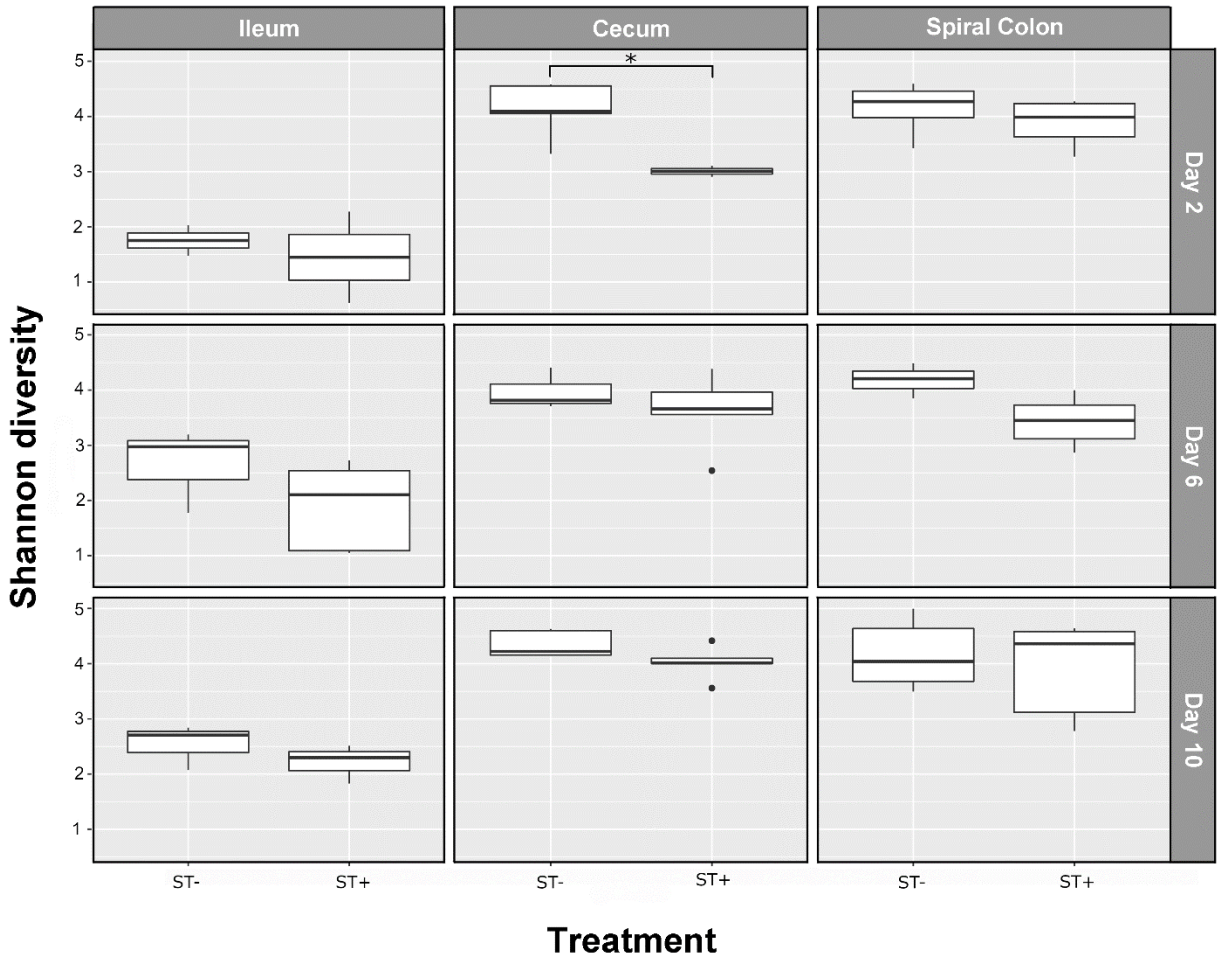

Figure 4.16 Alpha-diversity of microbiota in digesta from the ileum, cecum and spiral colon of animals inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). Samples were obtained from piglets at three time points post-inoculation. Values are expressed as means ± standard error. Boxes with an asterisk above indicate significant difference (*P < 0.050) between treatments.

