Gut microbiome and immune system changes in heat-stressed pigs

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Rising global atmospheric temperatures are expected to negatively affect farmed animals, especially pigs. To study the likely effects, pigs were exposed to chronic heat stress and their growth, changes to the intestinal microbiota diversity and selected immunological changes in the gut lining were investigated. Six test pigs were subjected to a temperature of 35 ± 2°C and six control pigs to a 'normal' temperature of 23 ± 2°C (both at a relative humidity of 75-85% for 21 days, in climate-controlled pens). Bodyweight, rectal and forehead temperatures were measured, and fresh faeces collected daily from each pig. The rectal temperatures, on average, increased in HS pigs, reaching up to 40.5°C, as compared to 37.6°C in control pigs. Similarly, average forehead temperatures had increased to 39.3°C in heat-stressed pigs and were 36.7°C in the control pigs. The average body weight gains in heat-stressed pigs were 2.9, 1.2, and 0.35 kg, respectively at days 7, 14, and 21, significantly lower ($P < 0.05$) than for the control pigs. While faeces were collected daily, only samples collected on days 1, 7, 14, and 21 were genetically sequenced to observe for changes. In heat-stressed pigs, marked changes in major microbial phyla composition were observed. The number of Gram-positive *Firmicutes* decreased, whereas Gram-negative *Bacteroidetes* increased from day 7 onwards. More importantly, major Gram-negative potential pathogens such as *Campylobacterales*, *Proteobacteria,* and *Spirochaetes* had increased in heat-stressed pigs. This increase in Gram-negative bacterial numbers resulted in upregulation of the NF-κB-mediated pro-inflammatory cytokines IL-6 and IL-8 *via* the TLR4/NF-κB-signalling pathway, in the gut lining. Increases in IL-6/IL-8 cytokines cause fever, anorexia, and other metabolic changes. The microbiota and immunological changes resulted in diarrhoea and potentially exposed pigs to inflammatory bowel disease, which partly explains the lower bodyweights in the heat-stressed pigs. Such losses in production impose financial strains on farmers, while the presence of pathogens may adversely impact food safety for the ever-increasing population, dependent in-part on low-priced meats like pork. To investigate cross-species transfer, gut microbiota from heat-stressed and control pigs were transplanted into 'specific pathogen-free mice, and the two groups' gut microbiota sequenced on

days 1, 7, 14 and 21. Similar changes to that observed in the pig gut microbiota were evident in the infected mice after 21 days. This study, hence, confirms that heat stress causes compositional and associated immunological changes in the gut microbiota of pigs; and that microbiota changes that occur in one species can be transferred to another species to cause similar clinical signs, gut microbiota, and body weight changes in the recipient species.

Keywords: colonic microflora, colonic pathology, faecal transplantation, GIT microbiota gut immunity, heat-stress, IBD, immune system microbiota, immunology, pigs, stressors.

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Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Pork meat has been a significant source of protein for humans for thousands of years. As agricultural production has increased, so has the consumption of pork. Pig farming is an important livestock sector on almost every continent around the globe. Although some cultures do not consume pork (e.g. Muslims, Jews) because of their religious principles, the pig farming sector is one of the largest in world animal production (István & Viktoria, 2017). Developing countries tend to have higher meat production outputs [15-fold increase in Asia since 1961 (Roser, 2017)] due to low input cost production systems. Meat demand in developing countries continues to increase as higher incomes and urbanisation lead to changes in the type of food consumed, favouring increased dietary protein sourced from animals. In the last decade, pork consumption has risen substantially in many emerging economies, particularly in China and other fast-growing Asian countries (OECD, 2019).

Pigs (*Sus scrofa domesticus*) were first domesticated in the Near East around 8,500 BC and were subsequently brought into Europe by agriculturalists. However, in addition to being a source of food, pigs are increasingly in demand by biomedical researchers because the pig immune system has been found to be similar to that of humans. Hence, the pig has become an important animal model in studies researching disorders of the human immune system (Barrios-Rodiles et al., 2005; Dawson et al., 2013). However, the similarities do not end there. Since the pig anatomy and physiology is similar to humans, they are also increasingly used to study major human diseases such as infectious diseases, cardiovascular diseases, and obesity (Boluyt et al., 2007; Brambilla & Cantafora, 2004; Meurens et al., 2012; Rothschild, 2011; Swindle et al., 2011). Owing to their long connection with humans, there is considerable accumulated knowledge about pig farming and hence their convenience for use as an animal model. Given that the average litter size of pigs is 12 and the gestation period is 114 days, many studies related to family, gender, and sibling matching are feasible in pigs (Mair et al., 2014). A study by Dawson and colleagues has shown that the immune response in pigs displays about 80% similarity to humans' in nearly all the common parameters studied. In contrast, the human immune response has only about 10% similarity to mice (Dawson et al., 2013). In most instances, the results obtained from using genetically modified mice have failed to translate into clinically relevant human outcomes. Hence there is an increasing tendency for researchers to move away from using mice to using pigs so that their results have more comparable and scalable implications for human health.

The rise in global atmospheric temperatures causes heat stress (HS) in animals and markedly impairs efficiency at every stage of the production cycle (Lacetera, 2019). Pigs are especially sensitive to HS because they lack functional sweat glands and also possess a thick layer of subcutaneous adipose tissue acting as an effective insulation layer (Ross et al., 2015a). Currently, HS is one of the largest economic challenges for the global pork industry (Ross et al., 2015a; St-Pierre et al., 2003). Genetic selection for breeds with higher lean tissue – one of the main economically important production traits –has increased, but such a selection has also reduced thermal tolerance in pigs. This is partly mediated by the altered body composition (i.e., increased lean tissue) as synthesising and maintaining skeletal muscle generates metabolic heat (Baumgard & Rhoads, 2013; Pearce & Escobar, 2013). The modern pork production industry has been plagued with major problems, particularly those associated with defence against the infectious diseases caused mostly by intensive livestock breeding. For instance, reproductive and respiratory syndrome, swine epidemic encephalitis, porcine pseudorabies, swine fever, porcine epizootic diarrhoea, foot and mouth disease, porcine parvovirus disease, swine enzootic pneumonia (SEP) and atrophic rhinitis are opportunistic infections that have resulted in high economic losses to the pork industry in recent years (Shinkai et al., 2006). Polymorphisms and changes to immune molecules such as toll-like receptors (TLRs) occur, and these have a significant effect on the animal's response to pathogens, and consequent disease resistance/susceptibility (Lazarus et al., 2002). Furthermore, since pigs are a vital source of food for humans, research into pig farming methods and nutrition has gained critical momentum to develop and maintain safe and sustainable pork production (Rothkötter, 2009).

A group of symbiotic and pathobiont microbes, collectively called the ‘microbiota’, are present in the guts of mammals. These microbes have developed a symbiotic relationship with their hosts over millions of years of evolution and have created a conducive environment within the host, in which the microbes contribute to many of the host’s physiological processes in return for the host providing the conditions essential for microbial survival (Xiao, 2017). All pathogens and commensal microflora express pathogen-associated molecular patterns (PAMPs) on them. These PAMPs are detected by pattern recognition receptors (PRRs) that are expressed in immune cells. A variety of cellular receptor classes are included in the PRR family, consisting of TLRs, nucleotide-binding oligomerisation domain receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), membrane C-type lectin receptors (CLRs), and DNA receptors (cytosolic sensors for DNA) (Kumar et al., 2011). Certain soluble PRRs such as surfactant proteins and mannan-binding lectins can also bind to PAMPs. These PRRs are of vital importance in innate immunity as they protect against pathogens by recognition of PAMPs such as flagellin and bacterial nucleic acids (Nobuhiko et al., 2013). These PRRs are evolutionarily conserved and have germline-encoded host sensors (Kumar et al., 2011; Reynolds & Dong, 2013). It is well established that the pig gut microbiota plays a critical role in

maintaining metabolic homeostasis, as well as in a myriad of physiological, neurological and immunological functions including protection from pathogens and digestion of food materials - some of which would be otherwise indigestible by the pig (Lu et al., 2018). A rich and diverse gut microbial ecosystem (i.e. a balanced microbiota) is the hallmark of good health; while qualitative and quantitative perturbations in the microbial composition can lead to the development of various diseases (Trevisi & Pérez, 2017). Alternatively, diseases caused by stressors or other factors have been shown to impact the microbiota negatively.

The pig industry is currently facing an onslaught from a unique array of pathogens. The TLR4 is crucial to initiate an innate immune system response to endotoxins such as lipopolysaccharides (LPS), originating from the Gram-negative bacteria that usually threaten pig production (Xiao, 2017). As the world tackles the problem of rising atmospheric temperatures, pigs, being highly sensitive to elevated ambient temperatures, activate a TLR4-mediated signalling pathway in response to abnormal thermal stresses. The gut microbiota plays a significant role in host immunity, partly mediated through TLRs (Sun, 2015). Hence, understanding the mechanism of action of gut TLR4 has become imperative to the efforts being put in to alleviate the effects of heat stress in mammals.

1.1 Research aim

This project aimed to explore the response of the pig gut microbiome and the immune response to heat stress and the impact of transplanting faecal microbes from pigs on the microbiota of recipient mice.

1.2 Objectives

1. To investigate the response of the gut microbiota to heat stress in pigs.
2. To study the correlation between changes to gut microbiota composition and the profile of the intestinal TLRs in HS pigs.
3. To study the effects of inter-species faecal transplantation from HS pigs to specific pathogen-free (SPF) mice.

1.3 Hypotheses

1. Heat stress is detrimental to pig health and production.
2. Chronic heat stress causes changes to the composition of the pig gut microbiota.
3. Changes in the gut microbiota composition can modify the gut immune system.

4. Transplantation of faeces from HS pigs into mice causes similar changes in the gut microbiome of recipient mice.

Chapter 2

Literature Review

2.1 Introduction

'Microbiota' is defined as all microbes, including their genomes and extra-chromosomal elements, present in and on the host animal; their interactions within the gastrointestinal tract (GIT), skin, and genital environments (Dominguez-Bello et al., 2019; Kim & Isaacson, 2015). The terms 'microbiota' (the microbial taxa associated with the host) and 'microbiome' (the catalogue of these microbes and their genes) are often used interchangeably (Ursell et al., 2012). The organisms comprising the gut microbiota reside outside the mucosal layer and play a role in triggering the host immune response and in communication between the gut and the brain (Min & Rhee, 2015; Thaiss et al., 2016). The GIT is not only the largest interface between the external and internal environments of animals, but it also contains the largest amount and the greatest diversity of microorganisms. The GIT microbiota is defined as an ecological community made up of commensal, symbiotic and pathogenic microorganisms; including bacteria, viruses, parasites, fungi, archaea, and protists, that inhabit the mammalian gut (Peterson et al., 2009; Wang & Donovan, 2015). Such a mammalian GIT is estimated to host approximately 10^{14} bacterial organisms comprising 500–1,000 unique species, which form a synergistic relationship with the host (Ley et al., 2005; Savage, 1977; Xu & Gordon, 2003). Co-evolution of gut microorganisms with their hosts has led to the acquisition of microbial roles in digestion, nutrient utilisation, toxin removal, protection against pathogens and regulation of the endocrine and immune systems (Bäckhed et al., 2005; Hill & Artis, 2009; Ruth E Ley et al., 2008). Hence, the abnormal intestinal functions observed in germ-free (GF) animals have been attributed to the absence of these essential GIT microbes (Wu & Wu, 2012a).

A healthy intestinal microbial community is diverse, stable, resistant (to minor changes) and resilient (Levy et al., 2017). Human and mouse studies have shown that dysbiosis, which consists of disequilibrium of the microbial community in the GIT microbiota, is associated with the development of several acute and chronic inflammatory conditions, bowel diseases, metabolic syndromes, and diabetes (Brown, 2012). As hosts mature, the organisms comprising the microbiota begin to sense their environment through toll-like receptors (TLR) and short-chain fatty acid (SCFA) receptors on the gut epithelial surface enabling them to detect incoming nutrients and microbial components. Specifically, they sense structural molecules such as LPS, flagellins, and peptidoglycans; metabolites such as SCFAs; and microbial secretions such as toxins or polyphosphate chains (Madsen, 2011; Yu & Gao, 2015).

In the animal husbandry sector, immense potential has been described for manipulating the GIT microbiota to improve nutritional and immunological activities within the host to boost livestock productivity. However, the GIT microbiota has not yet been fully explored in many of the prominent livestock species. The study of pigs has great potential to inform human research due to the many similarities identified in the physiological attributes of these two species. Moreover, pigs exhibit similar susceptibilities and clinical manifestations to pathogens that are the etiological agents in certain human intestinal disorders (Meurens et al., 2012; Zhang, 2013). Xiao et al. (2016) reported that although the homology between human and pig microbiomes is quite modest at the gene level, it was significant at the level of KEGG orthology functions. This same study identified more similarity between human and pig microbiomes than between human and mice microbiomes; and reported that approximately 96% of the functional pathways described in the human gut microbiome are common to the pig as well (Xiao et al., 2016). Further, it has been shown that humans share more similarities with pigs in terms of anatomy, genetics, physiology, pharmaceutical bioavailability, and nutrient digestibility than with rodents (Isaacson & Kim, 2012). Hence, the pig is a superior model to rodents for studying human physiology and pathology as pertaining to enteric health.

However, it has not yet been determined qualitatively or quantitatively what constitutes a healthy microbial community in the GIT of pigs or other mammals. It is, therefore, imperative to conduct statistically powerful studies to characterise the microbiome diversity in pigs and to determine how this diversity can be utilised to improve the performance and health of pigs. Towards this end, a study recently reported that the genome comprising the microbiome of pigs, at various stages of development, contained more than twice the number of genes as that of the actual pig genome (Lu et al., 2018).

In this Ph.D. project, we examined how changes in the GIT microbiota may influence biochemical, physiological, immunological, and metabolic processes within the host and vice versa. Most of the microbiota studies in farm animals have focused on the effects that treatments with antibiotics, prebiotics, probiotics, and feed additives have on the animals; while fundamental studies focusing on how the host-microbe relationships affect the physiology and immunology of farm animals, are scarce.

2.2 Gastrointestinal tract

The GIT is a functionally and anatomically diverse organ comprising the mouth, oesophagus, stomach, small intestine, large intestine and anus (Saffrey, 2014). Culture-independent techniques have revealed that the GIT contains a dynamic microbial population with unique organisms located in the different organ sections (Kim et al., 2012; Looft et al., 2014), with the most diverse group of microbes inhabiting the colon in pigs (Maradiaga et al., 2018). Specifically, within the colon of normal

healthy adult pigs, the taxonomical composition of the bacterial community has been described as 35% *Firmicutes*, 21% *Bacteroidetes*, 3% *Proteobacteria* and 2% *Spirochaetes* (Allen et al., 2011; Lamendella et al., 2011; Looft et al., 2012). In the jejunum and ileum, *Proteobacteria* account for about 70% of the microbes, followed by *Firmicutes*, which are about 20%. In contrast, in the cecum and colon, the *Firmicutes* dominate with >75% and *Proteobacteria* are about 13% (Kim et al., 2012; Zhao et al., 2015). Nevertheless, high microbial diversity identified within the small intestine is thought to minimise the negative effects of certain pathogenic bacterial strains that are often found within this organ. The microbiota colonises the mucosal entry sites of pathogens, where it occupies biological niches and prevents invasion by foreign pathogens – known as 'colonisation resistance' (Thaiss et al., 2016).

2.3 Brain–gut–microbiome axis

Several studies have shown that GIT microbiota contributes significantly to maintenance of normal physiological and metabolic functioning of mammalian hosts (Fig. 2.3.1). Furthermore, the gut microbiota has been shown to contribute to neurophysiological regulation, which subsequently governs neurotransmission, cognition, and behaviour. This is achieved through regulation of the immune and endocrine systems *via* the release of bacterial metabolites (Sandhu et al., 2017). Although the brain-gut–microbiome axis has not yet been thoroughly examined in pigs; through analysis of this system in other mammalian species, we have hypothesised that this axis would also play a key role in pigs. Microbes in the gut communicate with the central nervous system through at least three parallel and interacting channels, which involve mechanisms encompassing the nervous system, endocrine system, and immune signalling. The brain can affect the community structure and function of the gut microbiota *via* the autonomic nervous system, by modulation of local gut motility, intestinal transit and secretion, and gut permeability. It is thought to be accomplished through the luminal secretion of hormones that directly modulate microbial gene expression (Martin et al., 2018). Through the release of metabolites, the gut microbiota communicates with a network of neuronal, glial, endocrine, and immune cells (Bohórquez & Liddle, 2015). Microbiota and their metabolites have, therefore, been described as associated with the modulation of behaviour and brain processes, including emotional behaviour, brain biochemistry, responses to stress and pain, and GIT functioning (Mayer et al., 2015). The latter is due to changes in intestinal permeability, mucosal immune functioning, and activity of the enteric nervous system.

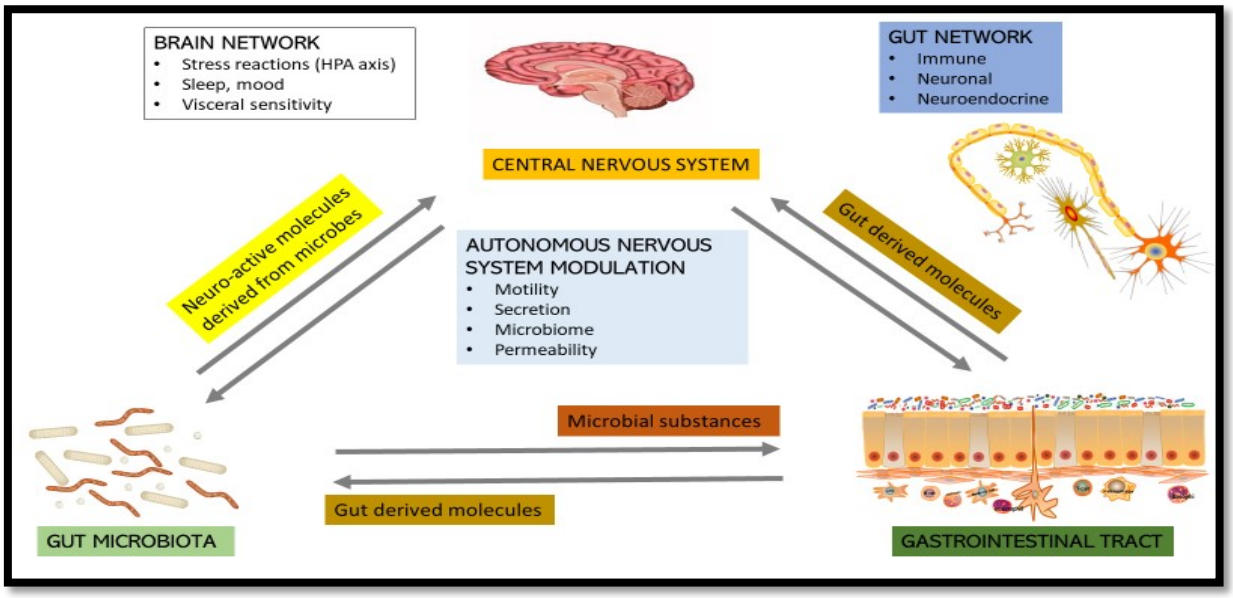

Figure 2.3.1 Brain–gut-microbiome axis.

A recent systems biology model postulated that circular communication loops exist between the brain and the gut microbiome and that perturbations to any of these factors can lead to dysregulation of the circuit. Many studies, primarily in rats and mice, have implicated alterations in the brain-gut-microbiome axis in the pathogenesis and pathophysiology of irritable bowel syndrome (IBS), obesity, as well as several psychiatric and neurological disorders (Martin et al., 2018). Similar studies relating to the brain-gut-microbiome in pigs have not yet been reported.

2.4 Birth-, age-, breed-, and genetics-related microbiota

2.4.1 Birth

The structure and composition of the gut microbiota in animals are determined by many factors (Fig. 2.4.1), such as genetics, age, phylogeny, diet, and surrounding environmental conditions during birth (Leser et al., 2002; Ruth E Ley et al., 2008). For example, in mammals, the initial exposure to microbes occurs at parturition in the birth canal. The mode of delivery, vaginal or Caesarean section (CS), along with the nutrition provided during early stages of life, have a significant influence on the intestinal microbiota. Furthermore, a recent study on the microbial composition of the umbilical cord found that maternal transfer is possible and that it may occur during gestation. Additionally, pigs born vaginally have a higher bacterial density and the higher concentration of SCFAs, including acetate, propionate, and butyrate, compared to pigs born *via* CS (Wang et al., 2013). Moreover, a study examined the effects of inoculating CS-born piglets with either a placebo containing simple-composition microbiota, or complex faecal microbiota from adult sows, which would mimic the microbial environment acquired through vaginal deliveries. The results showed that the adult pigs inoculated with the placebo contained a less diverse faecal microbiota compared to piglets inoculated with complex microbiota (Jansman et al., 2012). Further, the placebo pigs exhibited poor health and transient diarrhoea. These results suggest that the adult pig microbial community is

primarily influenced by the microbes that the piglet encounters during the earliest stages of its life. Pre-labour CSs were seen to decrease bacterial diversity and density in piglets compared to normal vaginal delivery (Siggers et al., 2008). Also, within the livers of piglets delivered *via* CS, lower expression of interferon (IFN), NKp80, and C-reactive protein (CRP) was observed (Hyde et al., 2010). Microbial colonisation, therefore, begins at birth and continues to diversify in the initial days of life based on exposure to environmental microbiota, which depends on the host habitat, diet, and physiology (Thompson et al., 2008).

2.4.2 **Age**

The density and species diversity of the gut microbial population in the different compartments of the GIT are in constant flux as pigs develop (Holman et al., 2017; Wenjing et al., 2015). The composition of the microbiota also becomes increasingly diverse with progression through the pig GIT (Pryde et al., 1999; Simpson et al., 1999). The *Lactobacilli*, *Bifidobacterium*, *Streptococcus*, *Bacteroides*, *Clostridium perfringes*, and *Escherichia coli* are major taxa identified in the pig GIT; however, the specific makeup changes with age (Dowarah et al., 2017). The earliest colonisers of the pig gut, between birth and 2 days, are primarily of the genera *Escherichia*, *Clostridium*, *Fusobacterium*, *Streptococcus*, and *Enterococcus*. It is further estimated that 34% of the total microbial population present at 6 h of age is from the family *Clostridiaceae*, which is seen to reduce to 1% by day 20, while *Enterobacteriaceae* are not detected during these early days (Petri et al., 2010). Rather, a steady increase in *Enterobacteriaceae* occurs from weaning (approximately day 28) to 5 days post-weaning; however, they are seen to decline after day 11 post-weaning significantly (Dou et al., 2017). Thus, the microbial community may differ between siblings in the first two days following birth but begins to stabilise by day 28. Moreover, by day 36, substantial similarities are observed in the intestinal microbial community of cohabiting non-sibling piglets, however, not necessarily between siblings if separated from the sow within 3 days of birth (Thompson et al., 2008). In the first 5 days after birth, the microbial community is dominated by strict aerobes and facultative anaerobes, which are gradually replaced almost entirely by strict anaerobes (starting from day 7 up to day 22) (Inoue et al., 2005). The first significant change in intestinal microbial diversity occurs in piglets on days 4–7 when the number of *Clostridium perfringens* organisms declines due to the activity of immunoglobulin A (IgA) inherited from the mother. The development of major immune system induction elements occurs approximately 2 weeks after birth, and by 4 weeks significant concentrations of secretory IgA are evident (Inoue et al., 2005). Hence, microbial intestinal colonisation affects susceptibility and tolerance to not only intestinal pathogens but also to systemic infectious and non-infectious diseases. The *Firmicutes/Bacteroidetes* ratio changes with increase in age (Kim et al., 2011) and has an impact on the breakdown of polysaccharides, nutrient absorption, gut permeability, and inflammatory response (Mathur & Barlow, 2015). *Bacteroidetes* have been

shown to participate in carbohydrate degradation; yet, in pigs, the proportion of bacterial species belonging to this phylum decreases with age, causing subsequent weight gain (Zhou et al., 2015). Further, *Prevotella* spp. have been shown to account for 26% of the bacterial content in the faeces of 10-week-old piglets, but only 4% in 22-week-old pigs. The faecal microbial composition continuously changes until the animal reaches 6 months of age, at which point it is seen to stabilise (Kim et al., 2011). However, as pigs move through their life, it is not only their age that changes but also the composition of their feed, weaning practices and the mixing patterns in their pens (Han et al., 2018). Hence, the relationship between the growth of pigs and the diversity of the intestinal microbiota remains complex.