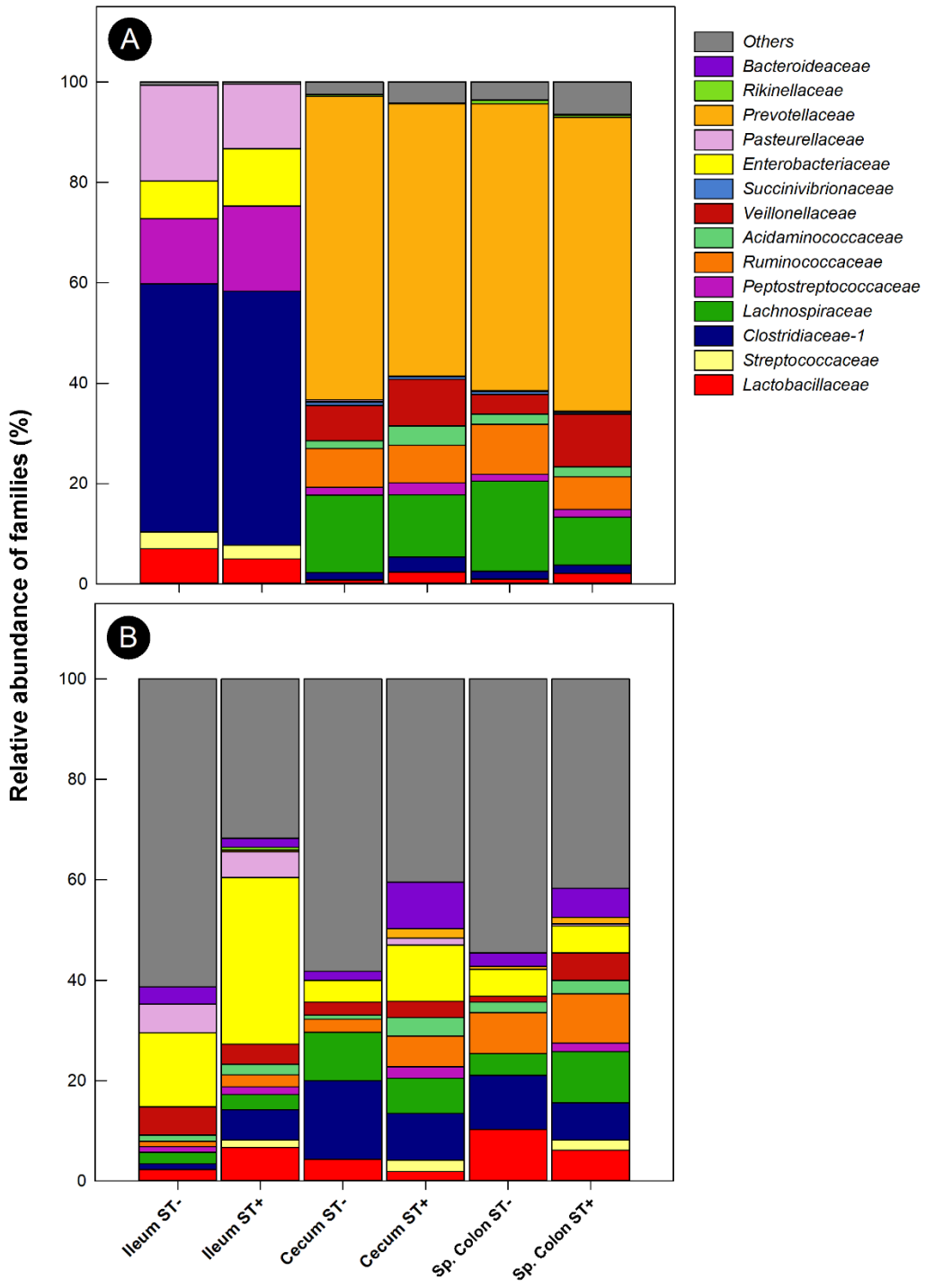

Figure 4.17 Spatial characterization of the main families of bacteria in digesta from the ileum, cecum, and spiral colon of piglets inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). (A) Next-generation sequence analysis; (B) culturomics. Data were combined across sample times (i.e. 2, 6, and 10 days post-inoculation).

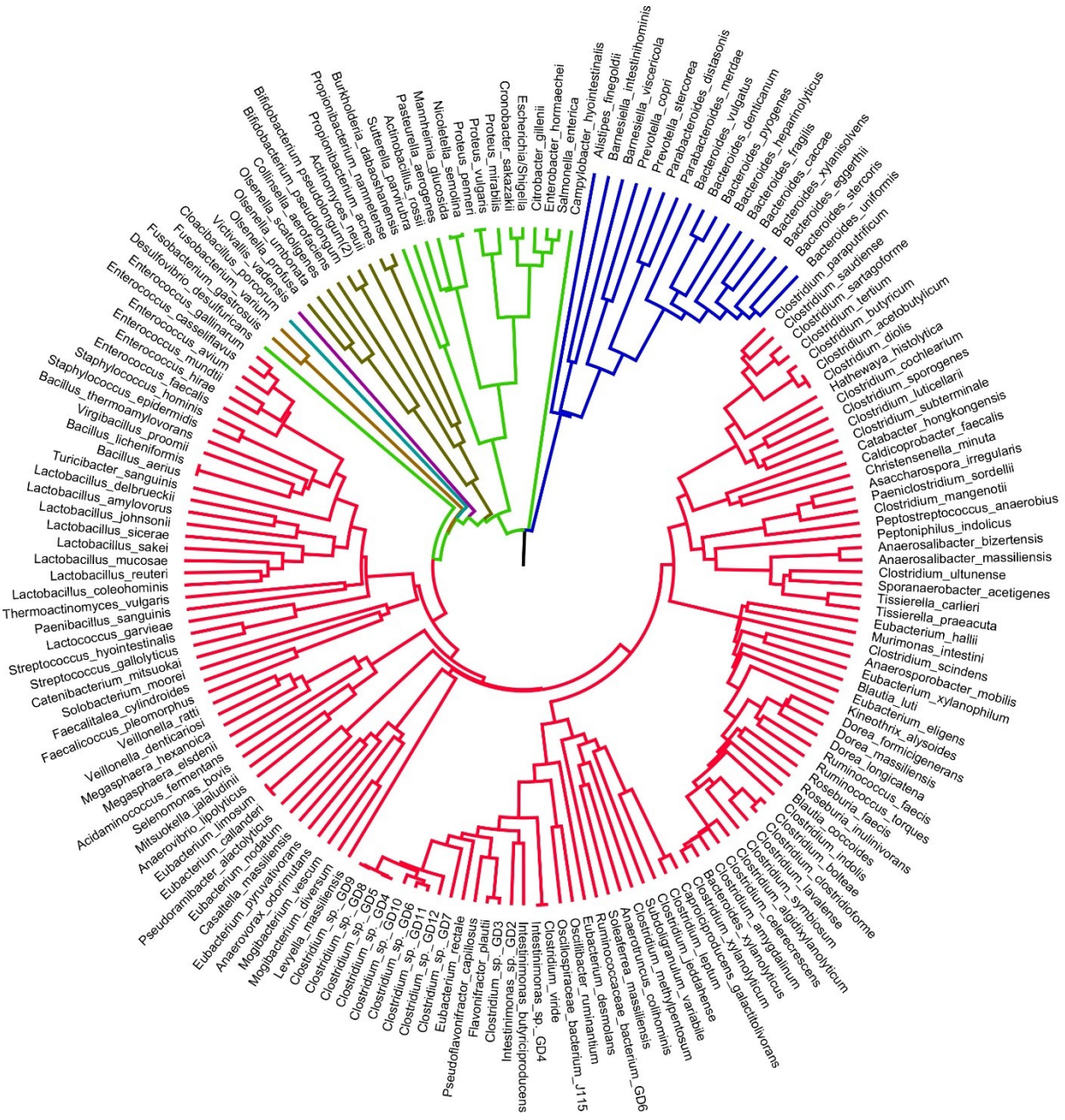

Figure 4.18 Phylogenetic Tree of the 16S rDNA sequence of bacteria isolated from the intestines of piglets. The species-level dendrogram was generated from the 16S rRNA gene with identities determined by matching with the database of the Ribosomal Database Project (RDP). Branch colors are: blue, Bacteroidetes; red, Firmicutes; green, Proteobacteria; light brown, Fusobacteria; light blue, Synergistetes; purple, Lentispiracea; dark brown, Actinobacteria.

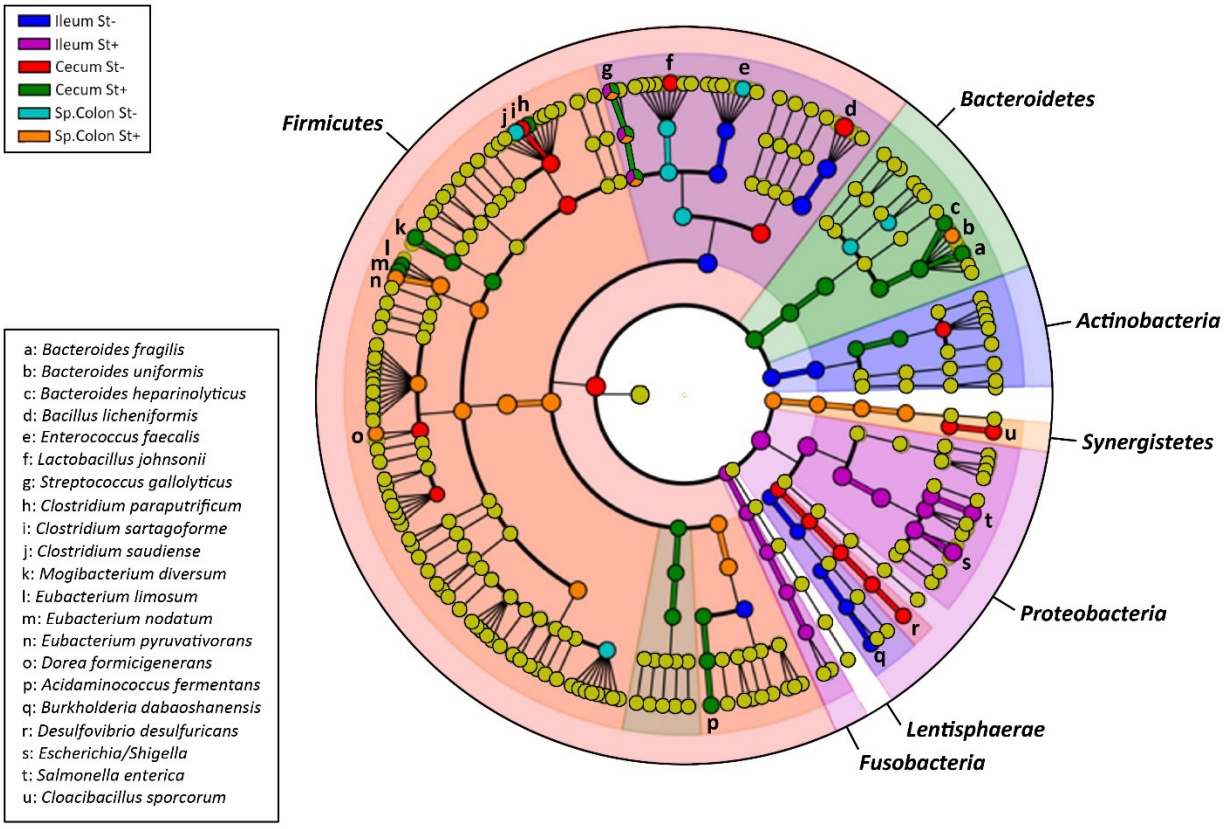

Figure 4.19 Cladogram illustrating abundance of bacterial species isolated from the ileum, cecum, and spiral colon of piglets inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). The cladogram background is color coded to illustrate relative changes in the abundance of isolated bacteria between treatments and intestinal locations. Moreover, 21 bacterial species that were differentially abundant in ST+ and ST- piglets are indicated with colored circles, and are labelled ‘a’ through ‘u’. Gold circles represent bacteria in which no difference in abundance due to infection by S. Typhimurium was observed.

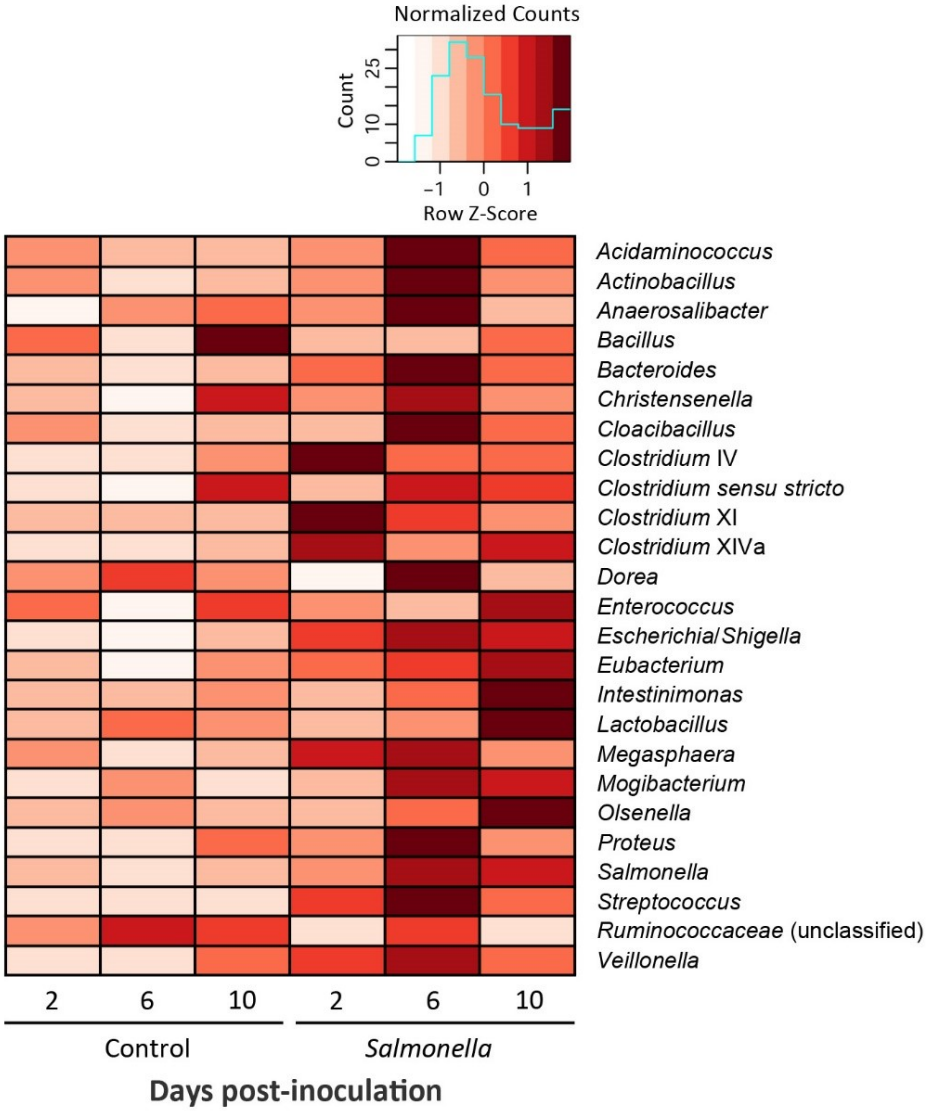

Figure 4.20 Abundance of bacteria isolated from the ileum, cecum, ascending colon, and spiral colon of piglets infected with *Salmonella enterica* Typhimurium (Salmonella) or buffer alone (control) at 2, 6, and 10 days post-inoculation. Cells are colored according to the number of bacteria recovered, and the distribution and color scheme of these counts are indicated in the "normalized counts" plot above the heatmap. The heatmap was generated using the *heatmap.2* function contained in the *gplots* package of R (Oksanen et al. 2019).