2.4.3 Genetics and Breed

The prevalence of *Firmicutes* and *Bacteroidetes* within the faecal bacterial community varies between certain pig breeds. Specifically, in Chinese Jinhua pigs 70.4% of the faecal bacterial population is composed of *Firmicutes* whereas 14.4% are *Bacteroidetes* (Hua Yang et al., 2018). Alternatively, western breeds such as the Duroc, Yorkshire, and Landrace contain 39.6%, 42.0%, and 45.6% *Firmicutes* and 57.0%, 51.4%, and 47.6% *Bacteroidetes*, respectively (Pajarillo et al., 2014a, 2015). It is well established that host genetics and the lean/obese nature of a breed of pig play an important role in the GIT microbiome/metabolome profiles. Significant differences have been observed in Meihua piglets (fatty-type, slow-growing Chinese breed) and Landrace piglets (lean-type fast-growing European breed) in the production of SCFAs and secondary bile acids, including deoxycholic acid and lithocholic acid, all of which are naturally occurring within the GIT lumen, however, at high concentrations can cause oxidative/nitrosative stress, DNA damage, apoptosis, and mutation (Ajouz et al., 2014). Specifically, accumulation of SCFAs and secondary bile acids was found to be higher in the colonic lumen of Landrace piglets (Yan et al., 2017). Moreover, a comparative study showed that Jinhua pigs exhibited better growth performance, lower diarrhoeal rates, and lower immune activation in response to challenge with an enterotoxigenic *E. coli* (ETEC) K88 species compared to Landrace pigs. The Landrace pigs also had a higher overall proportion of *Lactobacilli* spp., as well as a higher ratio of *Lactobacilli* to *E. coli*, and more tight junction proteins (Gao, 2013). The *Lactobacillus*, *Bacteroides*, *Prevotella*, and *Ruminococcus* species were found to increase in abundance throughout the colonisation process; however, the exact proportions were dependent on the pig breed (Bian et al., 2016). Additionally, when different purebred pigs were cohabitated for several weeks, their gut microbial communities shared more similarities yet retained the distinguishable breed-specific proportions (Pajarillo et al., 2014a). It has also been reported that the abundance of the methanogenic anaerobe, *Methanobrevibacter smithii*, significantly increased over

the first 14 days of life in two pig breeds, namely, Meishan (obese) and Yorkshire (lean) (Su et al., 2014). However, the level in the lean breed was found to be significantly higher than that in the obese pigs. Methanogens such as *M. smithii* help to remove hydrogen and carbon dioxide, forming methane, thereby preventing the accumulation of hydrogen in the gut and the subsequent decrease in microbial fermentation efficiency and energy yield. Hence, methanogens are an important component of the gut microbial community (Armougom et al., 2009). In a study by (Guo et al., 2008) on Bama miniature pigs, it was found that obese pigs contained approximately 61% fewer *Bacteroidetes* and approximately 56% fewer *Bacteroides* spp. than lean pigs. Hence, the authors concluded that elevated proportions of *Bacteroidetes* and *Bacteroides* species negatively impacted body weight (Pryde et al., 1999; Simpson et al., 1999).

The housing conditions, rearing density, environmental temperature, and time of sampling can also all significantly affect diversity of the intestinal gut microbiota (Liu et al., 2016). For example, the intestinal microbiota diversity has been reported to change three times from birth to after weaning in young piglets (Inoue et al., 2005).

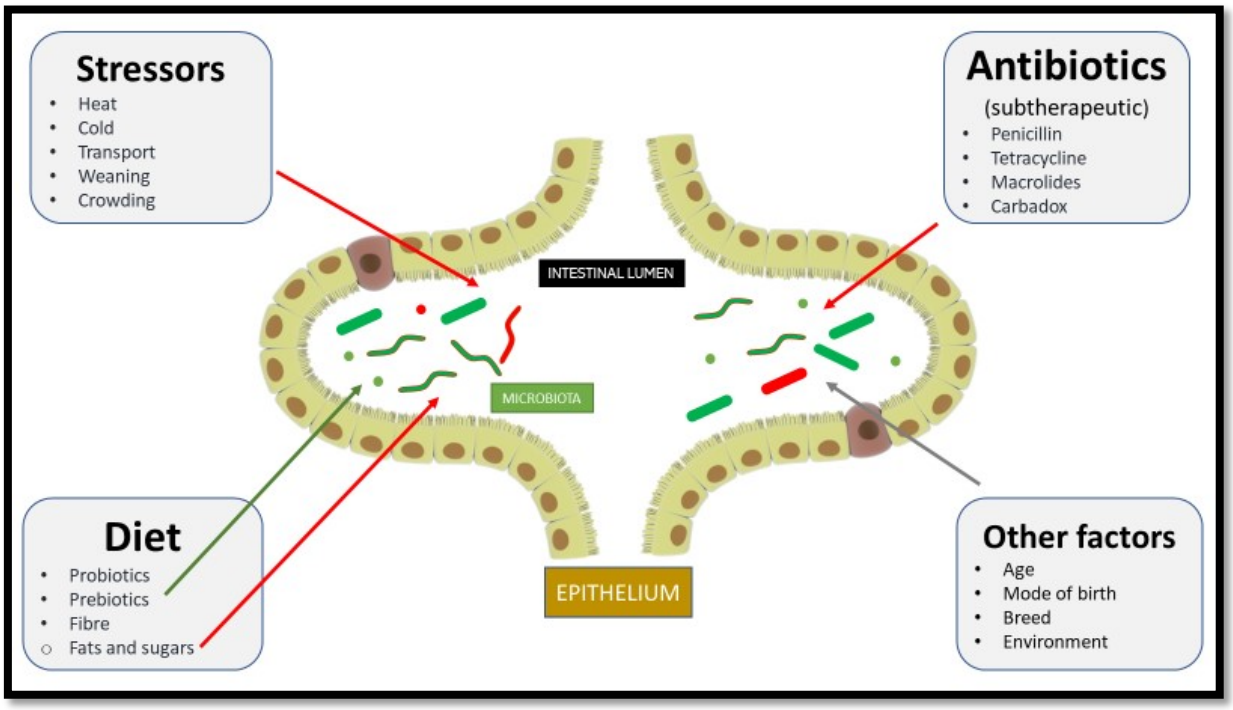

Figure 2.4.1 Factors affecting the gut microbiota in pigs.

The diversity of gut microflora can be affected by a variety of factors. Sub-therapeutic doses of antibiotics used in commercial farming can negatively affect the microbiota. Similarly, different stressors such as high temperature, transportation, weaning, and overcrowding can also change the diversity of the microbiota for the worse. The inclusion of probiotics, prebiotics, and fibre appear to nullify these effects and improve diversity. Other factors, such as the age of the animal, its mode of the birth, breed, and the environment it lives in, can influence the microbiota.
Legend: Potentially pathogenic microbes are depicted in red and beneficial (and other commensals) microbes in green. The red arrows indicate a negative impact and a green arrow a positive impact. The grey arrows are

an indication that different factors can affect the microbiota differently, either positively (e.g., if animals are bred in a healthy, growth-conducive environment) or negatively (e.g., following caesarean birth, devoid of any natural mother's microbiota).

2.5 Effect of diet on the gut microbiome

The diet of the sow affects the piglet microbiota and the fermentation end-products profile (Leblois et al., 2017). Diet significantly impacts gut microbial diversity and is extremely important in maintaining health by preventing the development of dysbiosis, an etiological factor in many chronic diseases (Doré & Blottière, 2015). For dietary nutrients to be efficiently metabolised, a population of healthy GIT microbes is highly important, as this can lead to improved digestion and efficient absorption/utilisation of nutrients *via* the pig gut mucosal membrane. When the GIT immune system, which accounts for approximately 70% of the total immune cell population, is activated in response to a stressor, a diverse set of specialised immune cells and signalling molecules are produced, sometimes at the expense of digestive efficiency (Liao & Nyachoti, 2017). A recent study (Yang et al., 2018b) found that *Ruminococcaceae* spp., which produce SCFAs and *Lactobacillus* spp., which produce lactic acid, have an important role in suppressing swine feed intake, whereas *Prevotella* may have the opposite effect. These authors, therefore, postulated that *Prevotella* may be the keystone bacteria for porcine appetite control (Hui Yang et al., 2018). These results suggest that the pig intestinal microbial community may have a vital role in the host's feeding behaviour; and thus, modulation of gut microbiota can be beneficial for the control of feed intake in the swine industry (Hui Yang et al., 2018). Interestingly, a recent study showed that the largest change in bacterial composition occurs in pigs that are between day 21 and 33 of age, which is the period of time that the animal transitions from a primarily milk-based diet to one containing solid feed. These results were consistent across all examined GIT sites, namely, duodenum, ileum, cecum, and colon (De Rodas et al., 2018).

2.5.1 Dietary fibre (DF)

Dietary fibre is a feed constituent that cannot be digested efficiently by monogastric digestive enzymes. However, it selectively stimulates the growth and activity of one or more bacteria within the GIT, resulting in microbiota-fermented DF in the distal colon. The main products of such bacterial fermentation are short-chain organic acids (SCOA) such as lactate, acetate, propionate, and butyrate. These SCOA assist in the development of the digestive tract by influencing proliferation of gut epithelial cells (Montagne et al., 2003). Further, the acidic properties associated with SCOA act to impede the growth of enteric bacterial pathogens such as *Salmonella*, *E. coli*, and *Clostridia* (Pickard et al., 2017*)*. Additionally, soluble non-starch polysaccharides stimulate the growth of commensal gut microorganisms, which increase SCOA production, thereby lowering the pH in the colon (Bach et al., 2012). Conversely, the inclusion of insoluble non-starch polysaccharides such as pectin, cellulose,

gums, and hemicelluloses in the diet can serve to increase the villus length and delay GIT transit time, thereby allowing a longer period for degradation of fibrous material by microbiota in the colon (Lindberg, 2014).

It is well established that lactobacilli supplementation in neonates aids the early development of stable gut microflora, stimulates the immune system, and prevents diarrhoea (Dowarah et al., 2017). Recently, it was also reported that by including xylanase in pig diet, the faecal and ileal counts of beneficial lactobacilli could be increased while simultaneously reducing the *E. coli* counts (Balasubramanian et al., 2018). The GIT microbial community adapts to variations in the host animal's diet, although the host diet also influences the distribution of microbiota within the GIT (Leser et al., 2000). Specifically, it has been reported that a reduction in *Firmicutes* occurs within the colon of pigs fed with DF consisting of non-starch polysaccharides (Tian et al., 2017). These *Firmicutes* are capable of energy extraction from the diet and store fat in adipose tissue. The *Firmicutes* also act to convert polysaccharides into SCFAs, which can interact through a variety of host mechanisms to increase adipose storage (Stephens et al., 2018). Thus, reduction in their numbers following the feeding of DF consolidates our understanding of the positive effects that these fibres exhibit in controlling obesity in higher mammals such as the pig and humans. In another study, when pigs were fed 'low fat and high fibre' (LF) or 'high fat and low fibre' (HF) diets, the gene copy numbers for *Lactobacilli* spp., *Bifidobacterium* and *Faecalibacterium prausnitzii* were observed to be higher in LF-diet-fed pigs, while HF-diet-fed pigs contained more *Enterobacteriaceae* (Heinritz et al., 2016). The LF diet containing higher amounts of DF was able to stimulate the growth of beneficial bacteria in the microbiota and increase the production of SCFA, especially butyrate. In contrast, the HF diet increased the number of potentially pathogenic organisms. Spurlock and Gabler (2008) presented a review of literature wherein swine were used as a model to study human obesity. Some breeds of swine such as the Ossabaw breed from the United States of America readily become obese in the absence of high-fibre diets (Spurlock & Gabler, 2008).

2.5.2 Dietary copper

In the swine industry, feed is commonly supplemented with copper (Cu) because of its antimicrobial properties and potential to promote growth. However, the nutritional requirement for Cu to swine varies from 5 mg/kg feed in piglets to 20 mg/kg in lactating sows (Committee on Nutrient Requirements of Swine, 2012). However, when weaned piglets were fed 175 mg/kg $CuSO_4$, the populations of lactic acid bacteria, *Lactobacilli* and *Streptococci* in the GIT were reduced (Hojberg et al., 2005). Such high amounts of Cu in the feed can also function to increase the content of unsaturated fatty acids, which can result in softer pork fat (Debski, 2016). High levels of dietary zinc (Zn) and Cu can also serve to decrease the commonly observed spike in plasma cortisol levels on days

9 and 19 when pigs are subjected to an LPS challenge. Further, high concentrations of dietary Zn, and particularly Cu, have been shown to reduce the diversity of ileal microbiota significantly. However, this effect was reversible, which suggests that microbiota diversity was restored following the removal of additional Zn and Cu from the diet (Namkung et al., 2006). Enterococci have been shown to develop resistance to antibiotics such as macrolides and glycopeptides, including vancomycin, following exposure to high Cu concentrations. Such resistant enterococci, which are a part of the *Lactobacillales* order and are quite frequently found in the gut microbiota of mammals including pigs, may get transferred to humans that consume the meat of such animals (Yazdankhah et al., 2014).

2.5.3 Prebiotics and probiotics

Probiotics, a group of microorganisms which 'when administered in adequate amounts, confer a health benefit on the host' (Daniel et al., 2017), can correct the imbalance of microbiota in the GIT and improve the overall health of humans and animals. Introduction of such beneficial microbes can serve to repair and reinforce the numbers of commensal microorganisms within the gut to restore or improve animal resistance to diseases. Simultaneously, probiotics also improve the efficacy of nutrient digestion, absorption, and utilisation with subsequent improvement in production performance (Kenny et al., 2011). However, the positive effects of probiotics in animals are strain-dependent (Maldonado-Galdeano et al., 2010). One study reported that in weaned pigs the lactobacilli counts in the GIT increased while *Clostridia*, *E. coli*, and *Enterobacterium* spp. counts decreased, following administration of probiotic therapy (*Lactobacillus rhamnosus* LPR together with *B. longum* BL999) (Wang & Donovan, 2015).

Lactobacillus, a component of the *Firmicutes* phylum, is a Gram-positive, facultative anaerobe or microaerophilic bacterium that improves feed conversion efficiency in animals. Furthermore, lactobacilli produce lactic acid, which elicits an inhibitory effect against *E. coli* and *Enterobacteria (Li et al., 2015)*. Administration of a cocktail of complex lactobacilli containing *Lactobacillus johnsonii* and *L. mucosae*, previously isolated from healthy pig faeces, was shown to promote a healthy gut by reducing the number of potential entero-pathogens such as *Clostridia* and *E. coli* (Chiang et al., 2015). Similar effects were observed in weaned piglets administered lactic acid bacteria (LAB) complexes containing *Enterococcus faecium* 6H2, *Lactobacillus acidophilus* C3, *Pediococcus pentosaceus* D7, *L. plantarum 1K8* and *L. plantarum 3K2* (Giang et al., 2010). Moreover, the administration of *L. salivarius* UCC118 WT was found to significantly decrease the number of *Spirochaetes* in the GIT of pigs. Also, administration *of L. salivarius* UCC118, which is well studied for its probiotic properties, positively influenced *Firmicutes* genus members, while production of *bacteriocin Abp118* by *L. salivarius* affected Gram-negative microorganisms, even though *Abp118* is not normally active *in vitro* against this group of microorganisms. Hence, this strain has the potential

to significantly affect pig microbiota through a partial bacteriocin-dependent mechanism (Riboulet-Bisson et al., 2012). *Lactobacillus reuteri* is also a probiotic strain that has been shown to alter the abundance of several bacterial taxa, such as *Enterobacteriaceae* including *E. coli*. This lactobacilli strain, which produces reutericyclin, increases the abundance of two strict anaerobes of phylum *Firmicutes*, while production of reuteran affects colonisation with enterotoxic *E. coli* without affecting other dominant members of the faecal microbiota (Yang et al., 2015).

Supplementation of weaning pig diet with probiotics functions is done to compete with pathogenic bacteria for nutrition resulting in competitive exclusion of the harmful bacterial strains (Lallès et al., 2007). The inclusion of specific probiotics, namely, *L. casei* ssp. *casei*, *L. reuteri* and *L. acidophilus,* during the suckling period and fortification of the piglet diet with probiotics and prebiotics during the post-weaning period serves to markedly improve growth rate and bodyweight gain. Higher counts of *Lactobacillus* spp. and lower *E. coli* counts in faeces were also observed in these animals (Wells et al., 2005). Further, in pigs, the diversity of anaerobic bacteria was found to increase from day 13 to day 16 after birth, with detection of dominant anaerobes such as *Eubacterium*, *Fusobacterium* and *Propionibacterium* (Inoue et al., 2005). This was attributed to the introduction of milk replacer from day 14 onwards. The same study also found changes in the intestinal microbiota after the introduction of the weaning diet beginning day 35 after birth.

Prebiotics, 'compounds found within foods which can induce the growth/activity of beneficial micro-organisms' (Hutkins et al., 2016), have been shown to promote the growth of specific groups of commensal GIT microbiota. Numerous metabolites are subsequently produced by these microorganisms, of which the SCFAs are transported across the epithelium by diffusion, a low-affinity transport mechanism such as HCO_3^-/SCFA exchange, a medium-affinity transport mechanism involving monocarboxylate transporter 1 [MCT1], or *via* high-affinity transport mediated by sodium-coupled monocarboxylate transporter 1 (SMCT1 or SLC5A8) into the colon (Sivaprakasam et al., 2016). There is also increased production of interleukin following supplementation of pig diet with prebiotics alone (Çetin et al., 2005; Yin et al., 2008) or in combination with probiotics, known as synbiotics (Krause et al., 2010; Smith & Jones, 1963). Further, lactulose (a prebiotic) supplementation of the feed of weaned piglets, orally challenged with *S. enterica* subspecies *enterica* serovar *Typhimurium,* served to improve the immunoglobulin IgG antibody responses as well as the total serum IgM and IgA levels (Pié et al., 2007). Lactose, which is a major sugar present in milk, acts as a prebiotic and can elicit the development of a highly diverse microbiota in the prenatal GIT of growing animals (Call et al., 2018). Although the mechanisms by which lactulose and other prebiotics affect the immune system are not fully understood, it is postulated that they may act indirectly by altering the indigenous microbiota of the GIT and causing changes in microbial metabolite production (Naqid et al., 2015).

Numerous previous studies have identified *Lactobacillus* as one of the core genera in the GIT of pigs, accounting for approximately 15% of 16S rRNA gene sequences from swine intestinal samples, irrespective of age (Qing et al., 2015). *Lactobacilli* occur in both the proximal and distal regions of the swine digestive tract and begin colonising soon after birth (Delia et al., 2012). Improvement in overall health, growth performance and an increase in the productivity of pig husbandry are some of the key benefits of administration of probiotic lactobacilli (Kenny et al., 2011). The LAB are also capable of suppressing microorganisms that are lethal to host health. The lactic-acid-related trophic chain in LAB is one of the major metabolic pathways in the mammalian gut (Katouli & Wallgren, 2005; Konstantinov et al., 2006b). Increased abundance of *Lactobacillus* spp. in the caecum of pigs directly correlates with high feed efficiency (Stafford et al., 2016). Recent research has also shown that certain strains of lactobacilli, namely *L. reuteri* ZLR003 and *L. salivarius* ZLS006, can increase the average daily weight gain, feed conversion ratio and nitrogen digestibility in growing pigs. They also help to significantly reduce total cholesterol, alanine transferase, aspartate transferase, blood urea nitrogen, and haptoglobin levels in serum (Zhang et al., 2018).

As has been suggested by several studies, administration of lactobacilli to pigs improves meat quality. Administration of *L. plantarum* ZJ316, which is a potential probiotic isolated from faecal samples of piglets to newly weaned pigs had promising results. Most notably, it served to improve several meat texture indices, promoted increased villus height, and appeared to inhibit the growth of opportunistic pathogens (Suo et al., 2012). Moreover, the administration of probiotics containing *L. amylovorus* into post-weaning pigs has been shown to increase monosaturated and polyunsaturated fatty acids in muscles, suggesting potential usefulness of probiotic administration in improving the fatty acid profile of pork (Ross et al., 2012). In addition, analysis of the immune-health-promoting properties elicited by *L. jensenii* TL2937 illustrated that the use of immunobiotic strains as supplemental additives to piglet feed significantly reduced tenderness while improving juiciness and palatability of pork, along with reducing backfat thickness (Suda et al., 2014). In pigs fed a diet high in calcium-phosphorus content, a 1.4-fold increase in lactobacilli was observed in the gastric pars nonglandularis (Mann et al., 2014).

Due to a high level of variation in growth and feed conversion between individual pigs in commercial production systems, it is difficult to measure the impact of probiotics on gut health accurately. To fully elucidate the effect of different variables, large-scale experiments are required; however, to date most of the studies have focused on assessing the effects that feed additives have on representatives of GIT health, including many immunological measures, in more controlled experiments (Table 2.5.1) (Roselli et al., 2017).

The following table 2.5.1 highlights pig studies in which the diet and environmental conditions were manipulated to determine their correlation with changes in the gut microbiota, and subsequent effects on the immune response.

Table 2.1 Pig studies that investigated gut microbiome and immune changes with different diet or environment

Experiment	Outcome	References
Piglets removed from sow and reared on bovine-based milk formula	Develop different microbiomes than littermates reared with the sow	(Inman et al., 2010)
	Differences in mucosal immune system components (rapid recruitment of antigen-presenting-cells [APCs], fewer T_{reg} cells, increased antibody responses)	(Lewis et al., 2012)
Pigs reared indoors and outdoors	Differences in the microbiome between the two groups	(Mulder et al., 2011)
	Differential gene expressions of MHC-dependent antigen-presentation in intestinal mucosa	(Schmidt et al., 2011)

2.5.4 Antibiotics

Feed and water on commercial pig farms are often supplemented with antibiotics to combat bacterial infections or promote growth. Although administration of antibiotics promotes piglet growth, it has a negative effect on the commensal bacterial population as it often leads to increased proportions of pathogenic species. These species inhibit the normal intestinal function (Dibner & Richards, 2005). Specifically, antibiotics such as penicillin, tylosin, chlortetracycline and antimicrobials like sulphamethazine have been shown to affect the composition of the gut microbiome in growing pigs (Allen et al., 2011; Kim et al., 2012; Kim et al., 2016; Looft et al., 2014). Moreover, simultaneous administration of multiple antibiotics, namely, chlortetracycline, sulfamethazine, and penicillin (ASP250), served to markedly increased the proportion of *E. coli* in the lumen and mucosa of the ileum compared to other gut compartments and faeces in pigs. Many of the functional changes within the metagenome were also attributed to an increase in *E. coli* (Looft et al., 2014). Additionally, a decrease in the number of LAB *Streptococcus* organisms, and a simultaneous increase in *Proteobacteria*, specifically in the *Escherichia* population, was observed following administration of ASP250 antibiotics to weaned piglets (Allen et al., 2011; Ichinohe et al., 2011). An additional study reported that short-term administration of low-dose antibiotics in feed caused an increase in the abundance and diversity of antibiotic-resistance genes specific for antibiotics that the animals had not previously been exposed to (Looft et al., 2012).

Furthermore, treatment with amoxicillin (600 mg/kg) was found to increase the abundance of faecal enterobacteria, while decreasing the proportion of LAB and the total bacterial viability as well as the total serum IgM concentrations within the jejunum (Bosi et al., 2011). Recently, it has been shown that therapeutic antibiotic administration alters the composition and metabolism of the microbial communities within the ileum and faeces (Gao et al., 2018). However, the ileal microbiota was found to be more susceptible to change than faecal microbiota. Specifically, *Lactobacillus* and *Bifidobacterium* spp. were found to decrease by an average of 3-fold and 508-fold respectively, in the ileum on days 2 and 13, and by an average of 45-fold and 72-fold, in the faeces on days 7 and 13, respectively. Moreover, the proportion of *Escherichia* and *Shigella* spp. were found to increase by 265-fold in the ileum between days 2 and 13, and by 36-fold in faeces between days 7 and 13 (Gao et al., 2018). This study also suggested that changes in microbiota are closely associated with changes in production of specific microbial metabolites such as SCFAs, which can be used as biomarkers for determining the stability of the gut microbial community. The levels of total SCFA including acetate, propionate, butyrate, and valerate extracted from faeces are regarded as effective indicators of intestinal health. In a recent study, pigs fed conventional diets (which included three types of antibiotics) contained 87 more antibiotic-resistant genes in the GIT compared to pigs fed organic diets, although the gut microbiota of both sets of pigs was not significantly different (DebRoy et al., 2017). Antibiotic-resistance virulence factors were identified in gene families unique to the swine faecal metagenome, exhibiting highest sequence homology to genes in *Bacteroidetes*, *Clostridia,* and *Methanosarcina* (Lamendella et al., 2011).

Due to excessive antibiotic usage in pig production, an increase in the development of immune tolerance has been noted during the early stages of life against a range of pathogenic microbial species. Also, antibiotics have been shown to suppress the systemic immune response in mice (Hill & Artis, 2009). Development of immune tolerance early in life can lead to inefficient immune response later in life when similar pathogens are encountered (Schokker et al., 2014).