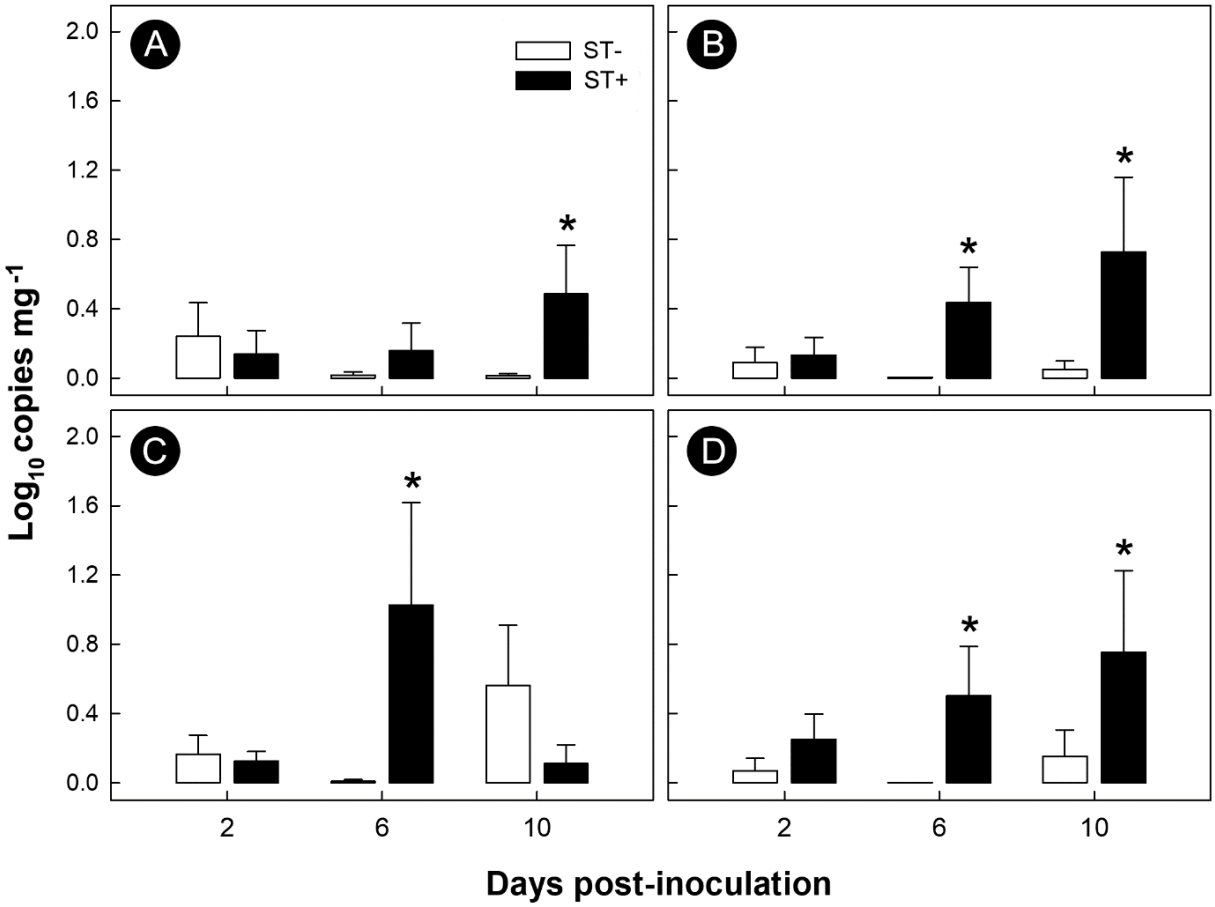

Figure 4.21 Densities of *Bacteroides uniformis* and *Streptococcus gallolyticus* in digesta of piglets inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-) as determined by quantitative PCR. (A) *B. uniformis* in the cecum; (B) *S. gallolyticus* in the cecum; (C) *B. uniformis* in the spiral colon; (D) *S. gallolyticus* in the spiral colon. Vertical lines associated with histogram bars represent standard error of the means. Histogram bars with asterisks differ (*P ≤ 0.124) between the ST+ and ST- treatments.

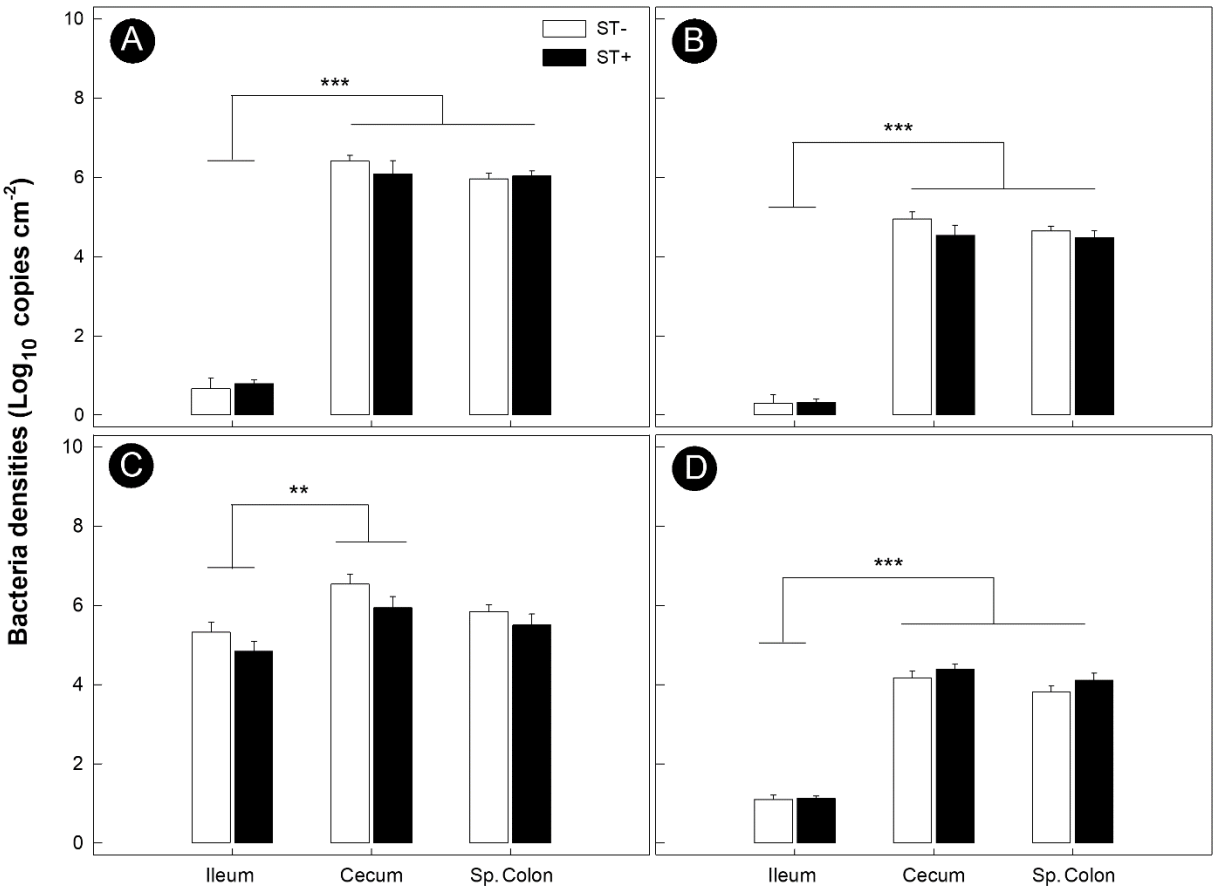

Figure 4.22 Bacteria densities within the digesta from the ileum, cecum, and spiral colon of piglets inoculated with *Salmonella enterica* Typhimurium (ST+) or medium alone (ST-). (A) *Prevotella*; (B) *Ruminoccus*; (C) *Clostridium* cluster I; (D) *Intestinimonas*. Vertical lines associated with histogram bars represent standard error of the means. Histogram bars indicated by asterisks differ (*P < 0.050; **P < 0.010; ***P < 0.001) between tissues.