2.6 Gut microbiome and intestinal physiology

Microbiota in the animal gut influences many physiological functions necessary for the maintenance of a healthy GIT. Within the GIT lumen, the microbiota assists in converting bile acids into secondary forms *via* de-hydroxylation, dehydrogenation, and deconjugation (Shapiro et al., 2014). The gut microbiota is involved in digestion of otherwise indigestible carbohydrates to produce SCFAs, which protect against epithelial injury, as well as in the synthesis of essential amino acids, regulation of fat metabolism, induction of intestinal motility, improvement in intestinal angiogenesis, and regulation of immune system activation (Hooper, 2004; Hooper & Gordon, 2001). The gut microbiota also serves to protect against colonisation by pathogenic bacteria through the production of anti-

microbial compounds, while also protecting the gut epithelial barrier from harmful effects of pathogens, thereby controlling the overgrowth of bacteria, and simultaneously reducing the susceptibility of pigs to enteric infections (Frick & Autenrieth, 2013).

2.7 Impact of the host on gut microbiome

The relationship between the microbiome and its mammalian host is one of the longest surviving symbioses, dating back to the beginning of multicellular life (R. E.; Ley et al.). The evolution of the microbiota within the host is driven by the need for each species to compete and survive within the host; natural selection alone will not make the microbiota useful to the host (Carey et al., 2016). At the same time, hosts, under natural selection, appear to select for organisms beneficial to them, and hence, the microbiota can be considered an ecosystem held on an ever-evolving leash by the host. The microbes are predictably controlled by the host, as there is a single host but many microbes. Thus, the host can influence the entire microbiome more readily while simultaneously benefiting from its components. Evolutionary theory has predicted that host-to-microbe effects are of larger importance for microbiome form and function. The host exerts control over the microbiota through immigration, compartmentalisation, monitoring and targeting (Foster et al., 2017). However, a recent study has also suggested that bacterial biodiversity within the pig GIT may be influenced by the genetic build-up of the host animal (Lu et al., 2018).

2.7.1 Stressors

Two-day-old piglets host a group of bacteria comprising primarily *L. amylovorus*, *L. reuteri*, *E. coli,* and *L. acidophilus.* Specifically, ileal samples of neonates and non-weaned pigs contain approximately 7×10^8 *L. amylovorus* and *L. reuteri* cells per gram of intestinal content (Konstantinov & Smidt, 2006). Multiple studies have shown that the composition of the GIT microbiota changes when the host animal encounters stressors.

2.7.1.1 Weaning

At the weaning stage, the dietary changes constitute a major stressor and cause changes to the gut microbiota. Due to this weaning stress, the lactobacilli community of the pig ileum undergoes significant change (Janczyk et al., 2007). After weaning, the quantities of *L. amylovorus* and *L. reuter*i decrease significantly to less than 10^3 within the ileum; and thereafter *Clostridia* and *E. coli* appear along with changes in the composition and metabolic activities of the predominant microbiota.

In a study of porcine faecal microbiota, samples from 15 commercial pigs were collected during the pre-weaning and post-weaning periods (Pajarillo et al., 2014b). The pre-weaning microbial community consisted primarily of the phyla *Firmicutes* (54%) > *Bacteroidetes* (38.7%) > *Proteobacteria* (4.2%) > *Spirochetes* (0.7%) > *Tenericutes* (0.2%). Although the same major phyla

prevailed post-weaning, the relative proportions varied, with *Bacteroidetes* (59.6%) > *Firmicutes* (35.8%) > *Spirochetes* (2.0 %) > *Proteobacteria* (1%) and *Tenericutes* (1%). Thus, *Firmicutes* and *Bacteroidetes* accounted for more than 90% of the faecal bacterial community during both the pre-weaning and post-weaning stages. However, although *Firmicutes* accounted for the initial prominent phyla, a shift towards *Bacteroidetes* was observed after weaning. Among the genera, *Bacteroides*, *Blautia*, *Dorea*, *Escherichia*, and *Fusobacterium* were determined to be most abundant pre-weaning; however, *Prevotella* and *Clostridia* increased in the post-weaning pig with a corresponding decrease in *Bacteroides* (Pajarillo et al., 2014b).

Mechanistically, during weaning, the piglet diet switches from easily digestible liquid milk to a less easily digestible, more complex solid feed. This change has significant consequences on the microbiota and the physiology of the GIT, which is still not fully mature. Other changes such as inflammatory response pathways are activated at this time in addition to hormonal changes, gastric motility reduction, small intestine atrophy, reduced height of villi, reduced absorption of nutrients, fluids, and electrolytes, and increased permeability to antigens and toxins (Lallès et al., 2007). It is possible that at least some, or all of the above changes are linked to modifications in piglet intestinal microbiota since it has long been established that dietary change is responsible for the etiology of post-weaning diarrhoea and enteric infections (Bomba et al., 2014).

2.7.1.2 Other stresses

In addition, growth of pathogenic *E. coli* occurs in pigs subjected to even mild handling stress (Dowd et al., 2007). The effect of stress on the microbiome is now recognised as a new field of study, called microbial endocrinology. The authors hypothesised a mechanism involving modulation of the transcription of virulence genes in a pathogen specifically *via* blocking with adrenergic antagonists (Freestone et al., 2008). A key cascade of reactions occurs in the hypothalamic-pituitary-adrenal (HPA) axis in response to stress conditions resulting in release of glucocorticoids from the adrenal cortex (Sudo et al., 2004). For example, when GF mice are subjected to restraint stress, they exhibit elevated adrenocorticotropic hormone and corticosterone levels (Lupien et al., 2009).

Interestingly, this process can be largely reversed by just one commensal bacterium, namely, *Bifidobacterium infantis* (O'Mahony et al., 2005). A crucial observation in this study was that the reversal of the HPA axis set-point was influenced by these commensal bacteria even in adults; however, this occurred only if the colonisation had taken place before the host reached 6 weeks of age and not in animals where colonisation had occurred after 14 weeks of age. It therefore appears that early-life signals elicited by indigenous bacteria seem to exercise a long-lasting programming effect on the HPA axis to enable the host responses to better cope with stressful situations in later life.

2.7.2 Infection and inflammation

Salmonella enterica is a pathogen that can induce substantial changes in the composition of the intestinal microbiota. For instance, disturbances in the porcine colon and cecal microbiota occur when challenged with *S. enterica* (Borewicz et al., 2015). The microbiota profiles in the *S. enterica*-challenged pigs were similar to each other yet varied markedly from the non-challenged controls. Statistically significant increases were observed in proportions of *Anaerobacter*, *Prevotella*, *Barnesiella*, *Pediococcus*, *Sporacetigenium*, *Turicibacter*, *Catenibacterium*, *Xylanibacter* and *Pseudobutyrivibrio* in the challenged pigs. Furthermore, in mice studies, inflammation has been shown to be induced in response to bacterial infection by species such as *Citrobacter rodentium* or *S. enterica* subspecies *enterica* serovar *Typhimurium*, or by chemical inducers such as dextran sulphate sodium (DSS), or in response to genetic deficiencies such as in the interleukin-10-deficient (IL-$10^{-/-}$) mouse model (Drumo et al., 2016; Hoffmann et al., 2009; Stecher et al., 2007). These factors function to change the composition of the intestinal microbiota by reducing both the quantity and diversity of resident intestinal bacteria. Similarly, the *Enterobacteriaceae* count has been shown to increase in mice following the induction of colitis by treatment with DSS (Lupp et al., 2007). Enteric infections caused by pathogens such as the porcine epidemic diarrhoea viruses, *Brachyspira hampsonii* and *Lawsonia intracellularis* also influence gut microbial composition and cause dysbiosis (Borewicz et al., 2015; Koh et al., 2015; Niederwerder, 2017). These viruses cause substantial reduction in the pig microbiota diversity to one dominated by the bacterial phylum *Fusobacteria*. In contrast, control pigs that were not exposed to the virus exhibited a rich microbial diversity with *Firmicutes* in the majority (Koh et al., 2015).

2.8 Impact of the gut microbiome on the host immune system

The host animal is subjected to many internal and external stresses during its lifetime. In pigs, defects in genes encoding various innate and adaptive immune cells result in dysbiosis and can induce development of pathogenic disorders of the GIT such as IBD (Knights et al., 2013). Further, external pressures including infections or exposure to antibiotics can introduce major disturbances to the microbiota, as is observed in IBD development (Keeney et al., 2014); which is now believed to occur as the result of disruption in communication between the host and intestinal microbiota. However, the molecular mechanism responsible for this communication breakdown is not fully understood. Nevertheless, it has been hypothesised to involve genetic susceptibility of the epithelial barrier and innate immunity, both of which are vital components in the host–microbiota relationship (Jostins, 2012).

Many approaches have been examined, including the use of GF animals, to demonstrate the critical link between gut microbiota and the host innate and acquired immune systems. Since GF animals are

reared in sterile conditions from birth and are not exposed to microbes during their life, changes that occur in the body on exposure to microorganisms from the external environment can be accurately monitored. The microbiota is known to influence not only the local intestinal immune system, but also systemic immunity (Smith et al., 2007; Wu & Wu, 2012b).

Gut bacteria monitor and regulate the immune system in a way that allows the immune system to distinguish between commensal microbes and pathogenic bacteria. A healthy GIT results from positive interactions between the microbiome and host. In this context, the epithelial barrier function and the mucosal immune system are vital components (Burkey et al., 2009; Pluske et al., 2018). The innate immune system and the gut are interdependent and therefore can be influenced by a system of interactions (Thaiss et al., 2014). The innate immune system relays signals to the host animal for functional adaption at the tissue level including influencing the composition and functional capabilities of the microbiota (Levy et al., 2015). It also acts to promote the growth of beneficial species to help maintain a stable community of microbes. Further, during an intestinal infection, fucosylated proteins are shed into the lumen of the intestine, which serve as an energy source for the GIT microbiota (Pickard et al., 2014). Thus, the innate immune system diverts its resources to aid the microbiota in times of perturbations of the intestinal ecosystem. For example, in *Yersinia enterocolitica* infection, signalling from TLR1 is necessary to preserve the composition of the commensal microbial community (Kamdar et al., 2016).

Antigen presenting cells (APCs) help protect the host against infections, while simultaneously maintaining immune tolerance to the commensal gut microbiota. The dendritic cells (DCs) of Peyer's patches in the gut wall generate high levels of IL-10 compared to DCs in the spleen when subjected to similar conditions (Iwasaki & Kelsall, 1999). Moreover, in GF animals, a reduction in the number of intestinal DCs is observed; however, not in splenic DCs. *Escherichia coli* (10^7 CFU of O83:K24: H31 *E. coli* and O86:K24: H31 *E. coli*) alone are enough to elicit a DC response in the GIT. In addition, fewer intestinal and systemic macrophages are observed in GF pigs resulting in reduced chemotaxis, phagocytosis, and microbiocidal activities (Haverson et al., 2007; Zhang et al., 2008).

Commensal bacteria function to regulate the immune response in host cells, primarily through the inflammatory cascade *via* the nuclear factor-kappa B (NF-κB) pathway (Neish et al., 2000). NF-κB regulates transcription by translocating to the nucleus and stimulating the production of inflammatory cytokines and recruitment of immune cells. This occurs only when NF-κB is unbound from IκB (inhibitor of κB). However, resident bacteria in the gut inhibit the NF-κB–IκB dissociation thereby halting the cascade since NF-κB is no longer able to enter the nucleus (Neish et al., 2000). Thus, commensal microbes and their products may be useful in therapeutics for inflammatory-based diseases such as IBD.

It is well-established that gut commensal microbes, upon colonising the neonatal mammals, activate the systemic immune system. This is achieved primarily by increasing the number of circulating antimicrobial-specific antibodies (Cebra, 1999). Since the food ingested by animals contains intact molecules that can retain their antimicrobial activity even after irradiation or autoclaving, it is difficult to generate antigen-naïve pigs. Moreover, the formation and development of the mucosal immune system in GF animals is very limited as compared to that in conventional animals that possess hypoplastic Peyer's patches. The GF animals also lack T_{reg} cells, and express minimal levels of heat shock proteins (HSPs) (Liu et al., 2014). The intraepithelial T lymphocytes in the GIT play a vital role in the defence system of the host. As compared to conventional pigs, GF pigs only have a fraction (approximately 35%) of the normal level of T-lymphocytes in the jejunum and ileum (Inoue et al., 2005). When the balance between host immunity and microbiota is disrupted, dysbiosis is created, which is a vital step in the progression of diseases such as diarrhoea and swine dysentery (Tamboli Cp Fau - Neut et al., 2004). However, when GF pigs are colonised with even a limited, defined microbiota, most of the functional immune system components including APCs, T-cells, and B-cells develop similar to that in conventional pigs (Inman et al., 2012; Sun et al., 1998).

Additionally, different strains of lactobacilli possess varied capacities to modulate the expression of host immune pathways. The presence of many lactobacilli species can lead to greater crosstalk between these microorganisms and the host immune cells. For proper development and function of the immune system to occur, communication between the microbiota and intestinal cells is pivotal. In piglets challenged with *E. coli* K88ac, one probiotic strain, *L. fermentum*, enhanced T-cell differentiation, increased pro-inflammatory cytokines as well as the proportion of $CD4^+$ lymphocytes in the ileum (A. Wang et al., 2009; A. N. Wang et al., 2009). Several studies have utilised different *Lactobacillus* spp. and strains in pigs to demonstrate their effects on the intestinal microbial communities and their beneficial activities following ETEC, *Salmonella* or rotavirus challenges. The specific *Lactobacillus* spp. studied include *L. plantarum*, *L. amylovorus* DSM 16698, and *L. reuteri* or *Lactobacillus rhamnosus* GG. The strain *L. amylovorus* DSM 16698 was employed in an experiment conducted on pig intestinal explants, where ETEC induced a higher level of TLR4, P-IKKα, P-IκBα, and P-p65; while *L. amylovorus* functioned to eliminate all these variations and simultaneously upregulated the expression of TLR4 regulators Tollip and IRAK-M (Roselli et al., 2017).

Pigs are the only animals that are susceptible to HRV (human rotavirus)-initiated diarrhoea. A study was performed with neonatal gnotobiotic pigs (born from near-term sows *via* CS, lacking a well-established microbiota) inoculated orally with probiotics, namely, *Lactobacillus rhamnosus* strain GG and *Bifidobacterium animalis lactis* Bb12, which are the primary bacterial species found in the gut of breastfed infants. This study sought to determine the impact that an attenuated (Att) HRV Wa strain vaccine had on B-cell responses. The Att-HRV-vaccinated piglets colonised with probiotics were

found to exhibit considerably lower faecal scores and reduced HRV shedding titres as compared to the uncolonised, Att-HRV-vaccinated piglets. Further, a reduction in HRV-associated diarrhoea was noted, which was correlated with the presence of a high number of intestinal IgA HRV antibodies and intestinal HRV-specific IgA antibody-secreting cells in probiotic-treated piglets compared to uncolonised, vaccinated pigs (Kandasamy et al., 2014). Moreover, in another study, elevated levels of IL6 and IL10 were observed in ileal mononuclear cells (Zhang et al., 2014) in gnotobiotic pigs inoculated with gut microbiota from healthy human infants (*Firmicutes* and *Proteobacteria* accounting for approximately 98%). Colonisation with this microbiota also promoted development of the neonatal immune system by substantially enhancing IFN-γ producing T-cell responses and by reducing T_{reg} cell differentiation and their associated cytokine production in the Att-HRV-vaccinated pigs. Wu and Wu (2012) have further summarised many additional studies in GF rats, mice, and humans, that highlight the importance of a healthy microbiota for proper development and functioning of the acquired immune system in mammals (Wu & Wu, 2012b).

Many studies have been reported on colonisation of gnotobiotic pigs with intestinal microbiota. In a 2012 study, 24 gnotobiotic pigs were inoculated with Bristol microbiota (a novel simple porcine microbiota). These pigs exhibited no significant health problems and the Bristol microbiota successfully induced upregulation in the expression of serum immunoglobulins IgA and IgM. However, the level of IgG2 was much lower than in conventional pigs that have access to colostrum, which suggests a maternal influence on IgG2 production. Thus, the Bristol microbiota may be used to improve the formation, and subsequent development, of the intestinal mucosa and general immune system in neonatal pigs (Laycock et al., 2012).

Toll-like receptors are a class of proteins that are present in the cells of lymphoid and non-lymphoid tissues, but their expression levels differ in their respective locations. The TLRs play a crucial role in the activation of innate immunity and the induction of adaptive immunity. They are necessary for the recognition of PAMPs in pathogens of numerous animals, including pigs and humans (Adrian et al., 2000; Hirschfeld et al., 2000). Initially, scientists cloned the TLR genes as homologues of the *Drosophila* Toll gene, which had been recognised as an encoding receptor for Spätzle. The dorsal and ventral orientation of the embryo depends mainly on the signal transduction between Toll and Spätzle (Uenishi & Shinkai, 2009). Later, it was found that Toll and its signalling pathway were involved in secretion of the antifungal peptide drosomycin in *Drosophila* (Lemaitre et al., 2012). Soon after the involvement of Toll in the immune response in *Drosophila* was explained, the human Toll homologue gene TLR4 was cloned, and after the cloning of this TLR4, it was shown that TLR4 was a receptor molecule that responded to LPS (Hoshino et al., 2016; Qureshi et al., 1999; Ruslan et al., 1997). The signalling adaptors myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-protein-inducing interferon-β (TRIF) are recruited by TLRs to ligate to

signalling molecules *via* nuclear factor-κB (NF-κB). This ligation causes an increase in the production of various pro-inflammatory cytokines (i.e., IL1β, IL6, and TNF-α). It activates the classic pro-inflammatory signalling cascade, contributing to defence against pathogens in the host (Chunlong et al., 2015; Tak & Firestein, 2001).

Even today, most models tested are *in vitro* or murine models *in vivo* (Li et al., 2013). The expression and regulation of TLR functions differ between mice and humans, as has been established by the different expression patterns of TLR4 in monocytes and macrophages after LPS treatment (Bryant & Monie, 2012; Vaure & Liu, 2014). Hence, it can be inferred that the murine model may not be adequate for studies of human TLRs and highlights the need for alternative animal models. Although porcine, human, and murine TLR4 promoter sequences are similar, murine TLR4 promoter exhibits significant differences in the regulation of gene expression. In contrast, the porcine TLR4 promoter shares more standard features with the human TLR4 promoter (Thomas et al., 2006). It has been found that bacterial pathogens may express TIR domains to manipulate TLR signalling and to limit the NF-κB expression and hence decrease the innate immune response (Patterson & Werling, 2013). Given a substantial homology of swine TLRs with their human counterparts (80% for TLR1, 3, and 10; 85% for TLR7; and 73% for TLR8), shared TLR domains were found in pigs, post cDNA analysis and sequencing (Sang et al., 2008; Shinkai et al., 2006). More research into porcine TLR expression and function is needed to evaluate if the pig model can effectively mimic and predict human conditions and outcomes. Furthermore, when there occur polymorphisms and differences in the production of immune molecules such as TLRs, it has a significant effect on the animal's response to pathogens, and this is closely associated with disease resistance and susceptibility (Lazarus et al., 2002).

2.8.1 Characteristics, distribution and function of TLRs

Out of the half a dozen PRRs known in pigs and humans, TLRs are the most extensively researched PRRs. Toll-like receptors can either be on the cell-surface or intracellular. The TLRs on the cell-surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) can recognise non-nucleic acid molecules, while intracellular TLRs (TLR3, TLR7, TLR8 and TLR9) can detect nucleic acids. These TLR proteins can be quite large in size. In pigs, the sizes of intracellular and cell surface TLRs ranges from 905 to 1050 and from 785 to 856 amino acids, respectively. Ten TLR genes (TLR1 to TLR10) have already been described and explicitly mapped to seven genomic regions on chromosomes (chr) 1, 8, 10, 13, 15 and X. The TLRs 1,6 and 10 are clustered within a 56-kb interval on chr 8, while TLRs 7 and 8 have been mapped within a 62-kb region on chr X (Shinkai; et al., 2006). The porcine intestinal epithelial cell was found to express TLR2, TLR4, and TLR9 (Burkey et al., 2009; Wilson et al., 2007). *In vivo*, TLR2 was found to be expressed in the porcine thymus, spleen, IPPs, mesenteric lymph nodes (MLNs), and palatine tonsil. It was also expressed to a lower extent on columnar membranous cells and innate

immune cells such as macrophages and epithelial cells but not on peripheral blood lymphocytes (Álvarez et al., 2008; Masanori et al., 2006). In pigs, the highest levels of TLR4 mRNA have been detected by PCR in the colon and spleen. However, lungs, small intestine, liver, kidneys, thymus, lymph nodes, brain (hypothalamus, hippocampus, cortex, and cerebellum), tonsils, ovary, and cornea have also shown the presence of TLR4 mRNA.

The expression levels of most TLRs are higher in splenic MNCs/DCs compared to ileal MNCs/DCs in conventional pigs, regardless of the age (either new-born, young or adult). The TLR expression levels are higher in the spleen than the small intestine, except for TLR5, for which similar expression levels were observed at both places. The lower TLR mRNA expression in PP DCs might be one of the mechanisms exploited by PP DCs to regulate immune responses to commensal bacterial inducing tolerogenic state (Davies et al., 2010). The upregulated TLR expression in splenic MNCs of adult pigs than young pigs, in contrast, may represent the more mature state of the immune system of the former group. Also, studies have shown that flagellin-induced activation of TLR5 on dendritic cells elicited the production of the cytokine IL22 to induce a protective gene expression program in intestinal epithelial cells (Zhang, 2014). This may indicate that TLR5 signalling is critical to induce antiviral effects in the gut and may contribute to the different expression profile of TLR5 compared to other TLRs. Of all TLRs recognising numerous microbial, synthetic, and endogenous ligands, only TLR5 appears to be specific for a single protein moiety, flagellin of invasive bacteria. This specificity may require the increased TLR5 expression in the gut mucosa versus spleen (Zhang, 2014).

TLRs are membrane glycoproteins capable of recognising extracellular or endosomal/endolysosomal PAMPs through leucine-rich repeats (LRRs). These TLRs can form either homodimers (TLR3-5, 7, 9) or heterodimers (TLR1 and TLR6 with TLR2) or complexes with other factors (TLR4 forming a complex with myeloid differentiation factor 2 [MD2] and CD14) for sensing pathogens. Once the ligand has bound, TLRs initiate a downstream signalling cascade of reactions *via* a cytoplasmic Toll/interleukin-1 receptor (TIR) domain (Fig. 2.8.1), which depends on the pathogen to be recognised and its location (Kumar et al., 2011). The LPS, lipopeptides or flagellins, which are extracellular PAMPs from bacteria, fungi, and protozoa, are recognised by TLR1, 2, 4, 5 and 6, which are expressed on the cell surface followed by downstream signalling through the MyD88, which translocate NF-κB to the nucleus and enable inflammatory cytokines production. TLR4 is also capable of signalling through TIR-domain-containing adapter-inducing interferon-b (TRIF) and the interferon regulatory factors (IRF) 3/7, inducing type I interferon (IFN) production to combat pathogens. Similarly, TLR10 is expressed on both the cell surface and within the cell inside epithelial cells. In this case, too, signalling is thought to involve MyD88. Regan et al. (2013) observed involvement of TLR10 in sensing and responding to the cell-invasive pathogen *Listeria monocytogenes* (Regan et al., 2013). Within the TLR family members, TLR2 and TLR4 can recognise some viral-envelope proteins (Lester & Li, 2014).

Classically, TLR3, 7, 8, and 9 are expressed within the cell and mainly recognise nucleic acids from viruses and intracellular bacteria in endosomes or endolysosomes. Signalling through TLR3 involves the TRIF and IRF3/7 pathway inducing type-I IFNs, while TLR7 and 9 signal *via* MyD88, activating further downstream IRF7, as well as NF-κB to induce the production of both type-I IFNs and pro-inflammatory cytokines (Kumar et al., 2011). TLR3, TLR7, and TLR10 get distributed in the cytoplasm, remaining stable in the endoplasmic reticulum (ER), from which they are transported to endosomes to bind with ligands (Taro & Shizuo, 2010). The TLR7 and TLR8 can recognise single-stranded RNA (ssRNA) sequences of RNA viruses (Heil et al., 2004), and TLR3 is involved in recognition of ssRNA and double-stranded RNA (dsRNA) viruses (Guillot et al., 2005). Alves et al. (2007) studied the role of TLR7 and MyD88 in porcine DCs by lentiviral-mediated RNA interference to reduce the expression of TLR7 and MyD88. In both knockdown models, IL-6 production was significantly decreased after stimulation with the TLR7-ligand imiquimod (R837) (Alves et al., 2007). Swine TLR7 and 8 have been found within the cells and mainly in the endoplasmic reticulum. Their stimulatory activity was analysed in transfected Cos-7 and HEK-293 cells using an NF-κB reporter assay. In contrast to humans, imiquimod could activate not only TLR7 but also TLR8 in pigs, showing altered receptor specificity across species (Zhu et al., 2008).