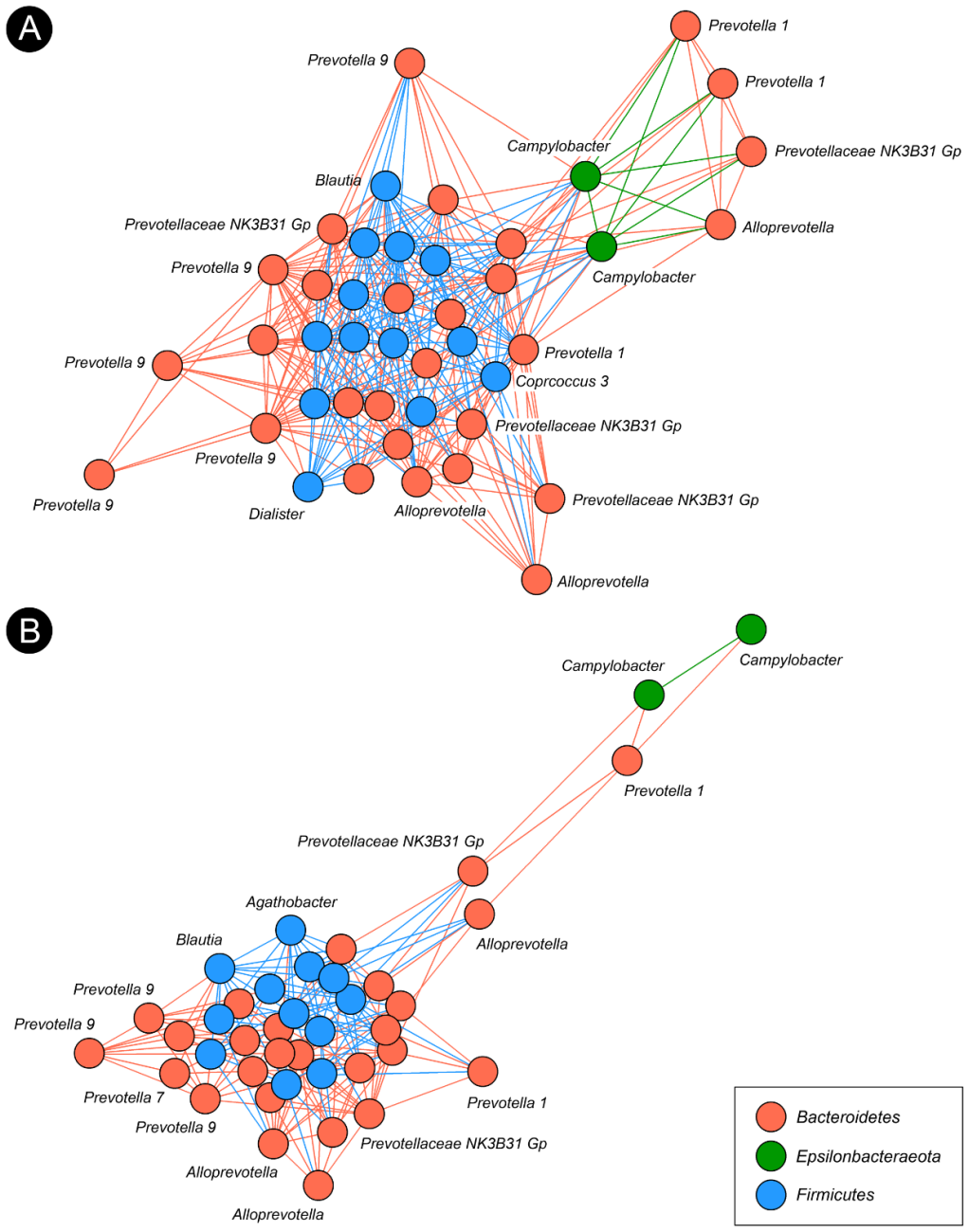

Figure 4.23 Microbial interaction networks determined by Bray-Curtis distances of Amplicon Sequence Variants (ASVs) from mucosal-associated bacterial of the spiral colon. (A) Animals inoculated with medium alone (ST-); (B) animals inoculated with *Salmonella enterica* Typhimurium (ST+).

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Chapter 5 General Discussion

Enteric diseases in livestock can have a significant impact on animal health, welfare and performance, and enteritis can reduce the net profitability in the swine industry by millions of dollars annually (Lochmiller and Deerenberg 2000; Alban and Stärk 2005). Moreover, it has been shown that many of these intestinal diseases are caused by the colonization of pathogens or a disruption in microbiota, as previously observed in in people, pigs, and chickens (Nurmi and Rantala 1973; van der Wolf et al. 1999; Gordon 2008). Interestingly, some organisms contained in the microbiota of infected animals are difficult to grow *in vitro*, and as such, it behooves researchers to develop better identification and isolation techniques of these enteric bacteria (Wade 2002; Vartoukian et al. 2010). In addition, the identification, classification, and functional evaluation of bacteria are also essential elements of the past, present, and future of microbiological research. As indicated, many bacteria have been previously cultured, particularly from enteric environments; however, vast amounts of bacterial species remain uncultured (Lloyd et al. 2018), and as such, it is important for researchers to continue the isolation and evaluation of function (i.e. metabolic, physiological, etc) of cultured bacteria. Studying both previously-cultured and -uncultured bacteria from a variety of enteric habits will expand our knowledge of habitat (specific location) and niche (functional role within a habitat) utilization within specific enteric locations, such as enteric crypts (Lee et al. 2013; Shepherd et al. 2018). This information could provide a better understanding of the underlying mechanisms of bacterial colonization and bacterial induced intestinal inflammation and disease within the host.

One critical enteric habitat of bacterial colonization in both farmed animals and humans is inflamed intestinal tissue within the GIT. Various methods exist for the evaluation of inflamed tissues, such as tissue cultures and 'gut on a chip' techniques (Possemiers et al. 2013; Kim et al. 2016). The *in vivo* functional colonization of inflamed gut by most autochthonous (resident) microorganisms, however, remains poorly understood using culture-dependant microbiological isolation methods (Kim et al. 2016). Inflammation is a complex physiological process and plays an important role in host defence. This process includes systemic responses such as increase body temperature, somnolence, production of acute phase proteins, systemic adaptive immune responses, etc. In addition, localized tissue responses are also active in this process, namely tissue edema, inflammatory cell chemotaxis with tissue infiltration, and the induction of a robust cell mediated or antibody immune responses (Garrett et al. 2010). Pro- and anti-inflammatory responses are constant and active processes within the GIT, influenced by the presence of pathogens and some autochthonous bacteria (Sansonetti, 2004). However, prolonged, low grade pro-inflammatory response can be detrimental to livestock performance

as metabolic energy is diverted from weight gain to maintaining localized tissue inflammation within the GIT. Most certainly, this interaction between inflammation and growth reduction is suggested to be one of the major factors in performance and livestock profitability. Indeed, historically it was shown that reducing inflammation by the addition of sub-therapeutic levels of antimicrobial agents to feed (antimicrobial growth promoters) enhanced animal performance and its believed that an attenuation in low grade intestinal 'physiological' inflammation was associated with improved weight gains (Niewold 2007; Brown et al. 2017; Kiarie et al. 2018).

In the context of potentially reducing bacterial associated enteric disease in animal agriculture, it is important for current research to better understand: 1) how we can improve the isolation of anaerobic bacteria from mammalian and avian animal samples; and 2) how we can better isolate and identify bacteria able to colonize inflamed tissues for future research and studies. Therefore, the aim of my thesis was to: 1) improve our understanding of the efficacy of traditional microbiological methods (i.e. direct plating and enrichment strategies) to isolate diverse communities of bacteria within a chicken model; 2) expanded evaluation of these comparisons to assess the efficacy of traditional isolation techniques as well as the use of Ichip and endospore selection methods to isolate diverse communities of bacteria in a pig model; and finally, to 3) induce acute inflammatory events within pigs as a model organism and identify candidate bacteria that are able to selectively colonize inflamed tissues that should be used for future studies (i.e. targeted delivery of anti-inflammatory agents co-administered with novel isolated bacteria). I hypothesized that the enrichment methods will outperform direct plating methods in both chickens and pigs by isolating more diverse and comprehensive collections of bacteria. Moreover, I expect that more modern isolation methods, such as the Ichip and endospore selection techniques, will also outperform these traditional isolation methods. In addition, I hypothesized that bacterial diversity will be greatly increased by localized host physiological events associated with tissue inflammation, and observable differences in bacterial isolation and identification will occur between culture-dependant and -independent methods. Lastly, I hypothesized that specific bacteria will be identified from inflamed tissues using a variety of methods, and that these bacteria may be able to selectively colonize within inflamed tissues of pigs.

In Chapter 2, the effectiveness of direct plating and enrichment isolation methods of enteric bacteria was determined. This research also assessed the bacterial diversity within different intestinal habitats of chickens. Through this study 899 bacteria were isolated from chickens, which included the isolation of 75 unique bacterial taxa. Of the bacteria isolated a vast majority (85%) were found to be members of the *Enterococcus*, *Escherichia*, *Proteus*, and *Lactobacillus* Genera. At present, the evaluation

of traditional and novel microbiological isolation techniques is being revisited with the study and application of culturomics. As such, it is important to further analyse how these methods could be used and adapted for poultry applications. At present, few studies involving the culturomics of chickens have been published using culture-based identification approaches. Of these the primary culturomics based study in chickens that employed culture based approaches was conducted by Ferrario *et al* (2017). Ferrario *et al*., demonstrated that the isolation of bacteria from the *Firmicutes, Bacteroides, Proteobacteria,* and *Actinobacteria* phyla, was possible from feral layer birds. In contrast, these results were not observed when I sampled industrialized broiler birds (i.e. birds raised similarly to production setting in Southwestern Alberta, Canada) as the isolation of bacteria from *Firmicutes* and *Proteobacteria* phyla were solely isolated from industrialized broiler birds (Ferrario et al. 2017; Moote et al. 2020). The bacterial populations differed from our study and this variation could be associated with the dissimilarities in diet of feral layer birds as compared to the diets of the industrialized broiler birds housed within a simulated production setting at the Lethbridge Research and Development Centre. One specific variation between the microbiota between these studies was that feral birds were found to have a diverse range of *Lactobacillus* spp., as 16 species recovered compared to two species in broiler birds whereas the industrialized broiler birds in our study were found to have a more diverse population of *Clostridium* spp., as 10 species were recovered from broiler birds compared to one from feral layer birds (Ferrario et al. 2017; Moote et al. 2020). Evaluation of different media used to culture bacteria showed that *Lactobacillus* spp., were most abundant isolated on MRS medium; a finding that was expected as this medium was designed to effectively grow *Lactobacillus* spp. bacteria (de Man et al. 1960). This observation was further supported as the diversity of non-*Lactobacillus* spp. bacteria isolated from this medium was found to be the lowest of those evaluated. In contrast, the isolation of *Escherichia/Shigella* [this Genera annotation was provided by the Ribosomal Database project (Cole et al. 2014)] was more commonly isolated on minimal culture medium, which is likely indicative of the well-known cross feeding and nutrient scavenging metabolic characteristics of these organisms (LaSarre et al. 2017). When isolating bacteria on medium containing 100 µg/ml Gentamycin it was found that members of *Clostridium* spp., were commonly isolated. In contrast, the lack of isolated *Bacteroides* spp., isolated was not expected, potentially indicating a lack of *Bacteroides* spp. present within the microbiota of these birds (Livingston et al. 1978). Finally, *Clostridium* spp. were also isolated using enrichment strategies, particularly enrichment on mucus or blood. In summary, a diverse collection of cultured bacteria was isolated from chickens using direct plating and enrichments strategies. Although the collection was dominated by four Genera (*Enterococcus*, *Escherichia*, *Proteus*, and *Lactobacillus*) there

were also a variety of *Clostridium* spp. isolated within these birds. This collection of bacteria represents a useful resource of bacteria that can be used to evaluate colonization within the enteric habitats of broiler birds.

In Chapter 2, I also evaluated the bacterial abundance and community structure when isolating bacteria from enteric or environmental samples (litter and drinking water). This work demonstrated that enteric samples from each intestinal segment (crop, ileum, jejunum, cecum) were found to be similar regardless of luminal or mucosal region of the gut that was evaluated. In addition, bacterial diversity (Shannon's index) was not found to differ between communities of bacteria isolated from various GIT segments and environments samples. However, the composition of bacteria was found to differ when comparing the communities of bacteria isolated from the various intestinal regions evaluated. As an example, the composition of bacterial communities (i.e. community structure) within the crop were analogous to the small intestine while the composition of cecal bacteria were unrelated to bacteria within the crop and small intestine (Figure 2.6). Specifically, bacterial communities isolated from the ceca of birds were found to be most similar to bacteria present in litter. This result was expected as the litter is often in direct contact with digest from the ceca, largest intestine and cloaca, an observation that has been determined previously (Cressman et al. 2010). In contrast, bacterial communities isolated from the drinking water, were found to be different in composition from other collected samples and these bacteria were dominated by the presence of *Escherichia/Shigella* spp., which are commonly isolated from surface water (Lyautey et al. 2010). Taken together, these observations suggest that the composition of bacteria changes within the small intestine and ceca of birds and may be associated with movement of digesta along the intestinal tract (Gong et al. 2007). This data underscores the importance of isolating bacteria along the entire GIT, as this would provide the most comprehensive information on bacterial community structure with the intestinal microbiota.