The team of Calzada-Nova et al (2010) found that when plasmacytoid dendritic cells (pDCs) were stimulated with both imiquimod and the TLR9 ligand, there was an enhanced production of various cytokines (IFN-a, IFN-c, IL-2, IL-6, IL-8, IL-12, and TNF-a) after CpG ODN. The CpG ODN additionally increased the expression of chemokine receptors on monocyte-derived DCs (MoDCs) and blood DCs (bDCs) also altered the general DC morphology of cultured sorted pDCs as compared to unstimulated cultures (Calzada-Nova et al., 2010). A detailed study including different CpG ODNs revealed that the three classes of CpG ODN (A–C) induce various cytokines. All three CpG ODN classes trigger upregulation of the IFN-c inducible protein 10 (IP-10), which suggests its use as a biomarker for TLR9 immune activity induced by CpG ODNs in pigs (Dar et al., 2010).

2.8.2 The multi-morphology of TLRs

The TLRs on the cell surface are highly polymorphic, especially within the ectodomains that recognise the pathogens, thus allowing the host to widen the scope and variety of molecules that it can recognise (Clop et al., 2016). Single-nucleotide polymorphism (SNP) studies carried out for the determination of the genetic diversity of the TLRs not only identified marked differences between wild boars and domestic pigs but also between different pig breeds, with several clusters of variation (Bergman et al., 2010). Also, SNP studies found 'hotspots' of variation in various functional domains. Many SNPs were in the LRR domain, but these SNPs were biased in the analysed animals. Hence the breeding did not seem to impact the heterogeneity of TLRs (Shinkai; et al., 2006). Together with

these genetic studies, swine TLR expression patterns and function were analysed. In pigs, TLR4 is located on *Sus scrofa* 1 (SSC1) V10.2 (289,776,058 bp to 289,785,087 bp) gene. The genomic structure of swine TLR4 has now been identified, and the distribution of SNPs for five TLRs in pigs has been exhaustively described (Shinkai; et al., 2006; Thomas et al., 2006). For TLR4, 13 single nucleotide polymorphisms (SNPs) were widely distributed in 11 pig breeds, seven of which were non-synonymous. Out of the 34 SNPs identified in TLR4 using pigs of European commercial breeds and some traditional breeds (*n* = 259), 17 SNPs were in the non-coding regions and 17 SNPs were found in the coding regions (Palermo et al., 2009). Polymorphisms in the TLR4 gene have also been identified as potential genetic markers for disease susceptibility in pigs (Uenishi & Shinkai, 2009).

2.8.2.1 Toll-like receptor 4 (TLR4)

The TLR4 specifically recognises LPS and lipoteichoic acid (structurally similar to LPS) of Gram-negative and Gram-positive bacterial cell walls (Kopp & Medzhitov, 2003). The TLR4 is the primary receptor for the LPS component of Gram-negative bacteria. Recently accumulated data has revealed that the lack of, or mutations in TLR4 are capable of crippling immune responses to pathogens that produce these ligands, and hence implying that polymorphisms in the coding sequence or in the promoter of TLR4 can underlie different resistance/susceptibility patterns to infectious diseases. It recognises structures from mycobacteria, fungi, parasites, and viruses, as well as endogenous molecules such as heat-shock proteins, fibrinogen, fibronectin or defensin (Kumar et al., 2009). Mice deficient in TLR4 were found to be LPS hypo-responsive (Takeuchi & Akira, 2009). LPS detection requires other molecules along with TLR4. The LPS binds to LPS-binding protein (LBP), which is present in the serum, and this LPS–LBP complex is consequently recognised by CD14, which transfers it to a receptor complex formed by TLR4 and MD2, undergoing oligomerisation and activating intracellular signalling pathways (Miyake, 2004).

When the structure of TLR4–MD2 in complex with LPS was studied, it was found that five of the six lipid chains of LPS bind to the hydrophobic pocket of MD2, and the remaining lipid chain that is exposed to the surface on MD2 associates with TLR4 (Kim et al., 2007). The positively charged residues of TLR4 interact with the phosphate groups. The formation that results from this, a receptor multimer composed of two copies of the TLR4-MD2-LPS complex, initiates signal transduction by recruiting intracellular adaptor molecules. LPS-binding protein (LBP) and CD14 are some of the additional proteins that are also involved in LPS binding (Akashi-Takamura & Miyake, 2008). The LBP, as the name suggests, binds to LPS, and CD14 is a glycosylphosphatidylinositol-linked, leucine-rich repeat-containing protein binding to LBP, delivering the LPS-LBP complex to the TLR4-MD2 complex.

The TLR4 is also expressed in M cells and gut-associated lymphoid tissues (Vaure & Liu, 2014). The porcine neonatal intestinal epitheliocytes (PIE) consistently produce TLR4 and MD-2 mRNAs, with a

high level of TLR4 protein being detected. Exposure of PIEs to LPS enhances the expression of TLR4, and consequently pro-inflammatory cytokines, and chemokines (Moue et al., 2008). Among the 10 swine TLR genes, TLR4 has the highest number of reported genotypes to phenotype associations. Missense SNPs within TLR4 have been linked to the expressions of IFNG, TNFA, IL-2, IL-4 and IL-6 in PBMC, and also to the presence of lesions in the lungs (Yang et al., 2012).

Figure 2.8.1 below depicts the different components of pathogens which can be detected by TLR4. The TLR4 is the only TLR that utilises all four adaptors and activates both MyD88- and TRIF-dependent pathways.

Figure 2.8.1 TLR4 intracellular signalling pathways.

TLR signalling is triggered by ligand-induced dimerisation of the receptors. TIR domains of TLR4 recruit TIR domain-containing adaptor proteins MyD88 and MAL (MyD88-dependent pathway) or TRIF and TRAM (MyD88-independent pathway). The MyD88-dependent pathway comprises the recruitment and activation of IRAKs (IRAK1, IRAK2, and IRAK4) and TRAF6 that induces TAK1 activation. TAK1 leads to MAP kinase- (MKK-) mediated activation of MAPKs (p38, JNK, and ERK1/2) and activation of the IKK complex. The MAPKs and IKK complex induces activation and translocation in the nucleus of transcription factors such as NF-κB and AP-1. The myD88-independent pathway involves TRIF and TRAM adaptor proteins and, *via* TRAF3, the recruitment of TBK1/IKKε, followed by the activation and translocation in the nucleus of the transcription factor IRF3. The MyD88-dependent pathway induces production of proinflammatory cytokines, and the MyD88-independent pathway induces the production of type-I interferons.

In weaned pigs, diarrhoea and oedema disease are the infectious diseases essentially causing most casualties, leading to enormous economic losses to the swine industry. The enteropathogenic *Escherichia coli* (EPEC) is the main pathogenic factor of these diseases, resulting in intestinal diseases through the release of endotoxin LPS. The TLR4 was the most strongly expressed TLR in the intestinal cells obtained from neonatal pigs (Moue et al., 2008). This can be attributed to the high incidence of

inflammation linked with weaning. Traditionally, TLR4 is known to detect Gram-negative bacteria. Still, the latest studies have identified other molecules that can bind to and activate TLR4, such as extracellular heat shock proteins like Hsp72 and Hsp90 (Chase et al., 2007; Poynter Sue et al., 2009). When released from cells, these HSPs may induce inflammation in a TLR4 and NF-κB-dependent mechanism. Increased circulating Hsp72 has been found in pathological conditions including renal disease, hypertension, atherosclerosis, and sickle cell anaemia (Asea, 2007).

Different researchers have found that piglets at different ages have various sensitivities to pathogens. Piglets within one week of age are most likely to get acute diarrhoeal diseases such as yellow dysentery (caused by pathogenic *E. coli* K88) and white scour, which is very common for piglets of age 10–30 days and piglets at age 35 days of weaning time. These animals get easily infected by *E. coli* F18, causing diarrhoea (Pan et al., 2011). The immune system of the neonate is less developed than that of the adult, and this may extend to TLR expression (Pott et al., 2012). There are two crucial periods in the development of the immune system – one immediately after birth and one after weaning. In the first period, neonates are exposed to non-sterile environments. In the later stage, the organism undergoes extensive exposure to new antigens due to the introduction of solid food and non-milk-based diets (Bailey et al., 2005).

Additionally, GF animals are used to provide a comparative control to define how the microbiota and diet affect the developing immune system (Lee & Mazmanian, 2010). The role of TLRs in porcine bacterial diseases was analysed in *Salmonella* and hog cholera vaccines. Scientists (Burkey et al., 2009) found that pigs infected with *Salmonella enterica* subspecies *enterica* serovar *Typhimurium* (commonly called *Salmonella Typhimurium*) showed increased TLR2 and TLR4 expression 24 and 48 h later, *in vivo*. *In vitro*, the porcine jejunum epithelial cell line IPEC-J2 showed increased expression of TLR1, 2, 3, 4, 6, and 9 but the porcine ileum cell line IPI-21 expressed more TLR8 and 10 after stimulation with LPS from *Salmonella typhimurium* (Hüsser et al., 2011). One group used a plasmid encoding the pig IL-6 gene and 11 CpG motifs in combination with chitosan nanoparticles as an adjuvant to boost the porcine immune system against an attenuated classical hog cholera vaccine (Li et al., 2011). Inclusion of the adjuvant plasmid was shown to increase T-cell frequency, amount of antibody, as well as the serum levels of IL-2, IL-6, and IFN-c. Thus, they showed a possible role for TLR ligands as adjuvants in vaccines against pig bacterial diseases. TLR expression has also been assessed in various types of cells, such as alveolar macrophages, in response to many other important porcine bacterial pathogens, including *Streptococcus suis* (de Greeff et al., 2010). Fungal pathogens are also able to manipulate TLR signalling (Seeboth et al., 2012). T-2, a fungal toxin, was shown to decrease the production of the inflammatory mediators IL-1b, TNF-a, and nitric oxide (NO) in PAMs in response to LPS (*via* TLR4) and synthetic diacylated lipoprotein FSL-1 (*via* TLR2/6). This reduced pro-inflammatory response was associated with a decline of TLR mRNA expression. Interestingly, the

activation of TLR7 by ssRNA was not modulated by T-2 toxin pre-treatment. These data suggest that fungal pathogens might decrease pattern recognition of pathogens and therefore interfere with the initiation of an effective immune response (Seeboth et al., 2012).

Consumption of dietary fats increases levels of LPS in the blood, with accompanying changes in gut microbiota. Lipopolysaccharide, either from an exogenous source or endogenously produced in the gut, is known to induce glucose intolerance mainly by reducing hepatic insulin action (Cani et al., 2007). Antibiotics, which can change the gut microbiota in diet-induced obese mice, reduced blood LPS and partially restored glucose tolerance (Caricilli et al., 2011). Also, genetic targeting of either TLR4 or CD14 can virtually eliminate the impact of changes in the gut microbiota and the impact of increased LPS on the generation of the insulin-resistant phenotype (Cani et al., 2007; Caricilli et al., 2011). Hence, it has been proposed that changes in the gut microbiota would result in parallel increases in fatty acid harvesting and LPS leakage, both of which could act systemically to activate TLR4 and promote metabolic inflammation (Nathalie et al., 2011; Nicholson et al., 2012). However, the link between changes in the microbiome of the gut to the increase in blood LPS levels is not direct. One of the most consistent changes in the gut microbiota in obesity is the increase in the *Firmicutes*, accompanied by a reduction in the *Bacteroidetes* phylum (Ley et al., 2005; Peter et al., 2006). Most species belonging to the *Bacteroidetes* phylum are Gram-negative, whereas most of the species belonging to the *Firmicutes* phylum are Gram-positive. Because LPS are among the endotoxins of Gram-negative bacteria, it would be unexpected to find increased LPS levels in the blood of subjects with the obese-type gut microbial landscape. It is interesting to note that one of the outcomes of gut dysbiosis in obesity is a change in the expression of proteins of the enterocyte-tight junctions, resulting in increased gut permeability (Cani et al., 2008).

2.8.2.2 Mechanism of action

At first, TLR4 recruits TIRAP at the plasma membrane and later, enlists MyD88 to trigger the initial activation of NF-κB and MAPK (Kagan & Medzhitov, 2006). The TLR4 then goes through dynamin-dependent endocytosis and is transferred to the endosome, where it forms a signalling complex with TRAM and TRIF, rather than TIRAP and MyD88, thereby initiating the TRIF-dependent pathway leading to IRF3 activation as well as the late-phase activation of NF-κB and MAPK (Jonathan et al., 2008; Tanimura et al., 2008). In this way, TLR4 activates the MyD88-dependent pathway before the TRIF-dependent pathway. It is noteworthy that activation of both the MyD88- and TRIF-dependent pathways is essential for the induction of inflammatory cytokines *via* TLR4 signalling, which contrasts with other TLRs, which suffice with either the activation of MyD88- or the TRIF-dependent pathway for induction of inflammatory cytokines. It remains a mystery as to why the activation of either pathway alone is insufficient for the induction of inflammatory cytokines *via* TLR4 signalling (Taro & Shizuo, 2010).

Functional analysis of the CD14 gene may be conducive to investigate the molecular mechanism of diarrhoea and oedema disease in weaned piglets caused by EPEC since it is the main receptor for the recognition of LPS. The pig CD14 gene is located on chromosome 2 q28 and includes two exons and one intron and which encode for 373 amino acid sequences. They have structures highly homologous with humans, mice, rabbits, horses, and cattle. A group of researchers on analysis of the polymorphism of the pig CD14 gene, found that there were three potential polymorphic loci (-61, 587 and 1246) in the porcine CD14 gene (Liu et al., 2008). The relationship between three kinds of genotypes at the -61 site and parts of the immune traits like IgG and DTH were also analysed. In one study it was found that under stimulation induced by LPS, transcription of the CD14 gene in epithelial cells (IPEC-J2) of the pig's small intestine was significantly raised with time (Sun, 2015). This showed that LPS was released in the IPEC-J2 cells with *E. coli* infection and that the CD14 gene played an essential role in mediating the inflammatory reactions and immune response.

Table 2.2 Recognition of different components of pathogens by TLR4.

Component	**Source**	**Reference**
LPS	Gram-negative bacteria	(Vaure & Liu, 2014)
Mannuronic acid polymers	Gram-negative bacteria	(Flo et al., 2002)
Teichuronic acid	Gram-positive bacteria	(Yang et al., 2001)
F protein	Respiratory syncytial virus	(Evelyn et al., 2000; Haynes et al., 2001)

Resistin is a cysteine-rich adipokine and has been known to regulate glucose metabolism. Recently, it has emerged as a mediator in inflammation and immunity. Resistin levels are related to the expression of interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α) in inflammation. Toll-like receptor 4 (TLR4) has been reported to be a receptor for resistin in cells, such as leukocytes and peripheral blood mononuclear cells (PBMC). A recent paper by Li et al. (2018) found that pro-inflammatory cytokine expression in porcine alveolar macrophages (PAMs) is positively correlated with resistin (Li et al., 2018). Their results also showed that resistin induced expression of TLR4, intracellular molecules MyD88, TRIF-related adaptor molecules (TRAM) and NF-κB in PAMs. In contrast, inhibition of TLR4, MyD88, TRAM and NF-κB retracted the pro-inflammatory effect of resistin on PAMs. Also, they found that resistin promoted the production of pro-inflammatory cytokine in PAMs *via* the TLR4/NF-κB-mediated pathway (TLR4/MyD88/TRAM/NF-κB).

2.8.3 TLRs in diseases

2.8.3.1 TLRs and bacterial diseases

The TLRs 1, 5 and 6 are related to antibody responses after vaccination against *Erysipelothrix rhusiopathiae* or *Actinobacillus pleuropneumoniae* (Shinkai et al., 2012). The TLR4, 5, and 9 are upregulated on monocytes and DCs after stimulation with various TLR ligands like LPS, lipoteichoic acid (LTA), and cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODNs) (Raymond & Wilkie, 2005).

The involvement of TLRs in the pathogenesis of bacterial infections is being studied by disrupting individual TLRs or their adapter proteins. In both humans and mice, mycobacteria are eliminated within the granuloma, and this is mediated through activation of killing mechanisms within the infected macrophages. Mainly, CD4+ T-lymphocytes play an essential role in mediating these responses (Ulrichs & Kaufmann, 2006). The IL12-activated CD4+ T cells recognise *M. tuberculosis* antigens in the context of MHC II molecules and secrete IFN-α. This is a crucial cytokine in defence against tuberculosis as, along with a second stimulus, such as TNF-α, it stimulates the anti-microbial activity of infected macrophages thus accelerating phagosome maturation. The IL-12 facilitates microbial killing (Schaible et al., 1998; Via et al., 1998). IL-12 and TNF-α production are mostly induced by macrophages soon after innate recognition of mycobacteria through TLRs (Raja, 2012). In particular, *M. tuberculosis*-induced TNF-α production is primarily TLR2-dependent *in vitro* (Underhill et al., 1999). The TLR2, in association with TLR1 and TLR6, mediates responses to mycobacterial lipoproteins, lipomannan, arabinose-capped lipoarabinomannan (ara-LAM) and phosphatidyl-myo-inositol mannosidase (PIM) [reviewed by (Quesniaux et al., 2004)]. Although most purified mycobacterial products signal through TLR2, overexpression of TLRs allows cells to respond to the whole bacilli *via* TLR2 or TLR4 in a MyD88-dependent manner (Means et al., 1999; Quesniaux et al., 2004). But, primary mouse macrophages, which express physiologic levels of TLRs, were found to sense intact mycobacteria in a TLR/MyD88-independent manner (Shi et al., 2005). On top of that, DNA from mycobacteria contains stimulatory CpG motifs that activate TLR9 (Bafica et al., 2005). Activation of TLR by mycobacterial products leads not only to the synthesis of pro-inflammatory cytokines, including TNF-α and IL-12, but also to the production of nitric oxide, a metabolite with potent anti-mycobacterial activity in mice (Pecora et al., 2006; Underhill et al., 1999). Considering these, it is not unreasonable to speculate that deficiency in TLR signalling results in worsening of *M. tuberculosis* infection. TLR2 is also connected to the incidences of pneumonia caused by *Mycoplasma hyopneumoniae* (Uenishi & Shinkai, 2009).

The bacterium *Salmonella* has a minimum of four TLR activators: LPS, bacterial lipoproteins, flagellin and CpG DNA, activating TLR4, TLR2, TLR5 and TLR9, respectively. *In vitro*, cells like macrophages and

DCs sense *Salmonella* through these TLRs, inducing the production of cytokines also relevant in *in vitro* responses to *Salmonella*. Injecting *Salmonella* or LPS directly into the bloodstream causes shock. This led scientists to postulate that LPS was a significant virulence factor of this bacterium. C3H/HeJ mice, which carry a mutation in the TLR4 allele, are more resistant to i.p. or i.v. challenge with LPS or *Salmonella* than the closely related C3H/HeN mice, which have a wild-type copy of TLR4 (O'Brien Ad Fau - Rosenstreich et al., 1980). The timely response to oral infections with *Salmonella* requires TLR2, TLR4 (Weiss et al., 2004) and TLR5 (Uematsu et al., 2006) when mice are challenged with a lethal dose. Interestingly, TLR2 and TLR4, alone or in combination, are not required at lower infectious doses where only around 50% of the wild-type animals died and probably reflected a natural infection with *Salmonella* (Weiss et al., 2004). TLR5, as opposed to TLR4, seems to be lethal for the host in a *Salmonella* infection facilitating the migration of *S. typhimurium* from the intestinal tract to the mesenteric lymph nodes. This also suggests that TLR4 is the dominant TLR involved in the host response to *Salmonella* infection (Uematsu et al., 2006; Weiss et al., 2004).

Staphylococci too have several TLR activators. Bacterial lipoproteins and lipoteichoic acid (LTA) serve as TLR2/6 agonists (Hashimoto et al., 2006; Wardenburg et al., 2006), whereas CpG DNA signals through TLR10. Also, other PRR receptors such as NOD2 sense *S. aureus* through the recognition of peptidoglycan motifs (Girardin et al., 2003). Studies involving *S. aureus* mutant strains lacking lipoproteins demonstrated that lipoproteins, rather than LTA, are the major component inducing the innate inflammatory response to intravenous *S. aureus* infection in mice (Wardenburg et al., 2006). Neutrophil recruitment is the vital host factor that drives these protective host responses (Verdrengh & Tarkowski, 1997). To clarify the impact of TLRs in host responses to *S. aureus*, different studies have assessed the susceptibility of TLR2-, TLR10-, and MyD88-deficient mice to a broad range of *S. aureus* infections. In an experimental brain abscess model, TLR2-deficient mice exhibited only a moderately reduced expression of TNF and inducible nitric oxide synthase (iNOS) and no differences in neutrophil infiltration, bacterial burden and animal survival as compared to control animals (Kielian et al., 2005). Although, in nasal, cutaneous, and corneal infection models, TLR2 deficiency was associated with higher bacterial loads and a moderate increase in disease severity during the early stages of the infection (Miller et al., 2006; Sun et al., 2006). Especially in the corneal epithelium, infected TLR2-deficient mice showed reduced corneal inflammation paralleled with reduced levels of chemo-attractants for neutrophils and monocytes such as KC and MIP-2. Consequently, they diminished neutrophil infiltration to the site of infection (Sun et al., 2006). This observation implicated TLR2 in the mobilisation of neutrophils to the infected-corneal epithelium.

In contrast, in cutaneous *S. aureus* infection, TLR2-deficient mice show only slightly larger than normal lesions, minimal increases in bacterial counts and no defect in neutrophil recruitment (Miller et al., 2006). This also demonstrated that IL-1R signals are essential for the inflammatory response to

cutaneous *S. aureus* infections. As pointed out by the authors, other TLRs and non-TLR receptors in addition to TLR2 might be involved in the production of IL-1, the crucial inflammatory mediator at skin sites.

Figure 2.8.2 depicts the cascade of events that occur from the moment of recognition of ligand and development of an inflammatory response against different pathogens like bacteria, protozoa, and nematodes.

Figure 2.8.2 Recognition of ligand and onset of inflammatory response against bacteria, protozoan, and nematode parasites.

2.8.3.2 TLRs and fungal infection

It is difficult to pin-point the precise molecular nature of fungal PAMPs, which activate specific TLRs, because of the collaborative mechanism of TLR recognition and the plasticity of the fungal cell wall. Although fungal PAMPs are recognised by several TLRs such as TLR2/1, TLR4, TLR3, TLR2/6, TLR7 and TLR9, they are not the primary receptors which engulf pathogens. Hitherto, fungal PAMPs for cell-surface TLRs had been characterised for *Candida albicans* (mutants with specific cell wall defects have facilitated the identification of PAMPs), they are still unknown for other fungi. It has been found that TLR2 recognises fungal β-glucans of several fungal species (Netea et al., 2006; Sorgi et al., 2009; Viriyakosol et al., 2005). Besides, TLR2 also explicitly interacts with phosphor-lipo-mannans (PLMs), linear beta-1,2-oligomannoside structures that are exclusive to *C. albicans* (Jouault et al., 2003). The TLR2 is also stimulated by, as yet unidentified, ligands present on conidia and hyphae forms of *A. fumigatus* (Netea et al., 2003). TLR2/TLR1 and TLR2/TLR6 heterodimers are receptors for the

glucuronoxylomannan (GXM) component of *Cryptococcus neoformans* (Fonseca et al., 2010). Remarkably, *A. fumigatus* activates mouse but not human TLR2/6 heterodimers, whereas TLR2/1 heterodimers recognise *A. fumigatus* both in human and mice (Rubino et al., 2012). TLR4 is activated upon ligation of *C. albicans* O-linked mannans (Netea et al., 2003), as well as *C. neoformans* GXM (Shoham, Huang, Chen, Golenbock, & Levitz, 2001). Ligands for TLR4 are present on *A. fumigatus* conidia but not hyphae (Netea et al., 2003). Along with cell-surface PAMPs, nucleic acids liberated from fungi within the phagosome also stimulate or modulate the dynamic host response during infection. TLR3 is activated by double-stranded RNA from *A. fumigatus* conidia in lung epithelial cells (Beisswenger et al., 2012). Single-stranded RNA from *Candida* spp. are ligands for TLR7 in mouse bone-marrow dendritic cells (BM-DCs) (Biondo et al., 2012). TLR10-mediated sensing of fungal genomic DNA (gDNA) seems to be conserved across fungal species (Biondo et al., 2011; Miyazato et al., 2009; Nakamura et al., 2008; Ramirez-Ortiz et al., 2008), and the recruitment of TLR10 to fungi-containing phagosome is similar in several fungal species (Kasperkovitz et al., 2011). Recognition of gDNA from *A. fumigatus* and *C. neoformans* occurs at unmethylated CpG motifs (Nakamura et al., 2008; Ramirez-Ortiz et al., 2008; Tanaka et al., 2012). On the other hand, TLR10 detection of *Candida* gDNA does not seem to be restricted to these motifs (Miyazato et al., 2009).