To summarize the experimentations of chapter 2, an evaluation of the culturable bacteria from the GIT of chickens and their environment was conducted using direct plating and enrichment strategies. Of note, although *Bacteroides* spp. were expected to be cultured in this study, this bacterial species was not isolated. The inability to culture *Bacteroides* spp. in my study is potentially due to specific diets and housing environments of these birds, as this genera has been isolated within feral and housed birds previously (Salanitro et al. 1974; Ferrario et al. 2017). In addition, it was found that the diversity of bacteria resulting from different isolation methods varied, with MRS medium representing the lowest diversity of medium evaluated. In contrast, no differences were observed when comparing the bacterial communities cultured between GIT locations. Finally, to improve the fidelity of these studies,

laboratories could utilize screening methodologies such as a MALDI-TOF, or PCR descriptive methods (ex REP-PCR), to increase the diversity of the resulting bacterial communities.

In Chapter 3, I further evaluated and compared the effectiveness of various bacterial isolation methods. In particular, the direct plating and enrichment methods used in the previous chapter were highlighted and compared to more modern methods such as the Ichip and endospore bacteria selection methods. The specific hypothesis to be tested was that modern isolation strategies (Ichip and endospore selective methods) would outperform traditional isolation strategies at isolating diverse and novel (not previously cultured bacteria). To evaluate this hypothesis, a porcine model was utilized with bacteria being isolated from the ileum, cecum, ascending colon of animals. Employing these methods, a total of 1,523 bacteria isolates were recovered that represented 80 genera and 7 phyla. When comparing the diversity of bacteria isolated from individual methods, the application of different atmospheres (carbon dioxide or nitrogen) did not alter the composition of isolated bacteria (Figure 3.3). In addition, no changes to the Shannon's index were observed between the different isolation methods. In contrast, the use of Tyndallization (heat) to select for endospore forming bacteria trended towards yielding less diverse communities of bacteria as compared to enrichment broths, Ichips, or direct plating methods. This result was supported when comparing the phyla isolated from each method. Endospore selective methods only isolated three of the seven phyla in the collection, compared to enrichment strategies which isolated bacteria from all phyla contained in the entire collection (Figure 3.8). Further, when evaluating the bacterial composition of these communities obtained through different isolation strategies, the use of endospore selection methods, regardless of ethanol or heat treatment, were found to be most similar in bacterial communities compared than any other method. In addition, the use of Ichips as well as antibiotic enriched media to isolate bacteria were found to generate different bacterial collections, which is likely due to the selective nature of these isolation strategies (Figure 3.4) (Livingston et al. 1978; Nichols et al. 2010; Browne et al. 2016). These different isolation methods were further evaluated for their ability to isolate novel (previously unidentified) bacteria. Although the application of endospore bacteria selective techniques was suggested to be effective at isolating novel bacteria at high frequency by Browne *et al* (2016), this was not observed in my study. Conversely, Ichips and broth enrichment isolation techniques were capable of isolating previously uncultured bacteria at greater frequency than either ethanol or Tyndallization (heat) -treatment endospore selection methods. Finally, my research also demonstrated that endospore bacteria selection via ethanol exposure was less effective at eliminating non-vegetative cells than heat treated endospore selection due to the abundance of *Lactobacillus* spp. isolated using the ethanol exposure method (Figure 3.7). This result was

unexpected as Browne, *et al* (2016) showed that selecting for endospore forming bacteria with ethanol was highly effective at isolating previously uncultured bacteria. In contrast, the efficacy of enrichment broths to isolate novel bacteria as well as isolated diverse collections of bacteria is demonstrated in this study and supports previous research observations. Our research findings, however could be further expanded to determine impact of the incubation time of enrichment strategies to isolate diverse collections of novel bacteria from (Ziemer 2014; Lagier et al. 2015). In summary, enrichment broths were the best method for isolating the most diverse collection of bacteria including some previously uncultured bacteria. Although enrichment broths were an excellent technique for enteric bacterial isolation, the application of multiple bacterial isolation methods in concert was required to provide the most comprehensive repertoire of bacterial isolates from the porcine gut.

In Chapter 3, I also demonstrated the differences in abundance and composition of culturable bacterial communities isolated from the ileum, cecum, spiral colon, and ascending colon of pigs. Through these evaluations it was determined that the culturable diversity of enteric bacteria residing within pigs in general was not affected by the location evaluated through the GIT (Figure 3.11). The composition (community structure) of bacteria isolated from these sites, however, was found to differ between specific segments of guts as the bacterial communities of the ileum were found to be different in community structure than those isolated from the cecum, ascending colon or spiral colon of pigs (Figure 3.12). Bacterial genera that were more frequently isolated from the ileum included *Escherichia/*Shigella and *Proteus* spp., whereas *Clostridium sensu stricto* and *Bacteroides* spp., were more commonly isolated from the large intestine (Figure 3.14). In summary, it was found the culturable diversity of bacteria was not found to differ between the locations of the GIT sampled, however, the community structure was different between regions of the small and large intestine.

To summarize the findings from Chapter 3, when evaluating the <1,500 recovered bacteria, my data indicates that the isolation of novel bacteria can be readily accomplished when using enrichment broths and Ichip methods. In addition, the use of antibiotics (i.e. Gentamycin) are very effective at isolating specific bacteria, such as *Bacteroides* spp. The endospore selective methods, although widely touted as an effective method to isolate previously uncultured bacteria, my study suggests that in particular, the use of Tyndallization (heat treated) for endospore bacteria selection was relatively ineffective at isolating unculturable bacteria or diverse communities of bacteria. As such, this finding did not support my hypothesis that both endospore selective and Ichip were more effective at isolation of novel or diverse communities of bacteria when compared to traditional isolation techniques. It may, however, be possible to improve endospore identification of previously uncultured bacteria by applying in concert

other analytical techniques such as MALDI-TOF instrumental analyses. When comparing the locations of bacterial isolations along the GIT, it was determined that the culturable bacterial diversity did not differ between region, however the community structure of isolated bacteria was found to differ between samples collected in the small or large intestines of pigs.

In Chapter 4, I used the experience gained from the previous chapters to specially isolate and identify specific bacteria of swine that are associated with inflammatory events along the GIT. To induce intestinal inflammation, *Salmonella* Typhimurium phage type DT104 or Columbia broth was inoculated into Landrace x Duroc cross pigs representing infected (ST+) or control (ST-) treatment respectively. Body weight and temperature were found to be significantly different between ST+ and ST- treatments, with the body weight of ST+ animals found to be significantly different at all days evaluated. In addition, the body temperature of these animals was also found to be different at the early days of infections; 1- and 2-days (d) post inoculation (p.i.) (Figure 4.2). These results were expected as *Salmonellosis* decreases weights of pigs (Gradassi et al. 2013). In addition, tissue changes increased within all samples in the GIT of ST+ animals, with the exception of the upper small intestine (duodenum and jejunum). Tissue changes decreased over time as the resolution of tissue injury progressed until 10d p.i. (Figure 4.3). This observation is supported by previous research which shows that tissue injury in pigs can be observed 2d p.i. with *Salmonella enteric* var Typhimurium (Walsh et al. 2012). Histopathological changes and the expression of genes involved in immune function and inflammation were also increased ST+ animals (Figure 4.12). In summary, this challenge study effectively generated gut inflammation and the use of *Salmonella* to induce enteric inflammation in the pig model that can also be used to identify autochthonous bacteria able to co-colonize *Salmonella* within intestinal habitats of the GIT.