Since microbial pathogens are known to carry multiple classes of PAMPs, their recognition involves the simultaneous or serial activation of several PRRs from different families. Collaboration between PRRs and crosstalk between their signalling pathways enhances the specificity and coverage of PAMP recognition enabling a custom-made host response (van de Veerdonk et al., 2008). TLR2 transduces signals as a heterodimer recruiting either TLR1 or TLR6 (Adrian et al., 2000). However, the functional consequences of these TLR co-operations for fungal recognition are not yet adequately defined. Also, several molecules, including C-type lectins or other carbohydrate-binding proteins, have been identified as TLR2 co-receptors (e.g., Dectin-1, SIGNR1, and Galectin-3). It is interesting to note that depending on the co-receptor involved, co-ligation of TLR2 may either enhance a TLR2-dependent response (Smeekens et al., 2010; Takahara et al., 2012) or modulate its PAMPs specificity (Jouault et al., 2003). Dectin-1 has also been shown to synergise with TLR4 signalling (Ferwerda et al., 2008). The molecular basis of signalling pathway crosstalk is just starting to be studied [reviewed in (Hontelez et al., 2012)]. Dectin-1 signalling requires its clustering and the formation of a phagocytic synapse (Goodridge et al., 2011). Although, no physical interaction between TLR2 and Dectin-1 has been reported as yet, TLR2 co-immuno-precipitates with Galectin-3 following stimulation with *C. albicans* (Jouault et al., 2003). Interestingly, Galectin-3 also co-immuno-precipitates with Dectin-1 (Esteban et al., 2011), which suggests that Galectin-3 may mediate the cooperation between TLR2 and Dectin-1 signalling. TLR2 also co-immuno-precipitates with SIGNR1 (Takahara et al., 2012). Thus, the dynamic clustering and exclusion of PRRs from the phagocytic synapse may control and modulate signalling

crosstalk during the initial immune response to surface PAMPs. Subsequent liberation of fungal PAMPs, such as nucleic acids, occurs through fungal pathogen degradation as the phagosome matures. Figure 2.8.3 shows the pathways by which TLR4 participates in recognising fungal PAMPs.

Fungal pathogens and TLRs may promote further recruitment of PRRs (Kasperkovitz et al., 2011). Along with microbial PAMPs, host DAMPs arising from tissue damage such as S100B proteins are also released at the site of inflammation during infection. In a TLR2-dependent manner, low doses of S100B proteins promote fungal clearance and protect against inflammation-induced epithelial damage in the lungs of mice with *A. fumigatus* intranasal infections. In contrast, the TLR3/TRIF axis may reduce over-production of S100B proteins, hence preventing worsening of the inflammation reaction, promoting its resolution (Sorci et al., 2011). Host DAMPs may collaborate with PAMP-activated TLRs to control the outcome of the inflammatory response. *C. albicans* is uniquely recognised by TLR2 after antifungal treatment that targets and alters the cell wall (Roeder et al., 2004). Similarly, pre-treatment of *C. albicans* or *A. fumigatus* with antifungal drugs enhances their ability to stimulate TLR expression in human PMNs (Salvenmoser et al., 2010). From all these, it can be inferred that besides their direct fungicidal properties, antimycotics may also facilitate pathogen detection by the host and, hence, smoothen clearance.

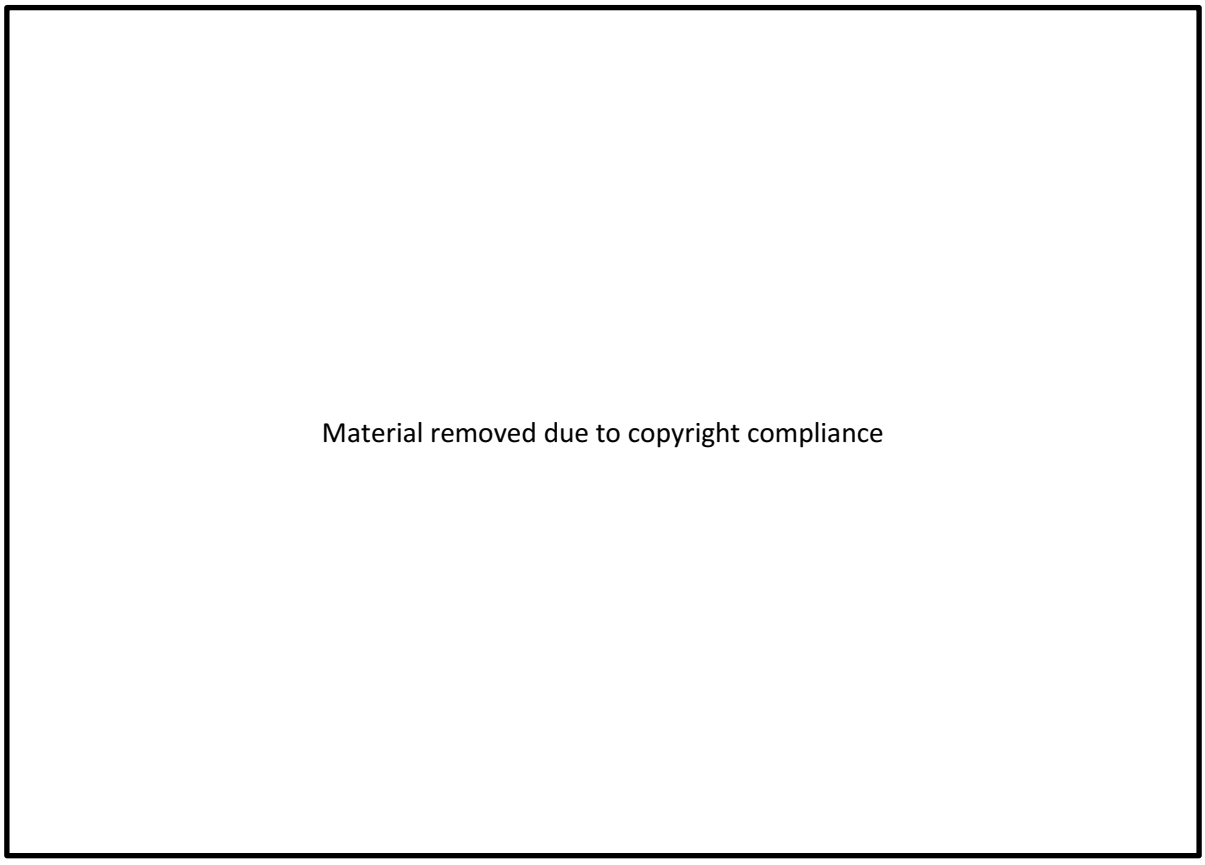

Figure 2.8.3 TLRs and fungal pathogens

TLRs on the surface, as well as in the endosome, participate in recognising fungal PAMPs. Activation of surface TLRs involves their homo- (TLR4) or hetero-dimerisation (TLR2/TLR1 or TLR6). The diversity of signalling pathways is increased by the involvement of co-receptors of the C-type lectin family (e.g., SIGNR1 and Dectin-1) or Galectin-3. Physical interactions between PRRs are represented by double-head arrows. The integration of simultaneously activated signalling pathways occurs at the level of intracellular adaptors, and transcription factors are shared between overlapping pathways. The resulting cytokine responses shape the activation of the adaptive response and ultimately modulate the outcome for the host. Adapted from (Biondo et al., 2012; Bourgeois et al., 2011; Takahara et al., 2012).

2.8.3.3 TLRs and parasitic infections

On infection, protozoan PAMPs activate innate immunity through TLR-induced signalling pathways that can induce either protective, regulatory, or pathogenic reactions (Marcela et al., 2012). The role of TLRs during infection with different protozoa has been analysed. *Leishmania* is an excellent example of the importance of TLRs in parasitic infections. TLR responses in a *Leishmania* infection have a protective role but also could serve to promote infection (Faria et al., 2012). The TLRs are vital in recognition of *Leishmania* species (Whitaker et al., 2008). They trigger the proper innate then acquired immune responses required for controlling *Leishmania* parasite (Pascale Kropf et al., 2004). Although *Leishmania* species express several PAMPs, few *Leishmania*-derived molecules have been reported to activate some TLRs, and the majority of the studies till now have focused on the activation of TLR2, TLR3, TLR4 and TLR10 (Faria et al., 2012; P. Kropf et al., 2004). Purified *Leishmania major* lipo-phospho-glycan induced the stimulation and upregulation of TLR2 on human NK cells. It also elicited leishmanicidal reactions *via* the release of different mediators like TNF-α, IFN-g, nitric oxide (NO) and reactive oxygen species (Th1 response) (Becker et al., 2003; Kumar et al., 2009). Also, TLR2 can induce the anti-leishmanial immune response through altered expression of TLR10 (Srivastava et al., 2013). However, *Leishmania* can down-modulate TLR2 responses in macrophages by inhibiting p38 MAPK, leading to decreased IL-12 and increased IL-10 production. Thus, TLR2 response leads to increased replication of *Leishmania braziliensis* and *L. amazonensis* in macrophages and subsequently increased disease pathogenesis (Chandra & Naik, 2008). Therefore, TLR2 stimulation by *Leishmania* can lead to positive or negative inflammatory signals. The figure 2.8.3 enlists the mechanisms utilised by parasites for the negative regulation of TLRs.

The TLR-mediated responses in helminth infections may occur through either the direct effect of the worms on the function of TLRs or from damaged cells resulting from parasitic activities. Alternatively, TLRs may be influenced by molecules from microbes co-localising at infection locations (Friberg et al., 2010). Early schistosomiasis is characterised by a moderate Th1 response. Out of the many TLRs, TLR2 and TLR4 are the most important sensors of parasite components during *Schistosoma* infection. Somatic and excretory/secretory antigens of worms activate TLRs in the early stage of schistosomal infection before egg deposition (Jenkins et al., 2005). Lyso-phosphatidylserine (lyso-PS) present in

Schistosoma eggs can induce the activation of DCs *via* a TLR – the 2-dependent mechanism and lacto-N-fucopentaose III, which is one of the most abundant glycans in schistosome eggs, promotes Th2 differentiation *via* a TLR4-dependent pathway (van Der Kleij et al., 2002). A group proposed that TLR2 was favourable for the parasite, while, on the contrary, TLR4 might be involved in the protection against this infection (Zhang et al., 2011). Another group of scientists showed that lyso-PS from *S. mansoni* eggs could stimulate DCs to induce IL-10-producing Tregs in a TLR2-dependent manner thereby regulating and enriching the chronic inflammation of *Schistosoma* infection (Layland et al., 2007). Alternatively, other TLR-related genes are generally decreased during *Schistosoma* infection; TLR1, TLR3, TLR7 and TLR10 being strongly repressed, with the appearance of the eggs at week 8 post-infection, and TLR3 showing most repression (Cheng et al., 2013).

Table 2.8.3 Mechanisms utilised by parasites for negative regulation of TLRs

Mechanism	Parasite	Reference
Inhibiting dendritic cells (DC) maturation	Schistosoma	(Zaccone et al., 2003)
DC maturation with silenced proinflammatory properties	*Heligmosomoides bakeri*	(Segura et al., 2007)
Regulatory T cell expansions	*Heligmosomoides bakeri, Trypanosoma cruzi*	(Kulkarni et al., 2011)
Excessive exposure to ligands	Chronic parasitic infections	(Hayashi et al., 2009)
Degradation of TLR by proteases	*Fasciola hepatica, Schistosoma mansoni*	(Donnelly et al., 2010)

2.8.3.4 TLRs and viral diseases

The TLR3 may play an essential role in the porcine innate antiviral response, and this has been suggested due to the observation that TLR3 expression *via* RNA interference reduces to trigger an increase in porcine reproductive and respiratory syndrome virus (PRRSV) replication (Miller et al., 2009; Sang et al., 2008).

Porcine epidemic diarrhoea (PED) is an extremely contagious and infectious disease, causing anorexia, vomiting, watery diarrhoea, dehydration, and loss in body weight. It results in high mortality, especially in neonatal pigs. The porcine epidemic diarrhoea virus (PEDV), which is the etiological agent in PED, targets the digestive tract in pigs. The PEDV distribution primarily happens through the faecal-oral route, but transmission through the air from the nasal cavity to intestinal mucosa is also possible (Wen et al., 2018; Yuchen et al., 2018). The host's first line of defence, the innate immune response, utilises PRRs for detection and to respond to PAMP molecules of the

invading PEDV. After PEDV invades host cells, the intra-cellular genomic nucleic acid, dsRNA, and proteins produced during replication mediate the TLR signalling pathways and the RLR signalling pathway in the natural immune response to exhibit congenital antiviral function (Jian et al., 2019).

Following a PEDV invasion, it is the TLR signalling pathway that is involved in the innate immune response. As we know, TLRs recruit MyD88 and TRIF, initiating downstream signal transduction, and producing inflammatory cytokines, chemokines, and antimicrobial peptides (Zhang & Yoo, 2016). TLR3 has a horse-shoe-shaped structure and primarily recognises the dsRNA viral genome during ssRNA virus replication. It has a large surface area, which promotes the recognition of viral dsRNA by viral-infected cells. The binding of dsRNA to the N- and C-termini of the outer convex surface of TLR3 enables the formation of homodimers through the C-terminal region. TLR3 relies on the TRIF pathway to activate IRF-3 and NF-κB signalling pathways and induce the expression of IFN-I and inflammatory cytokines. In plasmacytoid dendritic cells, TLR7 and TLR8 recognise viral ssRNA in the lysosome and activate NF-κB and IRF-7 through MyD88 to induce inflammatory cytokines and IFN-I, respectively. Also, autophagy is involved in the transfer of ssRNA to vesicles expressing TLR7. TLR9 recognises viral and bacterial DNA. Downstream signal transduction requires the degradation of TLR9 by cyto-protease. TLR9 recruits MyD88 to activate NFκB and IRF7, causing their phosphorylation and nuclear translocation (Kawai & Akira, 2011). Previous *in vitro* and *in vivo* research has found that TLR2, TLR3, TLR4, TLR7, and TLR9 are involved in PEDV-induced NF-κB activation in porcine intestinal epithelial cells (IECs), which suggests that the virus can use its surface glycoprotein and intranuclear nucleic acids to activate the innate immunity (Cao et al., 2015; Temeeyasen et al., 2018).

Beyond the basic characteristics and functions of TLRs, their roles in various viral, bacterial, and fungal diseases have been investigated. The PRRSV infection increased the expression of TLR3, 4, and 7 as well as the production of the pro-inflammatory cytokines IL-1b, IL-6, TNF-α, and IFN-c in tracheobronchial lymph nodes (Miguel et al., 2010). However, activation of TLR3, but not TLR4, could decrease the infectivity of PRRSV in porcine alveolar macrophages (PAMs) (Miller et al., 2009). A comparison of high (HP) and low pathogenic (LP) PRRSV strains revealed that relative to LP strains, HP PRRSV strains suppress ERK phosphorylation at early time points and impair LPS- and poly I:C-stimulated TNF-α release (Hou et al., 2012). The HP PRRSV strains also lead to high serum levels of pro-inflammatory cytokines, but lower IL-10 levels compared to LP PRRSV strains (Zhang et al., 2013). The more potent inflammation induced by HP PRRSV likely contributes to increased tissue damage and clinical signs. Furthermore, HP PRRSV strains have decreased amounts of SARM1, a TIR-domain containing adaptor protein involved in TLR signalling transduction (Zhou et al., 2013), which may decrease the ability to mount an immune response against PRRSV. Hüsser et al. showed that TLR3 is involved in sensing classical swine fever virus (CSFV) but not food and mouth disease virus (FMDV), vesicular stomatitis virus (VSV), or influenza A virus (IAV) (Hüsser et al., 2011).

The I329L is a novel gene isolated from African swine fever virus (ASFV). It interferes with TLR3-stimulated activation. TLR3 is one of the most densely glycosylated of the TLRs, and ORF I329L is expressed in the cell membranes and at the cell surface. These are locations where many receptors and adaptor molecules involved in innate immunity are found. It was also seen that I329L inhibited ds-RNA-stimulated activation of NFκB and IRF3 – both necessary in the innate antiviral response. The expression of I329L protein also inhibits the activation of CCL5 and IFN-β. Subsequently, activation of IRF3, which is mediated by expression of TRIF, was inhibited by I329L, whereas overexpression of TRIF degenerated the inhibition of reporter activation induced by I329L in a dose-dependent manner. Hence it can be concluded that I329L could be targeting at the level of TRIF, a major adaptor molecule in the MyD88-independent pathway (Oliveira et al., 2011).

The pseudorabies virus (PRV) is a porcine virus, part of the *Alphaherpesvirinae* subfamily of Herpesviridae. Viruses have been found to affect genes such as oxidative-stress response genes, genes involved in the phosphatidylinositol 3-kinase/Protein Kinase B (PI3K/Akt) signalling pathway, and interferon- and interleukin-related genes (Huang et al., 2014). Interferon-γ inducible protein-10 (IP-10), also known as chemokine (C-X-C motif) ligand 10 (CXCL10), is a CXC chemokine from the chemokine superfamily. It acts as a chemoattractant for T cells, monocytes, and natural killer (NK) cells and has angiostatic and antitumour activity (Loetscher et al., 1996; Tusher, 2001). Direct or indirect activation of *IP-10* is possible by pathogen TLRs (Luster, 2002). Exogenous *IP-10* resists viral replication after herpes virus infection of human neurons and enhances the cellular immune response. *IP-10* plays a crucial role in immune response, such as mediation of the development of inflammatory diseases and contribution to viral clearance, which mainly depends on the recruitment and activation of innate immune responses such as chemoattraction of NK cells and other immune cells. Host cell death programme is directly initiated due to *IP-10* expression to limit viral replication and the subsequent spread of the viral infection (Huifang et al., 2005). This indirect antiviral action of IP-10 is affected through the immune-mediated destruction of host cells. Huang et al. (2014) found that PRV replication was substantially decreased in PK-15 cells with overexpression of IP-10 (Huang et al., 2014).

As is true for all pathogens, foot-and-mouth disease virus (FMDV) is recognised by the immune system, causing an increased immune response, which is primarily mediated by type I and type III IFNs. FMDV uses many strategies to overcome the strong antiviral response induced by cytokines, including: (a) inhibit IFN induction at the transcriptional and translational level, (b) inhibit protein trafficking; (c) block specific post-translational modifications in proteins regulating innate immune signals; (d) modulate autophagy; (e) inhibit stress granule formation, and (f) *in vivo* modulation of immune cell function (Medina et al., 2018).

As PAMPs, viral RNAs are recognised by three types of receptors: endosome-associated TLRs, cytosolic RNA helicases known as RIG-I like receptors (RLRs), and NOD-leucine-rich repeat-containing receptors (NLRs). Also, viral RNA interacts with a family of cellular enzymes like dsRNA-dependent PKR, oligoadenylate synthetase proteins (OAS), and others, causing a signalling response limiting propagation of the virus (Dempsey & Bowie, 2015; Rathinam et al., 2012; Yoneyama et al., 2015). The TLRs interact with RNA in the extracellular milieu or inside endosomes. Amongst the TLR family, TLR3 recognises dsRNA, while TLR7 and TLR8 sense ssRNA (Medina et al., 2018). Signals sensed through TLRs are transduced through interactions with adaptor proteins including TIR domain proteins (i.e., TRIF, TRAF, etc.), and with MyD88. Successive TLR-driven signals lead to the nuclear translocation of nuclear factor κB (NF-κB, p65/p50) and IRF3/IRF7, key transcription factors for IFN and proinflammatory cytokines (Ikushima et al., 2013). In pigs, primary FMDV infection occurs inside epithelial crypts of the oropharyngeal tonsils (Stenfeldt et al., 2016). The micro-anatomic and phenotypic characteristics of the distinct regions of lymphoid-associated epithelium that support primary FMDV infection are highly similar in pigs and cattle.

Intensification and globalisation in the modern swine industry have led to the emergence and global spread of pathogens in swine, partly caused by regular movements of pigs, feed, and pork products at local, national, and international levels (Drew, 2011). For example, the PED virus spread from China to the United States in 2013. Within one year, the virus had impacted ∼50% of US breeding herds, resulting in the deaths of at least seven million piglets (Goede & Morrison, 2016). African swine fever (ASF) emerged in Eastern Europe from sub-Saharan Africa in 2007 and currently is causing high mortality outbreaks and restricting international trade throughout the region. The risk of the virus spreading to countries currently not affected is noteworthy, as shown by its recent 2018 introduction to China, the world's largest producer of pork (Ge et al., 2018). Finally, the potential importance of livestock pathogens for human public health was demonstrated by the H1N1 'swine flu' pandemic in 2009, which originated from influenza A viruses circulating in pig populations (Smith et al., 2009).

2.8.4 TLRs and non-infection diseases

Both TLR2 and TLR4 may mediate immune dysfunction in HS pigs. Heat stress induces an inflammatory response, which releases pro-inflammatory cytokines modulated by TLRs, stimulating hepatocytes to produce innate immune proteins for protection. The upregulation of HSP90B1 (heat shock protein) may help maintain the proper folding of TLRs during HS. The TLR signalling pathway is activated under heat stress in mammals, including pigs (Yanjun et al., 2016).

One of the major non-infectious diseases affecting pigs and other mammals is IBD, which is mainly classified into Crohn's disease or ulcerative colitis (UC). When a genetically predisposed host

encounters a luminal antigen, either a bacterium or food, the immune response to that can be dysregulated, causing IBD (Strober, 2006). Numerous reports have shown that TLR mutations and dysfunction are contributing factors in the predisposition to and maintenance of IBD (Montero-Vega & de Martín, 2009). In hosts with IBD, expressions of TLR3 and TLR4 are differentially modulated in the intestinal epithelium, meaning TLR3 is significantly downregulated in Crohn's disease (not in UC) and TLR4 is strongly upregulated in both conditions. TLR5 expression remains unchanged in IBD.

Results from some human studies have suggested that TLR2 and its co-receptors, TLR1 and TLR6, could be involved in the initial immune response to bacteria in the pathogenesis of IBD (Pierik et al., 2006). A vital immune stimulatory effect mediated by TLR9 is induced by non-methylated CpG motifs that are found in bacterial DNA. In animal models of colitis, CpG administration was able to confirm disease activity (Florian et al., 2010).

When an acute tissue injury occurs, many cells die by necrosis and release their intracellular content, and the matrix turnover leads to the creation of many breakdown sub-products. Recent studies have found that these endogenous sub-products can act as 'danger molecules', signalling *via* TLRs and stimulating the innate immune system by promoting inflammation (Miyake, 2007). It has also been found that by recognising micro-organisms and endogenous harmful stimuli, TLRs induce the expression of several genes that are involved in wound healing response and in tissue regeneration for the recovery of structural and functional integrity of injured organs (Kluwe et al., 2009; Rakoff-Nahoum & Medzhitov, 2008). Also, TLRs and their ligands have been found to control mesenchymal stem cell functions, inducing them to differentiate into mesodermal cell lineages, support and regulate haematopoiesis, regulate the stem-cell niche, and participate in the repair of tissue damage imposed by normal wear and tear, injury, or diseases (Pevsner-Fischer et al., 2007).

2.8.5 TLRs during growth

The maternal–foetal interface is an immunologically unique site that promotes tolerance to the allogeneic foetus, while simultaneously maintaining host defence against possible pathogens. About the immune system, normal pregnancy consists of three different immunologic phases:

1. The pro-inflammatory environment during embryo implantation, placentation, and early stage of pregnancy.

2. Anti-inflammatory milieu during mid-pregnancy

3. The pro-inflammatory environment at the end of pregnancy (Mor, 2008)

Expression of all 10 TLRs, as well as various co-receptors and accessory proteins such as CD14, has been described in the mammalian placenta. It has been shown, using RT-PCR, that in cultured cells isolated from term placenta, both cytotrophoblast and syncytiotrophoblast-rich cells express TLR2, 3, 4, 5, 6 and 9 (Koga & Mor, 2010). The pattern of the response of the TLRs depends mainly on the stimuli. Some studies have found that the LPS did not induce apoptosis in trophoblasts, but Chlamydia heat shock protein 60 did so through TLR4 (Abrahams et al., 2004; Equils et al., 2006).

The expression of TLRs has also been reported in other types of cells in the placenta. In one study, TLR4 was shown to be expressed in Hoffbauer cells, a type of macrophage in the placental villi (Kumazaki et al., 2004. Ma et al. (2006) observed stronger expression of TLR2 in endothelial cells and macrophages and weaker expression in syncytiotrophoblast and fibroblast, while staining for TLR4 was most prominent in syncytiotrophoblast and fibroblast. From these, it can be inferred that not only immune cells but also trophoblasts and other types of cells within the placenta can respond to the invading pathogens, just like the innate immune system, in the physiological protection of the placenta (Kumazaki et al., 2004; Ma et al., 2006).

The TLRs family, being the main regulator of innate immunity, is involved in protecting the female reproductive tract against invading pathogens and is a key regulator in immunologic events during stages of normal pregnancy such as implantation or labour. The study of TLRs is so interesting because of its accessibility to the agonists and antagonists of TLRs and the possibility to stimulate or suppress TLRs' function (Amirchaghmaghi et al., 2013).

2.8.6 Prospects for TLRs to be used in anti-disease breeding and improve health

Recent progress in our understanding of the fundamental mechanisms that trigger metabolic inflammation has found potential therapeutic targets that can give rise to more effective and longer-term results. Scientists experimentally and clinically evaluated some of these potential targets with encouraging results. Inflammatory signal transduction through the IKK/NFB pathway is activated by both TLR4 and ERS (Hu et al., 2006; Krappmann et al., 2004). A substantial decrease in glucose intolerance and insulin resistance in animal models of obesity have been seen through the use of salicylates, which at high doses inhibit IK (Kim et al., 2001). One study found that although the total enhancement in glucose control was only marginally significant, it has put forward critical clinical proof of the concept that targeting the inflammatory activity generated by the TLR4 and ERS pathways has a positive impact on metabolic diseases (Goldfine et al., 2013). ERS has been successfully inhibited using chemical chaperones in isolated cell systems and experimental animals (Bonapace et al., 2004). In animal models of obesity, insulin resistance was consistently reduced using two distinct chemical chaperones (Ozcan et al., 2006), and even the hypothalamic inflammation seen in obesity was targeted by a chemical chaperone, which resulted in partial

correction of the obesity phenotype. Due to its biologically strategic position in connecting nutrient sensing to ERS, PKR has appeared as a potential target for the treatment of metabolic diseases (Nakamura et al., 2010). A current study tested two small molecule inhibitors of PKR that successfully improved metabolic inflammation and insulin resistance in animal models of T2D (Nakamura et al., 2014). As it is challenging to obtain small molecules that can act with specificity on defined targets, the early development of drugs that inhibit PKR could, shortly, allow for the evaluation of its safety and efficiency in humans (Velloso, 2014).

The targeting of TLR4-mediated inflammation signalling could prove to be a crucial method to counteract pathogen-induced damage. Probiotic bacteria are micro-organisms that can confer health benefits to the host, including prevention of inflammatory intestinal diseases (Finamore et al., 2012; Haller et al., 2010) . Although there is some evidence suggesting that these probiotic bacteria can inhibit activation of the TLR4 signalling pathway, the work is limited, with contradictory outcomes. TLR4 expression was seen to be downregulated by *Lactobacillus paracasei*, associated with a decreased cytokine and chemokine release against *Salmonella typhi* infection found in dendritic cells (Miriam et al., 2013). Similarly, *L. jensenii* was seen to reduce the mRNA level of proinflammatory cytokines by inhibiting the pathogen-induced TLR4 activation in porcine intestinal epithelial cells (Shimazu et al., 2012). In another study, however, it was found that *L. rhamnosus* and *L. plantarum* did not change the TLR4 expression either the secretion of IL-8 in cells infected with *Salmonella* (Vizoso Pinto et al., 2009). The treatment of porcine intestinal cells with *L. amylovorus* strain 16698^{T}, a new lactobacillus species isolated from the intestine of non-weaned piglets, protects against enterotoxigenic *E. coli* (ETEC) K88 infection by inhibiting pathogen adhesion and membrane damages through cytokine modulation (Roselli et al., 2007). This lactobacillus can reduce diarrhoea caused by ETEC, decrease colonisation of ETEC, and improve the weight gain of infected piglets (Konstantinov & Smidt, 2006). In a more recent study by the same laboratory, it was found that *L. amylovorus* DSM 16698^{T} and its cell-free supernatant impede the ETEC K88 induced activation of the TLR4 signalling pathway through modulation of the negative regulators Tollip and IRAK-M. It also causes the down-regulation of the extracellular Hsp72 and Hsp90, which are vital for the functioning of TLR4, causing reduced pro-inflammatory cytokine production (Alberto et al., 2014). Other than in piglets, the probiotic characteristics of *L. amylovorus* have been seen in an *in vitro* model that simulated the human upper GIT, which suggested a potential use of *L. amylovorus* for human health as well, along with animal health (Martinez et al., 2011).

It is expected that the genotype of porcine TLR4 will be of importance in future approaches aimed at improving genetic resistance to infectious diseases. A partial cDNA sequence for swine TLR4 was reported to have 72% and 63% amino acids similar to human and mouse TLR4, respectively (Mammalian Gene Collection Program, 2002; Smirnova et al., 2000). But several issues need to be

ironed out before we investigate TLR4 as a candidate disease-resistance gene in pigs. First, the genomic structure must be established, and enough flanking intronic sequences gathered to enable simple PCR amplification of the coding portions of the gene. After this, a basic knowledge of the promoter region must be obtained as an allelic variation that can significantly alter absolute levels and tissue-specificity of TLR4 expression. The group of S. C. Fung, in 2012, set out to report such necessary sequence data along with exon-specific PCR protocols, a comparison of the genomic organisation of the pig TLR4 locus with its known mammalian counterparts and expression of TLR4 in porcine tissues. They observed that the combined use of complement inhibitors and the inhibitor of the CD14 signalling pathway was somewhat effective in the prevention and treatment of inflammation caused by endotoxin LPS (Fung & Mollnes, 2012).

The host immune system and the bacterial populations residing in the gut have an intricate balance existing between them. When this balance gets disrupted, it causes dysbiosis. It may give rise to an inflammatory response (Nobuhiko et al., 2013). It has been found that PRR signals can sense pathogens and promote the induction of innate effectors, along with fostering autoimmune diseases under inflammatory conditions. During these times, the microbiota in the gut works to modulate the adaptive immune responses. In recent years, the complexity of the microbiome has slowly been understood in greater detail, mainly due to high-throughput techniques, such as metagenomics and 16S rRNA-based approaches. Although questions remain unanswered due to the uniqueness of the microbiota within each individual, especially at the species and strain levels (Holmes et al., 2012), research has shown that individuality of the gut microbiota composition is dependent majorly on host genetic factors, which change with genetic variations of the host (Benson et al., 2010; Hildebrand et al., 2013). Very recently, it was uncovered *via* metagenomic studies that some bacterial genes might very well be 'phenocopies,' which means that they can potentially functionally be able to perform certain activities of the host (Gosalbes et al., 2012). This leads us to a hypothesis that gut microbiota may, in practice, contribute to individual phenotypes.

One of the methods to test this would be the use of faecal microbiota transplantation (FMT). The use of FMT has had a high success rate in curing colitis caused by *Clostridium difficile* (van Nood et al., 2013). One study, where GF mice were colonised with a mouse microbiota or human microbiota, showed that the nature of the colonising microbiota had an effect on the host's initial T cell populations (Chung et al., 2012), thus presenting evidence for host-linked co-evolution of the microbiota and immune responses. These findings can pave the way for the use of the microbiome to improve mammalian health soon; however, our understanding of microbial communities and their interactions with the host is still minimal. Understanding how microbes behave during FMT could be helpful for the selection of optimal microbial features of a transplanted microbiota to ensure a successful outcome. Studies have found that the gut microbiota can activate TLR2/TLR4 on the

luminal surface of epithelial cells, which can improve intestinal barrier function by promoting the assembly of intestinal tight-junction-associated molecules, as well as by regulating the proliferation and apoptosis of epithelial cells (Cario, 2007; Rakoff-Nahoum et al., 2004). In a study, scientists fed exogenous faecal bacteria to new-born piglets up to day 11 (Hu et al., 2017). They observed that TLR2 and TLR4 of the recipient piglets on day 12 were increased. It is postulated that the exogenous microbial flora disturbs the original balance of microbiota. Hence, TLRs in the intestine of piglets significantly increased on feeding with exogenous faecal microbiota. But there were no significant differences in the expressions of TLRs on day 27 (as compared to day 12), which suggests that intestinal microbiota of recipient piglets was re-established, and new homeostasis gets formed. TLRs can sense commensal bacteria and this helps in maintaining homeostasis in the colon, but they do not elicit an inflammatory cascade and are necessary for the maintenance of the epithelial barrier and TLR-dependent intestinal homeostasis (Ben-Neriah & Schmidt-Supprian, 2007; Cario & Podolsky, 2005; Rakoff-Nahoum et al., 2004). Also, the induced TLR receptors in the intestinal epithelium stimulate an innate immune response, activating the PP to secrete antimicrobial peptides, leading to the induction of MUC2 (Hiroko et al., 2007; Ji et al., 2016).

Recent evidence in pigs has shown that nutritionally non-essential amino acids (NEAAs) have a vital role in intestinal growth and improving its functions, including mucosal growth and integrity, nutrient digestion and absorption, redox signalling, immune response, and microbial balance. As an example, N-acetylcysteine (NAC), an active precursor of cysteine, can constructively reduce inflammation, assuage oxidative stress, improve mucosal barrier function, and amend intestinal damage in piglets challenged with LPS, indomethacin, acetic acid, or PEDV. The underlying mechanisms whereby NAC effects on intestinal functions may be mediated by TLR4/NF-κB, PI3K/Akt/ mTOR, EGFR, AMPK, and type I IFN signalling pathways, as well as glutathione-activated protein synthesis (Hou, 2018).

The TLRs are vital in the protective immunity against infection; inapt TLR responses can lead to acute and chronic inflammation and systemic autoimmune diseases. Studies have found that mice with defects in the negative regulation of TLR-mediated responses developed these diseases. Also, evidence that shows that endogenous molecules produced by dying cells, or in certain pathological conditions, stimulate TLRs, which results in the progress or even speeding up of inflammatory and autoimmune diseases, is increasing by the day (Taro & Shizuo, 2010). The expression, ligand recognition, and signalling pathways of TLRs, as well as the immune consequences of their activation, have been described at length elsewhere (Holger et al., 2007; Shizuo & Kiyoshi, 2004; Taro & Shizuo, 2010).

2.8.7 Inducible heat shock proteins (iHSPs)

Pigs have been shown to express high levels of iHSP in both the small and large intestines (Arnal et al., 2015; Arnal et al., 2014; Lallès et al., 2010). However, the distal ileum was noted as having a higher relative concentration of iHSP proteins than the proximal colon in growing pigs, suggesting that higher microbial stimulation occurs in the distal region of the ileum (Liu et al., 2014). Oral administration of the broad-spectrum antibiotic amoxicillin to sows during parturition affected the sow's faecal, and the piglet's gut microbiota as well as the level of gut epithelial iHSPs (Arnal et al., 2015). The association of gut commensal microbiota with iHSPs in growing pigs demonstrates that colonic iHSP70 correlates negatively with *Bacteroidetes* and *Prevotella brevis* colonisation, and positively with that of *Faecalibacterium prausnitzii*, the latter of which exhibits anti-inflammatory properties and has been found to be depleted in pig IBD (Malago & Van Dijk, 2002). These individual correlations are often difficult to interpret as direct cause-and-effect relationships; however, they all suggest intimate associations between iHSPs and the GIT microbiota in pigs. A systematic study carried out by (Lallès & David, 2011) showed that subjecting growing pigs to feeding or fasting for 1.5 days, or to fasting for 1.5 days followed by re-feeding for 2.5 days, that fasting induced an increase in iHSP27, but not iHSP70, throughout the small and large intestines. However, as soon as feeding was restored, so too were the intestinal and colonic concentrations of iHSP27; however, still with no influence on iHSP70.

The fig. 2.8.4 is a diagrammatic representation of how dietary nutrients and components from the commensal microbiota can function to induce production of HSPs, while reinforcing protection to the host against various stressors. Many different HSPs have been described as associated with the GIT mucosa and its function. A transient reduction in expression of HSP70, and in crypt depth, was noted in a study performed by Liu et al. (2014), who reported that modifications to bacterial colonisation during early life functions to control the intestinal architecture and function, at least for a short period. Furthermore, longterm site- and diet-specific effects are observed in the major immune components that serve to control intestinal homeostasis. The same study reported an association between the cytoprotective HSP72 and the relative abundance of *Lactobacillus* spp. in the small intestine, together with specific members of clostridial clusters IV and XIVa in the large intestine of pigs (Liu et al., 2014).

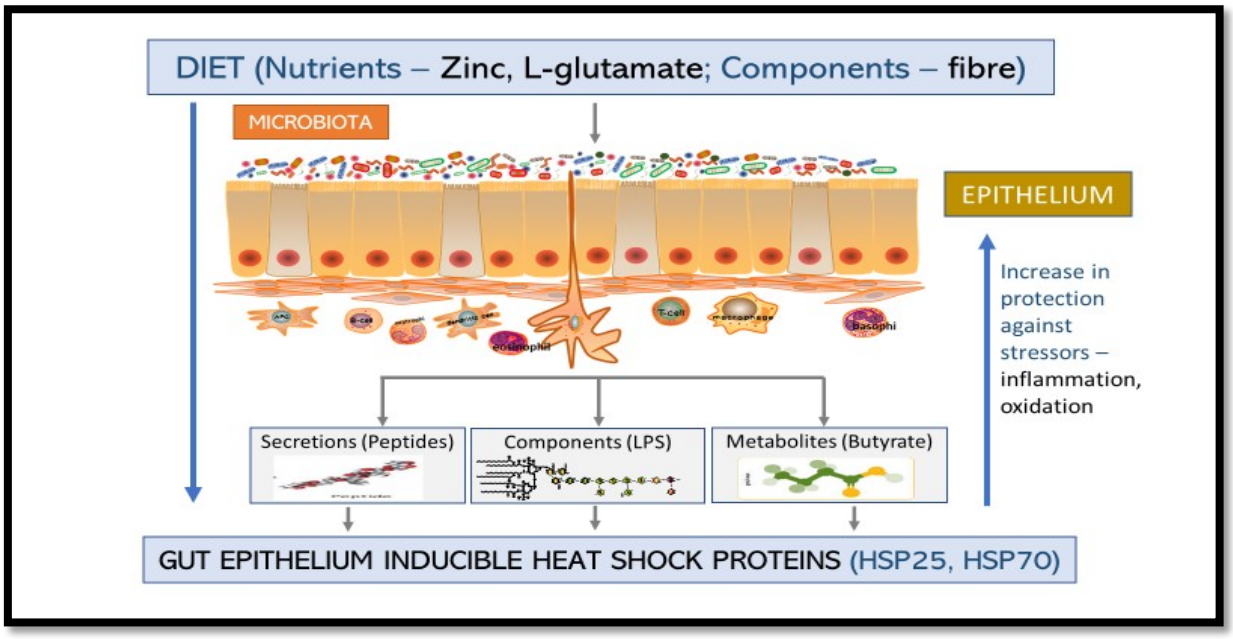

Figure 2.8.4 Interactions between dietary components and microbiota within a host.

Heat shock proteins can be induced by a broad spectrum of stimuli, including commensal microbes (Arvans et al., 2005). The physiological expression of molecular chaperones and HSPs is dependent on dietary components, commensal microbes, and resulting metabolites to which the mucosal surface is exposed (Liu et al., 2014). For example, the expression of ileal HSP27 has been correlated with inclusion of fibre in the diet. HSPs, in addition to being protein chaperones within cells, also function in immune responses, cell proliferation, apoptosis, and control of oxidation and inflammation. However, one of the most relevant functions of HSPs is their ability to regulate barrier function and minimise the adverse effects associated with inflammation and oxidative stresses on host cells. These proteins regulate the GIT barrier by controlling the expression of tight-junction proteins such as occludins (Willem van, 2015). In pigs specifically, a high concentration of HSPs is present in the small and large intestines; while an increase in duodenal and jejunal HSP70 is highly associated with foetal stress (Arnal et al., 2015).

2.8.8 Intestinal alkaline phosphatase (IAP)

The IAP is produced by enterocytes in the small intestine and secreted into the lumen and subsequently into the circulatory system, where it is involved in detoxification of microbial components by dephosphorylation (Fig. 2.9.2). The IAP confers many physiological properties including absorption of minerals and nutrients such as calcium and fatty acids, and control of GIT and systemic inflammation through detoxification of pro-inflammatory components produced by the microbiota, such as LPS and flagellin (Lallès, 2014). It is also directly involved in control of the gut barrier (Geddes & Philpott, 2008). Moreover, when IAP is bound to enterocytes it can function to delay the growth of potential pathogens such as *E. coli* and, hence, influence the microbiota

composition while restricting the translocation of *E. coli* into the body (Bates et al., 2007; Malo et al., 2010). Stressful conditions, such as weaning, significantly inhibit IAP production in pigs, which is responsible for development of many post-weaning disorders as well as an increased sensitivity of pigs to enteric infections (Arnal et al., 2014).

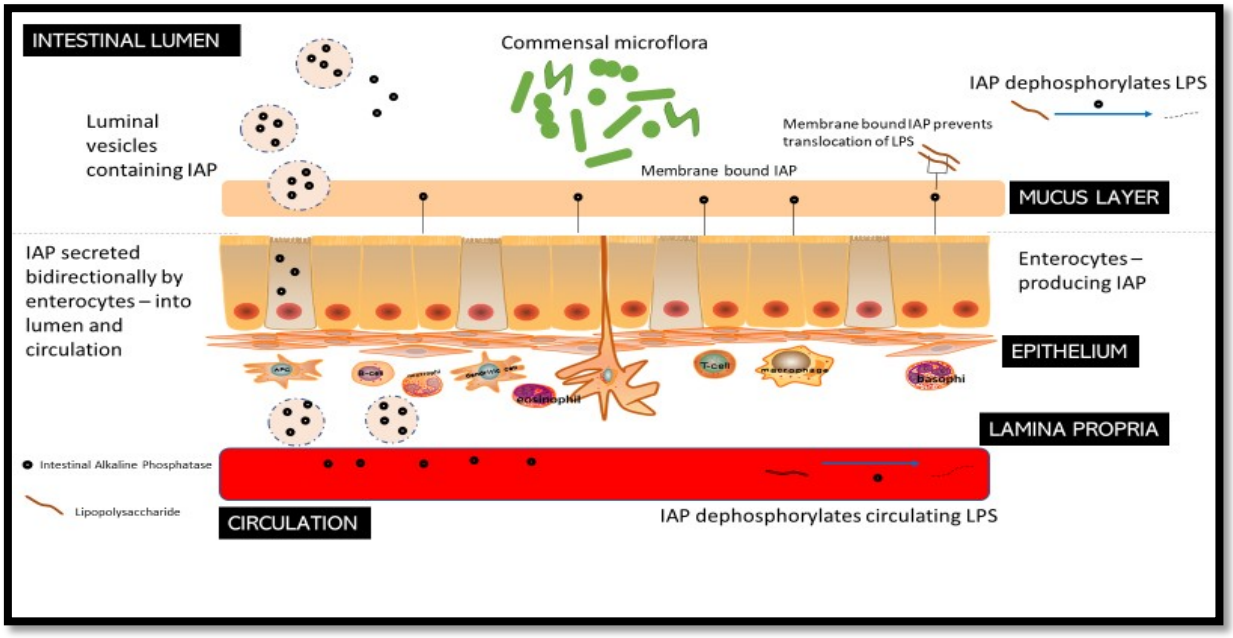

Figure 2.8.5 Intestinal Alkaline Phosphatase (IAP) – roles in the GIT.

IAPs secreted by the enterocytes travel bi-directionally into the blood circulation and to the intestinal lumen where they act to dephosphorylate LPS from Gram-negative bacteria. Membrane-bound IAPs also prevent the translocation of the LPS through the mucus layer.

Both the gene expression and enzymatic activity of IAP are influenced by the GIT microbiota, while IAP simultaneously impacts the composition of the gut microbiota by removing proinflammatory free luminal adenosine triphosphate (ATP), by reducing the level of inflammation, and by regulating intestinal surface pH (Mizumori et al., 2009). Thus, the expression of iHSPs in gut epithelial cells is proportionate to the number of microbes present along the GIT.

2.9 Mechanisms of interactions between microbiota and host components

It has been suggested that mammals possess a developmental window (2–3 weeks in pigs and a similar duration in other mammals) in which the developing host–gut–microbiota interactions are easiest to manipulate and during which time it is most susceptible to major disturbances (Thompson et al., 2008). Although the molecular mechanisms of these interactions are not clearly defined, limited data in pigs suggest that IAP and iHSPs have important roles in this process, especially in controlling inflammation and modulating gut function. These two components are involved in regulating antioxidant and anti-inflammatory reactions, by which they confer protection to the GIT epithelium (Lallès, 2016).

2.10 Conclusion

The resident GIT microbiota is unique for each species and has continually evolved over generations to become more functional and relevant to their current local environment and the host. Its prominent role in stimulating the maturation of the GIT and regulating the gut–brain axis, especially in young pigs, represents opportunities to design effective strategies to increase animal robustness (Trevisi & Pérez, 2017). There is, hitherto, no consensus on the definition of balanced or favourable microbiota even though knowledge on host–microbiota crosstalk is constantly being updated, revealing a highly complex scenario. Moreover, studies attempting to define the factors affecting the GIT microbiota and their subsequent roles in pig physiology and immunity are still in progress. Future studies will serve to inform the development of hypothesises for effective strategies to manage and restore intestinal homeostasis after an external perturbation, such as stress, early administration of an antibiotic, or a bacterial infection, all of which ultimately could be utilised to improve productivity, minimise stress and prevent diseases.

Chapter 3

Materials & Methods

Most of the materials and methods used in the project are described in this chapter. Certain methods specific to particular experiments are described in their respective chapters 4, 5 and 6.

All chemicals used in this study were purchased from accredited suppliers in USA and/or China.

3.1 Animals

The experimental protocols for management and care of pigs and mice were pre-approved by the Animal Care and Use Committee of Guangdong Ocean University, Zhanjiang, China (Permit No. 206-1108), where the experiments were conducted.

Pig study: Twelve pigs (Luchuan sows × Duroc boars; 6 males and 6 females), each initially weighing 15 ± 2 kg, were procured from a local supplier in Zhanjiang, Guangdong, China. They were pre-tested for diseases and symptoms and accepted only after found free of diseases. They were housed in two different pens for 1 week prior to commencing the experiment, for the animals to become acclimatised to their new surroundings.

Mice study: One hundred and thirty C57BL/6 strain mice were procured from an accredited supplier in Zhanjiang, China. The mice were housed, 5 to a cage (all males in one cage, all females in another), with circulating air (Exhaust Ventilated Closed-System Cage Rack, Guchuang Education Laboratory Animal Cage Co., China). Environmental conditions were: 12hr light-12hr dark cycles and 60-70% humidity.

3.1.1 Feed and water

Pig study: The pigs were fed a commercial diet (complete feed formula; Charoen Pokphand Group, China; 79% sorghum, 10% soybean meal, 7% calcium and protein supplement, 3.75% peanut meal, and 0.25% salt).

Mice study: The mice were fed with autoclave-sterilised commercial-grade chow (LabDiet® formulations 5K52, 6%; 5K20, 11%).

All animals had access to purified water *ad libitum* during acclimatisation and throughout the experiments.

3.2 Effect of heat stress on pig gut microbiota and physiology

Six pigs were subjected to temperatures of 35 ± 2°C and 75–85% relative humidity. The other 6 pigs acted as the controls and were kept at 23 ± 2°C but the same relative humidity as the test pigs. The two pens were maintained at the specific temperatures with use of heaters and coolers (Mitsubishi Electric Co., Japan).

The rectal and forehead temperatures of the pigs were measured on days 1, 7, 14 and 21 using a non-contact hand-held infrared thermometer (TMC testing services, Shenzhen, China). Fresh faeces were collected immediately and aseptically (by an experienced collector wearing Fisherbrand powder-free nitrile exam gloves) at each of sampling days 1, 7, 14 and 21. Faeces were stored in sterile test-tubes under liquid N_2 and later at −80°C until used for microbiome composition analyses.

3.2.1 Animal sacrifice

Pigs were sacrificed on day 21 by head-only electric stunning (Hubert Haas, TBG 100). Colonic faeces and colon tissue samples were collected and immediately stored under liquid N_2 and later at −80°C until used for subsequent transcriptomic and cell culture (subjecting IPEC-J2 cells to LPS) analyses.

3.3 Effect of heat stress on pig immune system

3.3.1 Morphological observations

The colonic tissue was fixed in 10% buffered formalin, embedded in paraffin wax, sectioned with a microtome (Microm HM 560, Microm International GmbH, Dreieich, Germany) at 5µm and stained with haematoxylin and eosin (H&E). Some sections were stained with 0.5% periodic acid solution (PAS). Image-Pro Plus, v 6.0 (Media Cybernetics Inc., Silver Spring, USA), was used to measure villus height, crypt depth and width (Nagao-Kitamoto et al., 2016). Photomicrographs of the colonic tissue were taken and Image-Pro Plus 6.0 software used to count the number of intestinal mucosal goblet cells per unit area (Lan et al., 2015).

3.3.2 Western Blot analysis

To detect the response of the critical proteins of the TLR4/NF-κB signalling pathway in the intestinal tissues, total protein in the colon was extracted with RIPA (radioimmunoprecipitation assay buffer) lysate. Cytoplasmic and nuclear proteins were extracted, and the protein concentration determined by the Bicinchoninic Acid (BCA) method, and SDS-PAGE (Sodium dodecyl sulphate – polyacrylamide gel electrophoresis) was performed, which entails the separation of proteins by their mass. The antibody was incubated after transfection and the electrochemiluminescence (ECL) method was used for luminescence detection and protein quantification; in which electron transfer produced between molecules due to the applied electric potential causes a light to be emitted.

3.3.3 TLR4, MyD88, and TRAF6

Using the paraffin sections of intestinal tissues prepared previously (described in section 3.3.1), TLR4, MyD88 (myeloid differentiation primary response 88 protein), and TRAF6 [tumour necrosis factor receptor (TNFR)-associated factor 6] positive cells were detected by immunohistochemistry (IHC), and p65 (NF-κB) activity was also detected (see 3.3.4). Results are expressed as means ± standard deviation. One-way ANOVA and Tukey's multiple comparisons test were performed on the data in SPSS v. 21.0 (IBM Corp., Armonk, NY, USA); $P \leq 0.05$ indicates a significant difference, and $P \leq 0.01$ a highly significant difference.