In Chapter 4, I also evaluated the changes to bacterial colonization in ST+ and ST- animals. When comparing the abundance of bacteria within the gut of pigs, the bacterial communities from ST+ and ST- treated animals did not vary between different regions of the intestine. In contrast, overall changes to bacterial diversity consistently showed that the bacterial community structure of the ileum differed from the cecum and other areas of the small intestine, which is aligned with my findings using culture-based detection methods described in chapter 3 (Figure 4.14). When comparing methodologies using culture-dependant and culture-independent analyses, it was determined that <55% of the bacteria identified (culture-independent methods) within the large intestine were found to belong to *Prevotella* spp. Culture-dependent methods, however, found only a small percentage of this organism present within the harvested tissues (Figure 4.17). Moreover, to isolate specific bacteria associated with inflamed tissues culture-dependant methods were effective at identifying specific bacterial communities

that were commonly isolated from inflamed or non-inflamed tissues. For instance, some *Bacteroides* spp., including *B. fragilis* and *B. heparinolyticus*, were more commonly isolated within the cecum of ST+ animals. In contrast, organisms such as *S. enterica* and *Escherichia/Shigella* spp. were more commonly identified within the ileum of ST+ animals. Of note, *Bacilli* were most abundant within the spiral colon of ST+ animals and the *Bacilli* class bacterium *S. gallolyticus* was isolated only from ST+ animals (Figure 4.19). Furthermore, *Bacteroides* spp. and *S. gallolyticus* represent unique inflammation specific bacteria that can selectively colonize habitats within the inflamed intestine as observed within tissue harvested from ST+ pigs. Specifically, at d6 and d10 p.i. the numbers of *B. uniformis* were found to be elevated in spiral colon and cecum of ST+ animals, respectively. This result was expected as *Bacteroides* are associated within the gut microbiota in intestinal samples from people following intestinal surgery (Brook and Hirokawa 1989). In contrast, *S. gallolyticus* was observed in greater abundance within the cecum and spiral colon of ST+ animals at both d6 and d10 p.i. (Figure 4.21). The observation of this organism colonized inflamed tissues was surprising. It has however, been shown that this bacterium can be isolated form abnormal gut, such as colon cancer, and its likely that neoplastic tissue undergo intermittent periods of inflammation are an ideal habitat for this bacterium. Indeed, it is possible that this bacterium is not a primary pathogen of the gut, but instead favours inflamed habitats within the GIT—a process that may be associated with secretion of bacteriocins (Aymeric et al. 2018). In my study, identification of increased abundance of *S. gallolyticus* within the cecum and spiral supported my hypothesis that unique populations of microorganisms can effectively colonize and reside inflamed segments of the intestinal tract of pigs.

In summary of chapter 4, intestinal inflammation was generated within a *Salmonella* challenged porcine model and was associated with physiological and histopathological changes as well as the expression of genes involved in immune function and inflammation. Using this model, this data supported my hypothesis that specific members of the porcine intestinal microbiota were more frequently isolated within inflamed tissues. Of these isolated organisms, *Bacteroides uniformis* and *Streptococcus gallolyticus* were further explored by quantitative PCR (qPCR) to validate observations made using culture-based methods. Of note, *S. gallolyticus* was isolated only from ST+ challenged pigs and this elevated abundance in ST+ animals was confirmed using qPCR. Interestingly, our information is in-line with other studies that showed *S. gallolyticus* has been isolated within inflamed tissue habitats in other species such as humans, and cattle; and the presence of this organism is commonly associated with inflammatory or inflammatory related events such as colon cancer and rumen acidosis (Russell and Hino 1985; Aymeric et al. 2018). In addition, and to the best of my knowledge, the observation of this

organism within inflamed tissues in swine has never been observed; instead, this organism has been observed to be associated with the microbiota of healthy pigs with higher feed efficiencies (Quan et al. 2019). Moreover, the ability of *S. gallolyticus* to utilize various niches within these intestinal environments likely leads to its colonization and growth within these altered gut habitats potentially exacerbating tissue injury associated with tumor development and ruminal acidosis. Future work could clarify whether *S. gallolyticus* is primarily associated with habitat utilization within inflamed gut or whether the microbe can also cause direct tissue injury through increasing host inflammatory mediators by or whether this organism is simply a resident bacteria favoring growth in these habitats (Pasquereau-Kotula et al. 2018).

In conclusion, culturomics is an important tool in both the advancement of research field of microbiology and to further understand the pathogenesis of infectious enteric disease in the livestock sector. Indeed, this methodology will provide researchers with information related the bacterial diversity within a sample and can be used to collect store and catalogue bacteria for future use (Lagier et al. 2015). These archived organisms can provide valuable information in understanding metabolic processes performed by different bacterial species and provide more insight into the etiology of animal diseases. In addition, any catalogued organisms are valuable to industry and the research community as they could function as to ensure the conservation and maintenance of members of the microbiota present with the microbiota of pigs. Indeed, it has been suggested that the ingestion of a consistent modern day diet in humans is associated with the loss of specific bacterial taxa, and as such pigs fed high energy rations are potentially susceptible to extinction of bacterial species of within their gut microbiota (Sonnenburg et al. 2016). My research has demonstrated important differences in efficacy of isolation methods used to culture bacteria (Chapters 2 and 3). Importantly, I have identified specific techniques that can effectively isolate unique communities of bacteria. When comparing the isolation of previously uncultured bacteria, it was suggested that the use of endospore selective methods to isolate unculturable bacteria should be employed after pre-screening and subsequent elimination of commonly isolated bacteria with MALDI-TOF technical analysis (Browne et al. 2016). In addition, my research also showed that the use of long-term enrichments was effective at isolating novel bacteria, which was consistent with previous observations (Ziemer 2014; Lagier et al. 2016). Further to this, it was determined that the effectiveness of enrichment methods was similar to the Ichip isolation methodologies as they increased both the potential of isolating unique bacteria and improving the diversity and novelty of bacterial collections. In addition, a *Salmonella* porcine challenge model was developed to induce enteric inflammation enabling the subsequent identification of bacteria that

colonize inflamed gut. In particular, the bacterium *S. gallolyticus* was isolated from the inflamed gut of pigs, and although not a classic probiotic bacterium, this candidate bacterium could be a novel probiotic, co-administered with encapsulated immunomodulating agents to reduce enteric inflammation.

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