3.3.4 Detection of p65 protein entry into the nucleus

From the intestinal tissue stored at −80℃, 8μm frozen sections were prepared and fixed in 4% paraformaldehyde for 10 min. They were rinsed three times with phosphate-buffered saline (PBS) for 2 min each time and blocked with 1% bovine serum albumin (BSA) for 1 h. The p65 primary antibody was diluted 1:100 and 100 μL of it was added to each section of the tissue and placed in a wet box at 4℃ overnight. It was again rinsed three times with PBS (pH 7.4) for 10 min each time. Alexa Fluor 647-labelled goat anti-rabbit IgG (H+L) antibody was diluted 1:1,000. This secondary antibody was added dropwise and then incubated in an opaque wet box for 1.5 h. The tissue was then washed three times with PBS (pH 7.4), 10 min each time. The tissue was immersed in DAPI (4',6-diamidino-2-phenylindole); blue-fluorescent DNA stain (LeaGene Biotechnology, Beijing, China) that exhibits ~20-fold enhancement of fluorescence upon binding to AT regions of dsDNA for 10 min, and rinsed with PBS three times, 10 min each time. The anti-quenching sealer (Elabscience E-IR-R119) has a strong anti-fluorescence decay effect and it is used to seal fluorescent tissue samples. This was mounted and photographed under a fluorescence microscope.

3.3.5 Microbial genomic sequencing and analysis

Total faecal DNA was isolated using the 'repeated bead-beating' method with a mini-bead beater (Biospec Products, Bartlesville, OK, USA) as described previously (Hu et al., 2016)(Yu & Morrison, 2004). Total genomic DNA from the colonic tissue cells was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, China; 51504) according to the manufacturer's instructions. The DNA concentration and purity were monitored in 1% agarose gels. The quantity of DNA was measured using a NanoDrop 1000 spectrophotometer, and the DNA concentration was diluted to 1 ng/μL using sterile water. The V3–V4 distinct regions of 16S rRNA genes were amplified using specific primers with barcodes. These PCR reactions were performed in triplicates in a total volume of 25 μL containing 5 μM of each of the primers, 10 ng of the DNA template, 4 μL 1×FastPfu buffer, 2.5 mM of dNTPs, and 0.4 μL of FastPfu polymerase (TransGen Biotech, Beijing, China).

The PCR procedure was as follows: initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 50 s, 55°C for 30 s, and 72°C for 50 s, and a final extension at 72°C for 6 min. The PCR products mixture was purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, USA). Amplicons produced from both control and test samples were sent to a commercial company (Majorbio, Shanghai, China) for sequencing on an Illumina HiSeq 2500 platform (Xiao et al., 2017).

The raw data from Illumina HiSeq high-throughput sequencing was pre-processed to obtain accurate and reliable results in the bioinformatics analyses. Following this, bacterial tags were grouped into OTUs (operational taxonomical units) at a sequence similarity of 97% by scripts of Mothur (v1.31.2) software (Schloss et al., 2009). Representative sequences of the bacterial OTUs were taxonomically classified by Mothur scripts based on the Ribosomal Database Project (RDP) database (Cole et al., 2009). The 'VennDiagram' of R (v3.0.3) software was used to draw the Venn diagrams which denote the number of common and unique OTUs among groups. Similarly, the package 'ade4' of R (v3.0.3) software was used for Principal Component Analysis (PCA) based on OTUs' abundance. The OTU representative sequences were compared with the microbial reference database. The species classification information corresponding to each OTU was obtained. Next, the sample community composition was calculated at each level (phylum, class, order, family, genus, species) generated by using QIIME (Quantitative Insights into Microbial Ecology) software.

The Chao, Shannon, and Simpson indices, which reflect alpha diversity, were calculated by Mothur (v1.31.2) and the corresponding rarefaction curves were drawn by R (v3.0.3) software. The QIIME (v1.80) software was also used to perform Beta diversity analysis based on weighted UniFrac distance and displayed as the principal coordinates analysis (PCoA). Heat maps were generated using the package 'gplots' of R (v3.0.3) software. By comparing the OTU representative sequence with the microbial reference database, the species classification information corresponding to each OTU was obtained. The sample community composition was calculated at each level (phylum, class, order, family, genus, species) and generated using QIIME. The species abundance tables at different taxonomic levels were then mapped using R language tools into community structure maps at each taxonomic level.

The software GraphPad Prism (version 6.0c), R (v3.0.3), Metastats, and Statistical Analysis of Metagenomic Profiles (STAMP) were used for the statistical analyses (Parks et al., 2014; White et al., 2009). Statistical comparisons of weighted UniFrac distances among groups were carried out using the analysis of similarities; package 'vegan' of R (v3.0.3).

In univariate analysis of gut microbiota and predicted Kyoto Encyclopaedia of Genes and Genomes (KEGG) biochemical pathways for each group, one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test was performed for the comparison of alpha diversities among

groups. Metastats software was used to identify the differentially abundant taxa (phyla, genera, and species) among groups. The significant differences between groups were analysed using the LEfSe [Line Discriminant Analysis (LDA) Effect Size] method.

3.3.6 Transcriptome sequencing and genomic sequence analyses

The enzymatic-free cryopreservation tube was pre-cooled in liquid N_2, and the intestinal tissue was quickly removed from the animal and cut into pieces of similar size to soybean granules. Using RNase-free water to prepare 1x PBS or saline, the tissue surface stains were quickly cleaned, and the surface liquid was absorbed and collected into the freezing tube. Then the freezer tube was rapidly transferred to liquid N_2 for cryopreservation and the samples were sent to a sequencing company (Majorbio, Shanghai, China) for sequencing. Eukaryotic mRNA sequencing is based on the HiSeq platform for sequencing all mRNAs transcribed from specific tissues of eukaryotes at a particular time. The total RNA was extracted from the tissue samples, the concentration and purity of the extracted RNA detected by Nanodrop2000, the RNA integrity detected by 1% SDS-PAGE, and the RNA Integrity Number (RIN) value determined by Agilent 2100. The eukaryotic mRNA 3' end has a structure of a polyA tail.

Magnetic beads with Oligo (dT) 15 primer (Santa Cruz Biotechnology) were used for A-T base pairing with the flo A, and the mRNA was isolated from the total RNA for transcriptomic analyses. The fragmentation buffer was added to randomly break the mRNA into small fragments of about 300 bp. Under the action of reverse transcriptase, six-base random hexamers were added, and the mRNA was used as a template to reverse the synthesis of one-strand cDNA, followed by two-strand synthesis to form a stable double-stranded structure. After connecting to the adaptor, short sequence fragments were sequenced using the Illumina HiSeq platform. An Illumina sequencing single-run can generate billions of reads. Hence, statistical methods were used for quality control of the measured sequences, which can visually reflect the quality of library construction and sequencing of the samples. The quality data after the quality control (reads) was compared with the reference genome to obtain the mapped data (reads) for subsequent analysis, and the quality of the comparison results of the sequencing was evaluated. Based on existing reference genomes, the mapped reads were assembled and spliced using the software Cufflinks, and compared with known transcripts, transcripts without annotation information, and functional annotations of potential new transcripts. Read Counts for each sample gene/transcript were obtained using 'featureCounts', using alignment to genome results and genome annotation files. This was then subjected to FPKM (fragments per kilo base) conversion to obtain standardised gene/transcript expression levels. After securing the number of Read Counts of the gene/transcript, differential analysis of the expression of

the gene/transcript between samples was performed on the multi-sample (≥2) project, and the differentially expressed genes/transcripts identified for study.

3.3.7 RNA extraction

To extract RNA from the pig tissue samples, about 0.5 g of the prepared intestinal tissue blocks were ground to a powder in liquid N_2. The mixture was transferred to a 1.5-ml centrifuge tube, and 1 ml of Trizol on ice added for 20 min. During that time, the centrifuge tube was inverted to avoid insufficient lysis due to tissue sedimentation at the bottom of the tube. Then 200 µl of NH_3Cl was added, the tube shaken vigorously to emulsify the mixture, then allowed to stand for 5 min at room temperature, before being centrifuged for 10 min at 4℃. Next, 200 µl of supernatant was transferred into a new centrifuge tube, and an equal volume of isopropanol added, and mixed upside down, letting it stand at room temperature for 10 min, then centrifuged for 10 min at 4℃. The supernatant was discarded, 1 ml of 4℃ pre-cooled 75% ethanol added, the tube gently inverted and allowed to stand for 2 min, before being centrifuged at 12,000g for 5 min at 4℃. The supernatant was then removed and dried at room temperature for 5 min before 20 µl of RNase-free double-distilled H_2O was added to dissolve RNA before storage at −80℃. From this extraction liquid, 2 µl was taken, and 1% SDS-PAGE performed to check its integrity. The 2 µl sample of RNA liquid was diluted 100 times, and DEPC water used as a blank control to detect the OD260/OD280 value. The sequences of synthetic primer pairs purchased from BBI Life Sciences, Shanghai, China, and were used in the PCR (for IL6, 8, 17 and control β-actin) as shown in Table 3.3.1 below.

Table 3.1 Primer sequences

Gene	Primer Sequences (sense and antisense)
IL-6	CACCGGTCTTGTGGAGTTTC
	GTGGTGGCTTTGTCTGGATT
IL-8	TTTCTGCAGCTCTCTGTGAGG
	CTGCTGTTGTTGTTGCTTCTC
IL-17	CCAGACGGCCCTCAGATTAC
	CACTTGGCCTCCCAGATCAC
β-actin	AAGTACTCCGTGTGGATCGG
	ACATCTGCTGGAAGGTGGAC

Based on the HiScript® Q-Select RT SuperMix for qPCR (+gDNA wiper) kit instructions, the genomic DNA was removed, and a reverse transcription reaction system was prepared on ice. The PCR machine was set at 25℃ for 10 min, 50℃ for 30 min, and 85℃ for 5 min. After the reaction was completed, the cDNA fractionation apparatus was stored at −80℃ until analyses.

3.3.8 Relative Quantitative Real-Time PCR

The reaction system was configured according to the ChamQTM SYBR® qPCR Master Mix kit for quantitative fluorescence PCR. The relative level of RNA expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

3.3.9 Cell culture

The intestinal porcine epithelial cell (IPEC-J2) model was a gift from Dr Bruce Schultz, Kansas State University, USA. Cells were cultured in a humidified incubator at 37°C and 5% CO_2 in a 25-cm, two-cell culture flask (Corning Inc., Corning, NY, USA). Cells were grown in Dulbecco's modified Eagle's medium [a nutrient mixture (DMEM/F12; Sigma Aldrich, St. Louis, MO, USA) containing 10% FBS (foetal bovine serum), penicillin (100 U/ml), and streptomycin (100 µg/ml)]. After growing the IPEC-J2 to sub-confluence in 24-well plates, the culture medium was removed, followed by washing twice with PBS.

3.3.10 The *in vitro* validation experiment of LPS

One gram of colonic faeces was weighed from each sample and washed three times with PBS for a final faecal mass: PBS volume of 1:9. The samples were diluted and pulverised using an ultrasonic cell pulveriser for 15 min (Φ6 horn amplitude ultrasonic treatment 1.5 s, interval 2 s, power 25%). After centrifugation at 5,000g for 10 min, the supernatant was collected for ELISA detection of LPS (lipopolysaccharide). IPEC-J2 cells were treated with 10 µg/ml LPS for 3 h. Western Blot analysis was carried out for expression of TLR4 and p65 in the nucleus detected.

3.4 Faecal transplantation of pig faecal matter into mice

All experiments on mice were conducted at an ambient temperature of 26 ± 1℃. Pseudo-germ-free animals (SPF grade) were obtained by feeding C57BL/6 strain mice (*n* = 130) with an antibiotic mixture of vancomycin (200 mg/kg), metronidazole (200 mg/kg), ampicillin and neomycin sulphate (200 mg/kg) (all sourced from Abbott Pharmaceuticals, China) consecutively for 5 days. The mice were allowed 1 week to acclimatise to their surroundings before the experiment began. Throughout the study period, all mice were maintained under a 12-h light/12-h dark photoperiod, and an air circulation cycle under an Exhaust Ventilated Closed-System Cage Rack. They had *ad libitum* access to autoclave-sterilised commercial grade-chow and water.

To study the effect of pig faecal transplantation on the intestinal microbiota and the expression of the TLR4/NF-κB signalling pathway in mice, 120 out of the 130 SPF mice were divided into four groups of 30 mice each. The mice were further divided into three treatment groups of 10 mice each (control – CF, heat stress – HF, and PBS groups). The CF group was fed with faeces from CON (control)

pigs, the HF group with faeces from HS pigs, and the remaining 10 mice were only fed PBS and no faeces. Ten additional mice were used as untreated controls and were fed only chow and water (neither faeces nor PBS).

The faeces collected from pigs in the previous experiment were homogenised with 1.5 ml of PBS and then 0.5 ml of the mixture was orally gavaged to each SPF mouse by intragastric administration. The PBS group of mice were given 0.5 ml of PBS only. At each of the pig faeces sampling days 1, 7, 14 and 21, fresh faeces were collected from the mice on sacrifice. The mice faeces were immediately stored under liquid N_2 at −80°C until further analyses. Similar to the pig experiments, the blood and intestinal tissue of mice were also collected for analyses of TLR4, TRAF6, and nuclear p65 upregulation at RNA and protein level, and upregulation of pro-inflammatory cytokines like IL-6, IL-8, and IL-17. All the groups of mice were periodically sacrificed after 7 days of FMT.

Chapter 4

Effect of Heat Stress on Gastrointestinal Microbiota of Pigs

4.1 Abstract

Heat stress effects on the physiology and GIT microbiota composition of pigs were investigated in this experiment. One group of 6 pigs (HS) were subjected to atmospheric temperatures of 35 ± 2℃ and another group of 6 control (CON) pigs were kept at temperatures of 23 ± 2℃, at 75–85% relative humidity for 21 days. The average forehead temperature of the HS group increased on day 3 to reach 37.77°C, which was 1.03°C higher than that of the CON group. The average rectal temperature of HS pigs on day 3 was 40.46°C, 2.84°C higher than for the CON group. By day 21, the average forehead temperature of the HS group had gradually declined but was still significantly higher than for the CON group. The average weight gains in the HS group on days 7, 14, and 21 were 6.41%, 61.30% (P < 0.01), and 88.71% (P < 0.01) lower, respectively, than those for the CON group. Changes in the GIT microbiota were studied by analysing the freshly collected faeces from the pigs on days 1, 7, 14, and 21. As the experiment progressed from day 1 to 21, the qualitative and also quantitative composition of the GIT microbiota changed. Major bacterial phyla such as the Gram-positive *Firmicutes* decreased in number whereas the Gram-negative *Bacteroidetes* increased. Other major potentially pathogenic phyla, such as *Proteobacteria* and *Spirochetes,* also increased through the course of the experiment. This increase in pathogenic strains within the GIT strongly affects not only the local physiology in the pigs, but also their immune system functions.

4.2 Introduction

The recent rise in atmospheric temperatures due to global warming has brought to light the detrimental effects of heat stress (HS) on animal welfare and livestock production. Pigs suffer from HS when the ambient temperature exceeds their thermal neutral zone (16–22℃ for growing–finishing pigs) (Pearce & Escobar, 2013; Van Heugten et al., 1996). Pigs are more sensitive than other livestock animals to HS, because they produce high metabolic heat, lack functional sweat glands, and exhibit rapid fat deposition (S.; Ganesan et al., 2016). Heat stress conditions in pigs cause a decrease in food intake, with consequent lower bodyweight gain, and reduced pork meat quality. These effects together can result in severe economic losses, as has been evident in the US swine industry, which lost over $300 million to HS in 2010 alone (S. C. Pearce et al., 2013).

The mammalian GIT plays host to a broad, diverse, complex microbial community (known as its ‘microbiota’) that can be considered as a separate independent organ owing to its influence on host physiology and its effects on health and disease (Bäckhed et al., 2005). The porcine GIT, which

consists of about 400 different phylotypes (Leser et al., 2002), is among the most densely populated microbial ecosystems, home to about 10^{14} cells, which is 10 times more than the total eukaryotic cells present in the host (Isaacson & Kim, 2012). The gut microbiome in animals constitutes a diverse variety of micro-organisms that influence many aspects of its host (O' Hara & Shanahan, 2006). These microbial communities begin to appear in specific locations within the body, most prominently in the gut, shortly after birth. They are highly host-specific and stable over time. Their roles in host physiology and health can be understood more clearly by studying their taxonomical composition and functional capacities (Ley et al., 2006). The health and performance of pigs are greatly influenced by the gut microbiome as it impacts the animal's physiological, nutritional, and immunological processes (Richards et al., 2005).

As well as playing a crucial role in maintaining the physiological, nutritional, and immunological functions of the pig (Fouhse et al., 2016), the gut microbiota is also important for the development of the intestine and the innate immune system (Collado et al., 2012; Ivanov & Honda, 2012; Matamoros et al., 2013). Microbial colonisation of the piglet gut by *E. coli* and *Streptococcus* spp. begins immediately after birth. After birth, an anaerobic environment is created for successive colonisers such as *Bacteroides, Bifidobacterium,* and *Clostridium*. The final microbial composition depends on what diet the piglet is exposed to and its surrounding environment. *Lactobacillus* spp., which are beneficial microbes, prevail in the small intestine of the milk-fed piglet until weaning, and play a significant role in disease prevention (Konstantinov et al., 2006a; Petri et al., 2010). Numerous studies of the gut microbial community have consistently shown that populations and diversity of constituent organisms are shaped by exposure to environmental microbes, diet, immunological stressors, host genetics, and ecological forces within the ecosystem itself. Although gut microbes have been studied for decades, most of the work related to their structure and functional roles has been carried out using culture-dependent techniques. In recent years, the development of culture-independent techniques such as 16S rRNA gene sequence analysis has revealed the diversity and structures of many hitherto unknown communities within many animal gut environments (Lamendella et al., 2011; Ruth et al., 2008).

Many studies have elucidated the adverse effects of HS on the physiology of pigs (Baumgard & Rhoads, 2013; Bernabucci et al., 2010; Hicks et al., 1998), but very little is known about how HS affects the microbiota within the pig gut. The substantial interest in the scientific community in gut microbiota studies employing pigs as models is because pigs are very similar to humans in terms of both anatomy and nutritional physiology (Wilson & Obradovic, 2015; Zhang, 2013).

The present study has focussed on exploring changes in microbial diversity and taxonomical composition of the bacterial community in the GIT of pigs exposed to persistent HS. This knowledge

will improve our understanding of the role of different gut organisms in the physiology of the host animal and how the GIT microbiota affects the immune system function of pigs.

4.3 Materials and methods

4.3.1 Animals and management

For this study 2-month-old pigs (Luchuan sows × Duroc boars; 6 males and 6 females), each initially weighing 15 ± 2 kg, were used. These animals were housed in two pens at the Guangdong Ocean University Animal Facility, Zhanjiang, China. Six pigs (HS) were placed in a pen with a temperature set at 35 ± 2℃ and 6 (CON) in another pen at an ambient temperature of 23 ± 2℃. Relative humidity in both pens was 75–85%. The animals were allowed an environmental adaptation period of 1 week before the start of the experiment. All animals were fed twice a day on a commercial diet (a complete feed formula from Charoen Pokphand Group, China) throughout the experiment at 5% of their metabolic bodyweight. The diet was free of antibiotics or probiotics, which have the potential to alter the intestinal microbiota. Water was available *ad libitum* throughout the experiment and acclimatisation period. Rectal and forehead temperatures were measured with a contact thermometer every day, and bodyweights were recorded on the sampling days 1, 7, 14, and 21. Animals were continually observed for diarrhoea and other clinical signs. The experimental protocol on the management and care of the animals was reviewed and approved before-hand (Approval #206-1108) by the Animal Care and Use Committee of Guangdong Ocean University, Zhanjiang, China.

4.3.2 Body temperature and weight

The forehead and rectal temperatures of the pigs were measured daily with a hand-held infrared thermometer. The animals were closely monitored throughout the day and all pigs were observed for diarrhoea and body weight changes. At each sampling time (days 1, 7, 14, 21), three pigs each from the control and treatment groups were electrically shocked and sacrificed.

4.3.3 Faeces

On sampling days 1, 7, 14, and 21, fresh faeces were immediately collected aseptically by a collector wearing disposable plastic gloves, the samples labelled and stored at −80℃ for subsequent microbiome and transcriptomic analyses and cell culture (subjecting IPEC-J2 cells to LPS). Faecal shape and colour were recorded daily and scored, as shown in Table 4.3.1. The Diarrhoea Index (DI) was calculated according to the scores for six pigs sacrificed at each sampling time:
Diarrhoea Index = sum of scores / 6 (Rossi et al., 2012)

Table 4.1 Diarrhoea Index

Point	Degree of hardness/softness
1	Hard, dry, and blocky
2	Hard
3	Soft, but lumpy
4	Smooth and soft
5	Watery
6	Watery, yellow, foamy

4.3.4 Genomic analyses

4.3.4.1 Microbial genomic DNA extraction

Total faecal DNA was isolated using the 'repeated bead-beating' method with a mini-bead beater (Biospec Products, Bartlesville, OK, USA) as described by Hu et al. (2016) and Yu & Morrison (2004). Total genomic DNA was extracted from the samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Hamburg, Germany) according to the manufacturer's instructions. The DNA concentration and purity were determined on 1% agarose gels. The quantity of DNA was measured using a NanoDrop 1000 spectrophotometer, and the DNA concentration was diluted to 1 ng/µL using sterile water so that samples fell within the detection range of the instrument (Xiao et al., 2017).

4.3.4.2 Sequencing data analysis

The V3–V4 distinct regions of 16S rRNA genes were amplified using specific primers with barcodes. The PCR reactions were performed in triplicates in a total volume of 25 µL containing 5 µM of each of the primers, 10 ng of the DNA template, 4 µL 1×FastPfu buffer, 2.5 mM of dNTPs, and 0.4 µL of FastPfu polymerase (TransGen Biotech, Beijing, China).

The PCR procedure was as follows: initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 50 s, 55°C for 30 s, and 72°C for 50 s, and a final extension at 72°C for 6 min. The PCR products mixture was purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, USA). Amplicons produced from both control and test samples were sent to a commercial company (Majorbio, Shanghai, China) for sequencing on an Illumina HiSeq 2500 platform (Xiao et al., 2017).

The raw data from Illumina HiSeq high-throughput sequencing was pre-processed to obtain accurate and reliable results in the bioinformatics analyses. Following this, bacterial tags were grouped into

OTUs at a sequence similarity of 97% by scripts of Mothur (v1.31.2) software (Schloss et al., 2009). Representative sequences of the bacterial OTUs were taxonomically classified by scripts of Mothur (v1.31.2) based on the RDP database (Cole et al., 2009). 'VennDiagram' of R (v3.0.3) software was used to draw the Venn diagrams, which denote the number of common and unique OTUs among groups. Similarly, the package 'ade4' of R (v3.0.3) software was used for PCA, based on OTUs abundance. The software QIIME (v1.80) built-in scripts were used for construction of the genus-level phylogenetic trees and were imaged using R (v3.0.3) software (Caporaso et al., 2010).

The changes in the OTUs of the porcine faeces were monitored, and the copy number of the 16S rRNA gene fragments of *Firmicutes*, *Bacteroidetes*, *Bacteroides,* and other significant bacterial genera was determined using real-time PCR.

The Chao, Shannon, and Simpson indices, which reflect alpha diversity, were calculated by Mothur (v1.31.2), and the corresponding rarefaction curves were drawn by R (v3.0.3) software. QIIME (v1.80) was also used to perform Beta diversity analysis based on weighted UniFrac distance and displayed as the PCoA. Heat maps were generated using the package 'gplots' of R (v3.0.3) software. By comparing the OTU representative sequence with the microbial reference database, the species classification information corresponding to each OTU was obtained. The sample community composition was calculated at each level (phylum, class, order, family, genus, species) and generated using QIIME software. The species abundance tables at different taxonomic levels were then mapped using R language tools into the community structure maps at each taxonomic level.

4.3.5 Statistical analyses

The body temperature data was subjected to a *t*-test using SPSS (v21.0) software, and the results were expressed as mean ± standard deviation. $P \leq 0.05$ indicates that the difference is significant, and $P \leq 0.01$ suggests that the difference is highly significant.

For faecal genomic data, the software GraphPad Prism (v6.0c), R (v3.0.3), Metastats (White et al., 2009), and STAMP (Statistical Analysis of Metagenomic Profiles) were used for the statistical analyses (Parks et al., 2014). Statistical comparisons of weighted UniFrac distances among groups were carried out using the analysis of similarities (ANOSIM), package 'vegan' of R (v3.0.3). One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test was performed for the comparison of alpha diversities among groups. Metastats software was used to identify the differentially abundant taxa (phyla, genera, and species) among groups. The STAMP software was used to detect the differentially abundant KEGG pathways among groups with false discovery rate correction.

4.4 Results

4.4.1 Physiological changes

The forehead and rectal temperatures of the pigs were measured daily. The HS pigs exhibited a higher body temperature immediately after the commencement of exposure to HS. The bodyweights were taken on sampling days 1, 7, 14, and 21 (Figs 4.4.1 and 4.4.2). Although both sets of pigs were fed the same quantity of feed, the HS pigs gained less weight over the 21 d as compared to the CON pigs (Fig. 4.4.3).

The average forehead temperature of the HS group increased on day 3 to reach 37.77°C, which was 1.03°C higher than in the CON group. The average rectal temperature on day 3 was 40.46°C, which was 2.84°C higher than that of the CON. By day 21, the average forehead temperature of the HS group had gradually declined but was still significantly higher than in the CON group. The weight gains on days 7, 14, and 21 respectively in the HS group were 6.41%, 61.30% ($P < 0.01$), and 88.71% ($P < 0.01$) lower than those for the CON group (Fig. 4.4.4).

The pigs were monitored daily for the occurrence of diarrhoea. Diarrhoea was observed in HS pigs from day 6 onwards. The DI of HS pigs was 1.5, 2.8, 5, and 2.5 on sampling days 1, 7, 14, and 21, respectively (Fig. 4.4.4). The shape and colour of each pig's faeces was recorded daily and a group score was determined, based on the DI method. The scores of the six pigs in each group were summed and divided by 6. A score of >3 was considered as diarrhoeal. The HS pigs were therefore regarded as diarrhoeal on day 14.

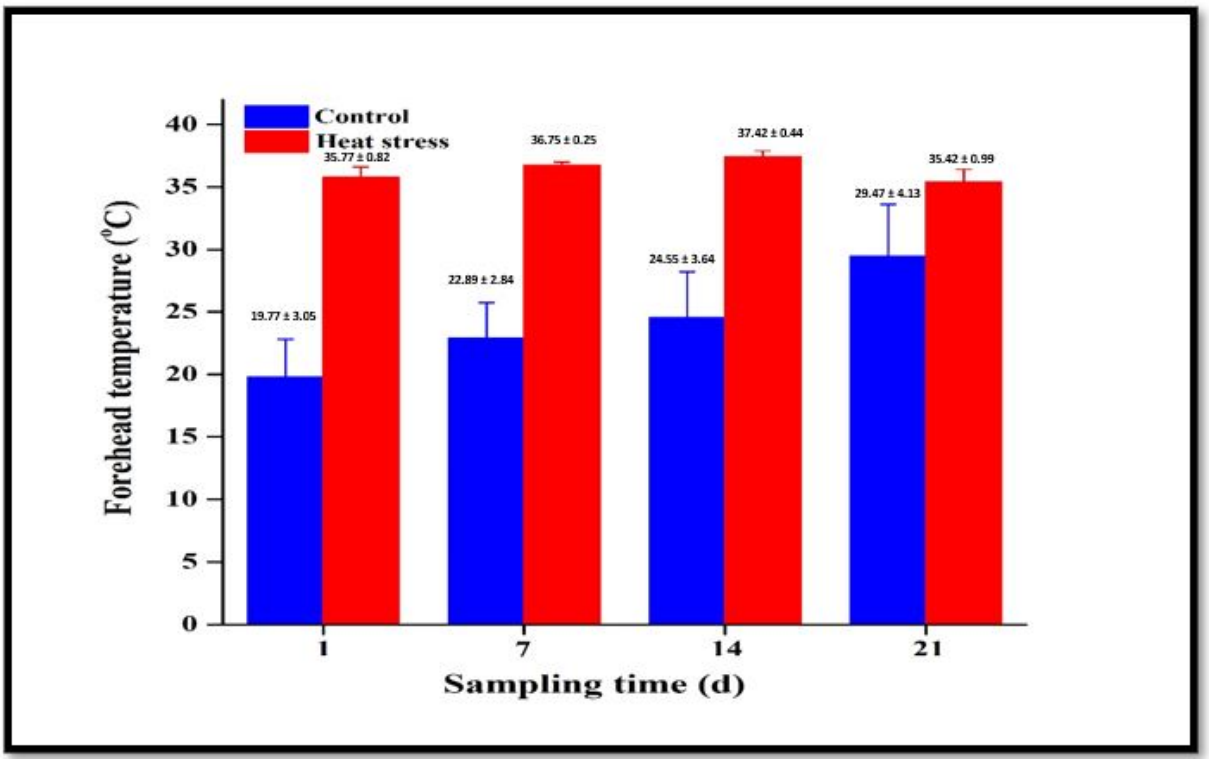

Figure 4.4.1 Average forehead temperatures for each sampling day. Results are group means ± standard deviation.

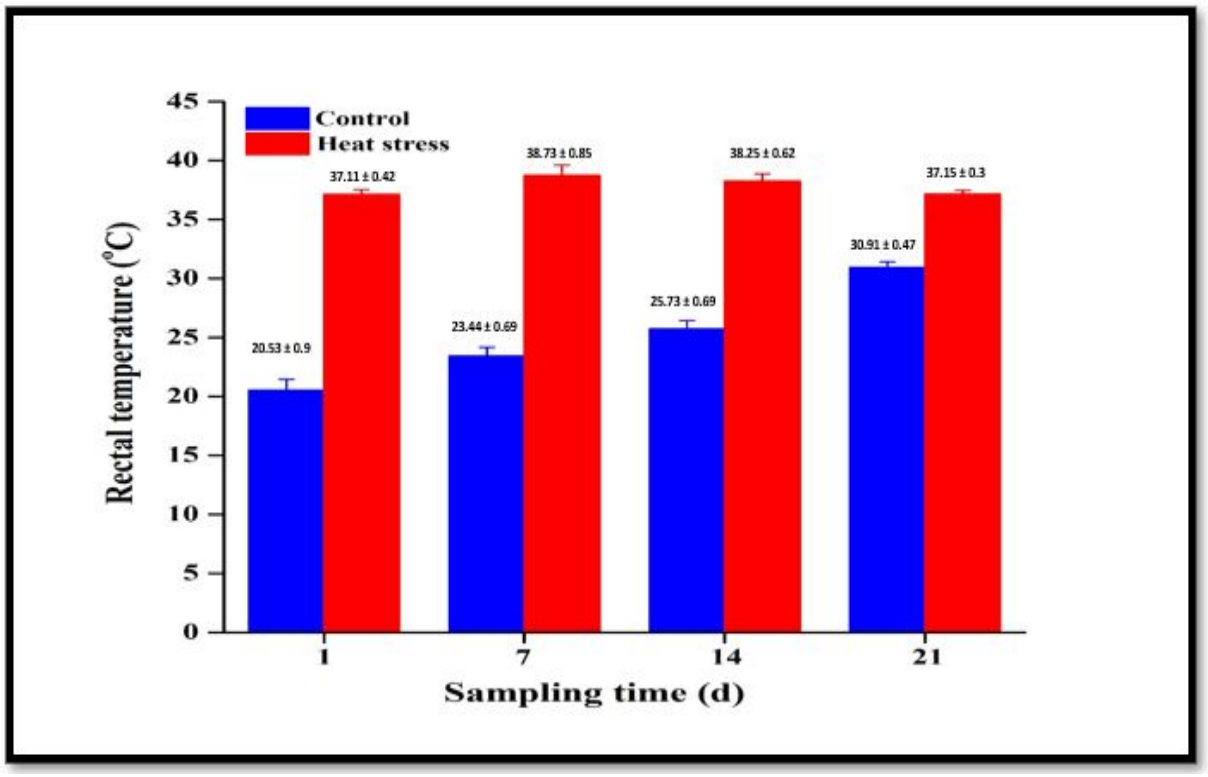

Figure 4.4.2 Average rectal temperatures for each sampling day. Results are group means ± standard deviation.

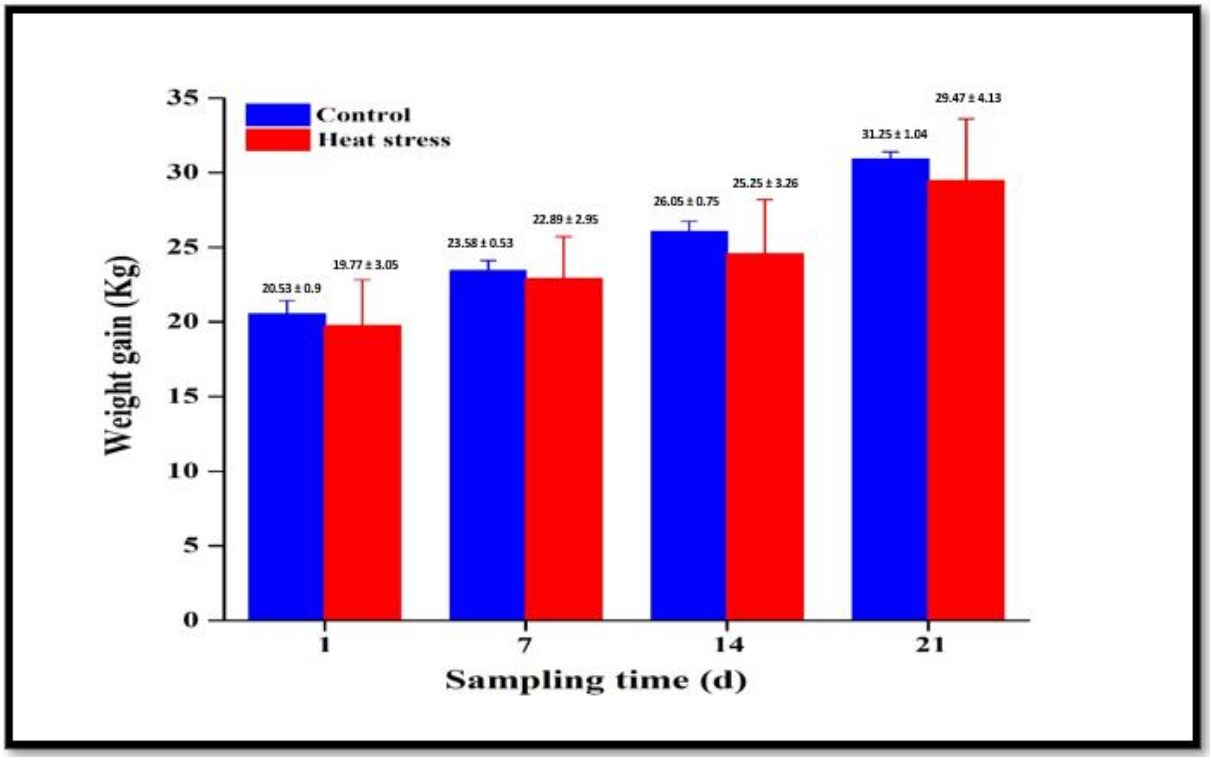

Figure 4.4.3 Average body weight gain. Results are means ± standard deviation.

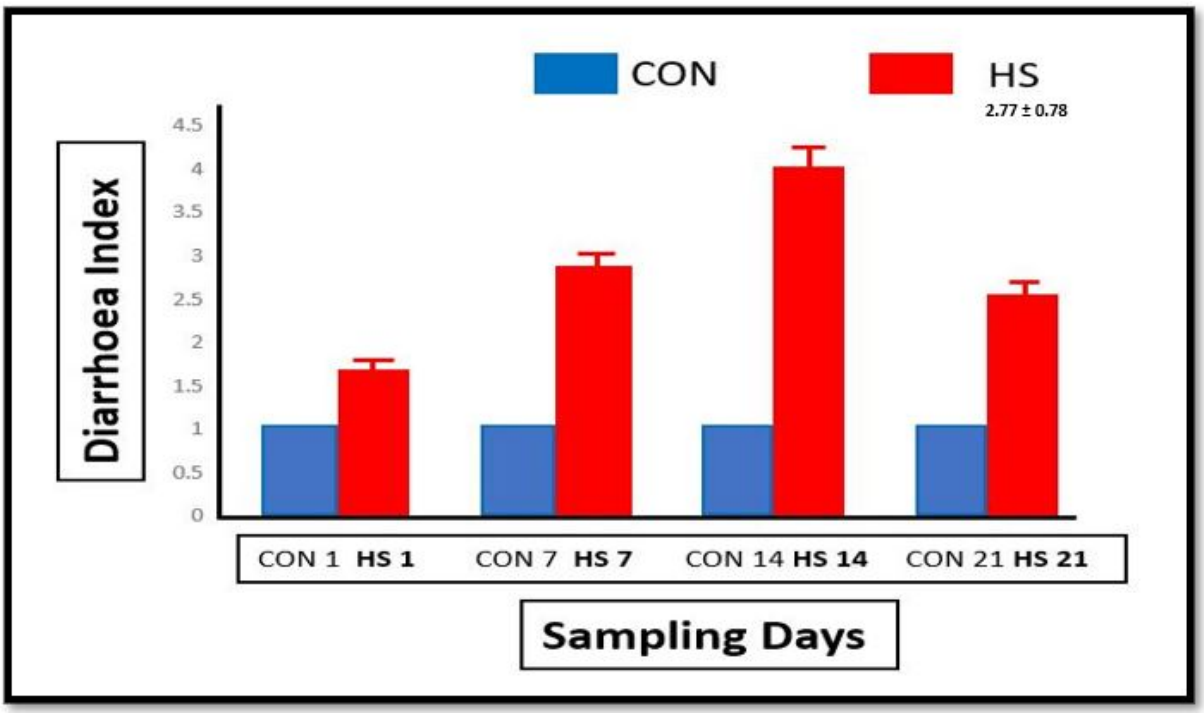

Figure 4.4.4 Diarrhoea Index. Results are means ± standard deviation.

4.4.2 Gut microbiota composition and diversity changes

Bacteroidetes and *Firmicutes* have been found to be the two most predominant phyla in the large intestine of pigs (Pedersen et al., 2013), and this is consistent with our results. The proportions of *Firmicutes* decreased on exposure to HS from day 1 to day 21. In contrast, the abundance of *Bacteroidetes*, *Bacteroides,* and other Gram-negative genera such as *Proteobacteria* and *Spirochetes* increased with HS over time. The *Cyanobacteria*, *Actinobacteria,* and *Saccharibacteria* OTUs were found in substantial numbers in HS pigs.

A total of 9,249,898 pairs of Reads were obtained by sequencing faecal samples from HS and CON pigs, and 5,362,329 Clean tags were generated after the two-end Reads were spliced and filtered, resulting in an average of 54,165 Clean tags per sample. A small fragment library was built for sequencing. Operational Taxonomic Units (OTU) Clustering by filtering Reads and species annotation and abundance analysis was performed to reveal the species composition of the sample. Fig. 4.4.5 depicts the rank abundance of different species on different days of the experiment.

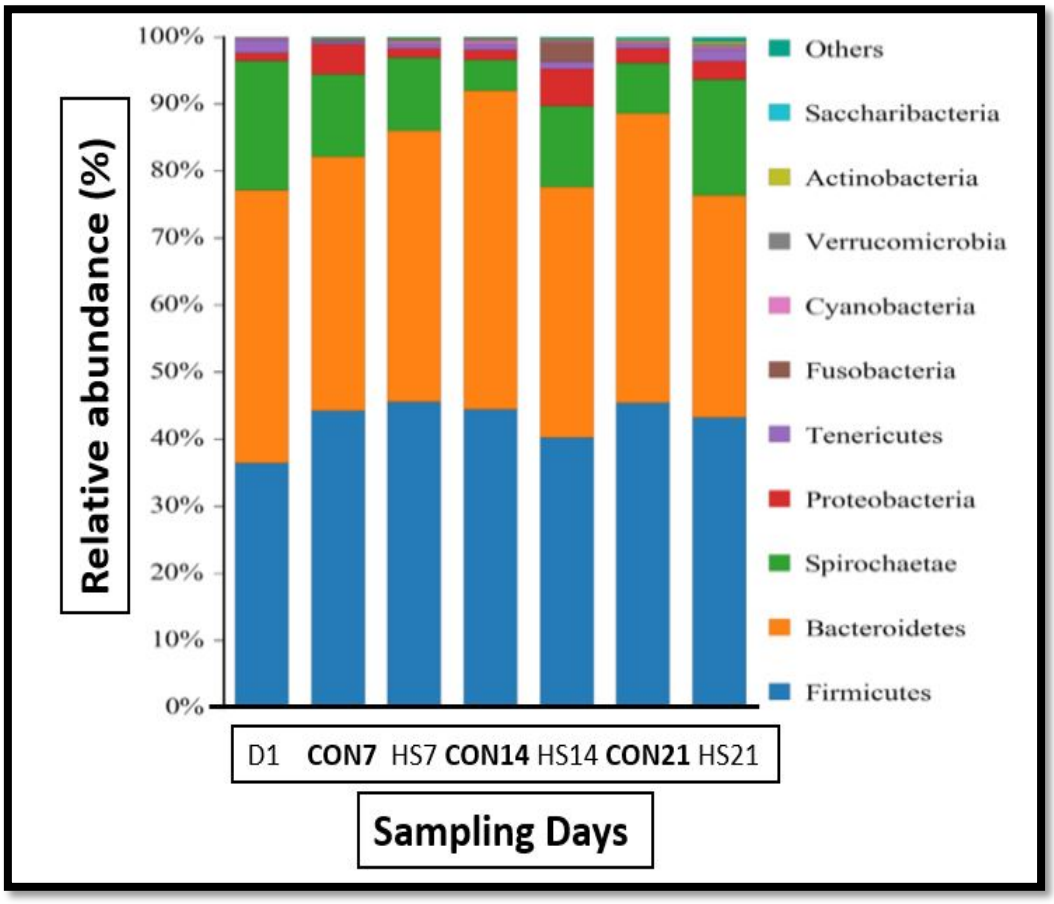

Figure 4.4.5 Relative abundance graph for each sampling day.

4.4.3 Alpha and beta diversity results

On evaluating the alpha diversity to explore the change in dynamics of GIT bacterial communities due to HS, it was evident that the Chao1 index, which reflects the species richness and estimates the diversity from abundance (i.e., the importance of rare OTUs), had significantly decreased over time (Fig. 4.4.6). Similarly, the Shannon Index, which determines species richness and evenness, also considerably reduced in the HS group with time from day 1 to day 21 (Fig. 4.4.7). In contrast, the Simpson Index, increased with the progression of HS (Fig. 4.4.8). Thus, the HS pigs displayed continuously decreasing alpha diversity in their gut bacterial communities as the HS persisted.

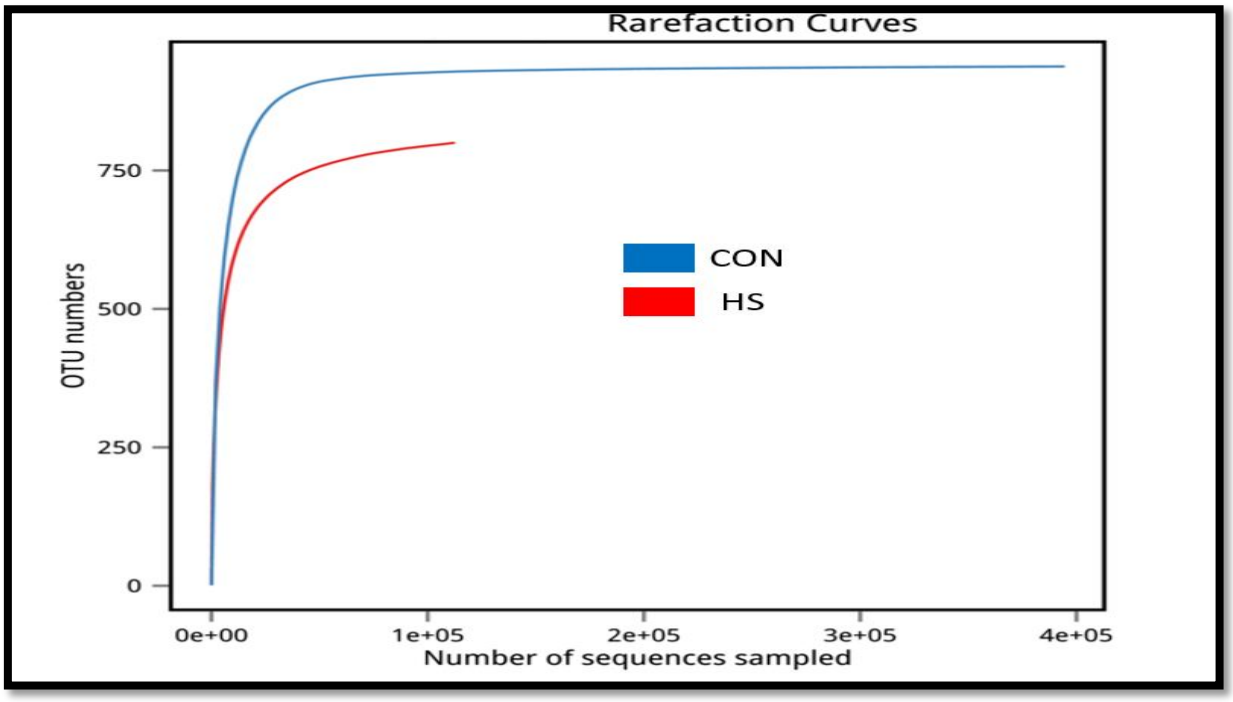

Figure 4.4.6 Rarefaction curves.

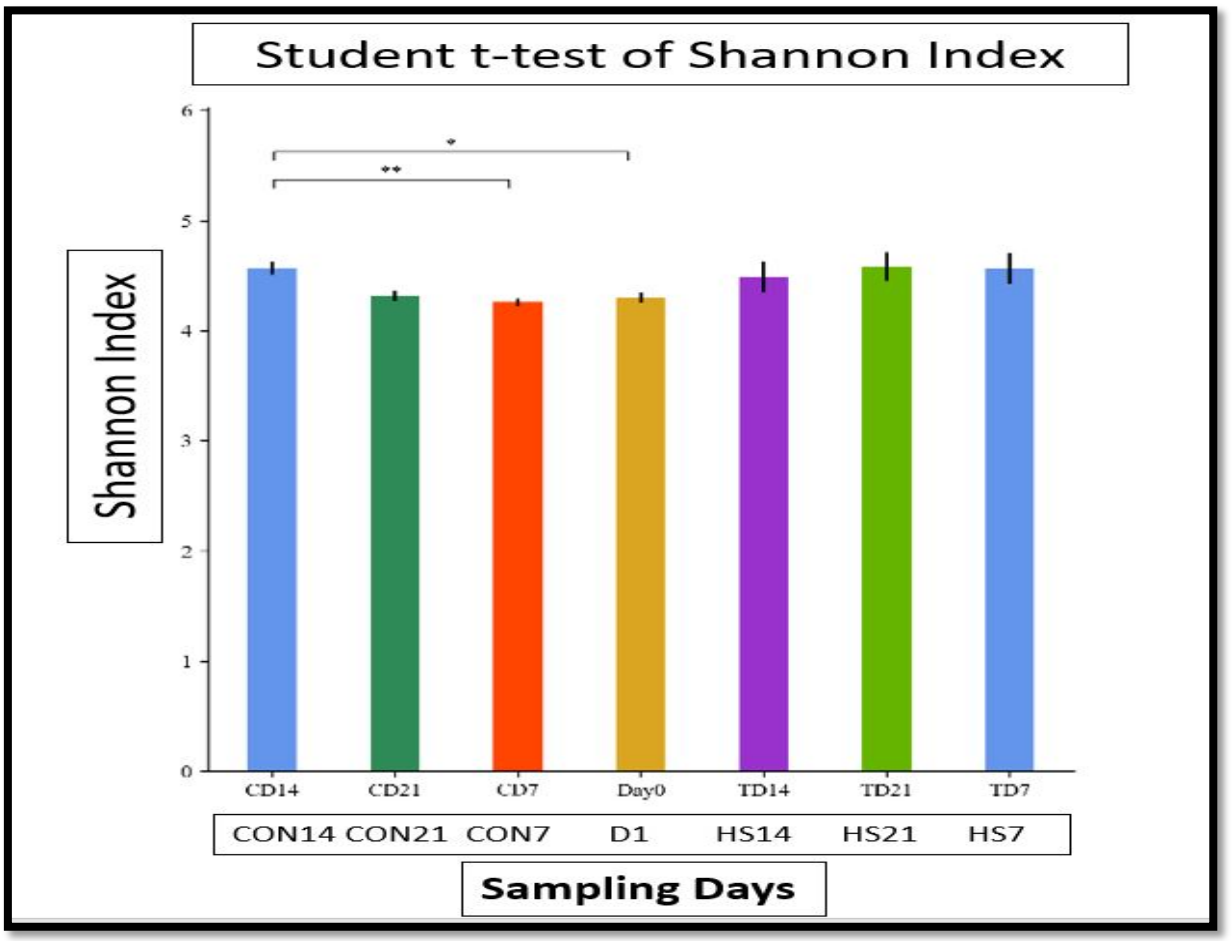

Figure 4.4.7 Shannon Index.

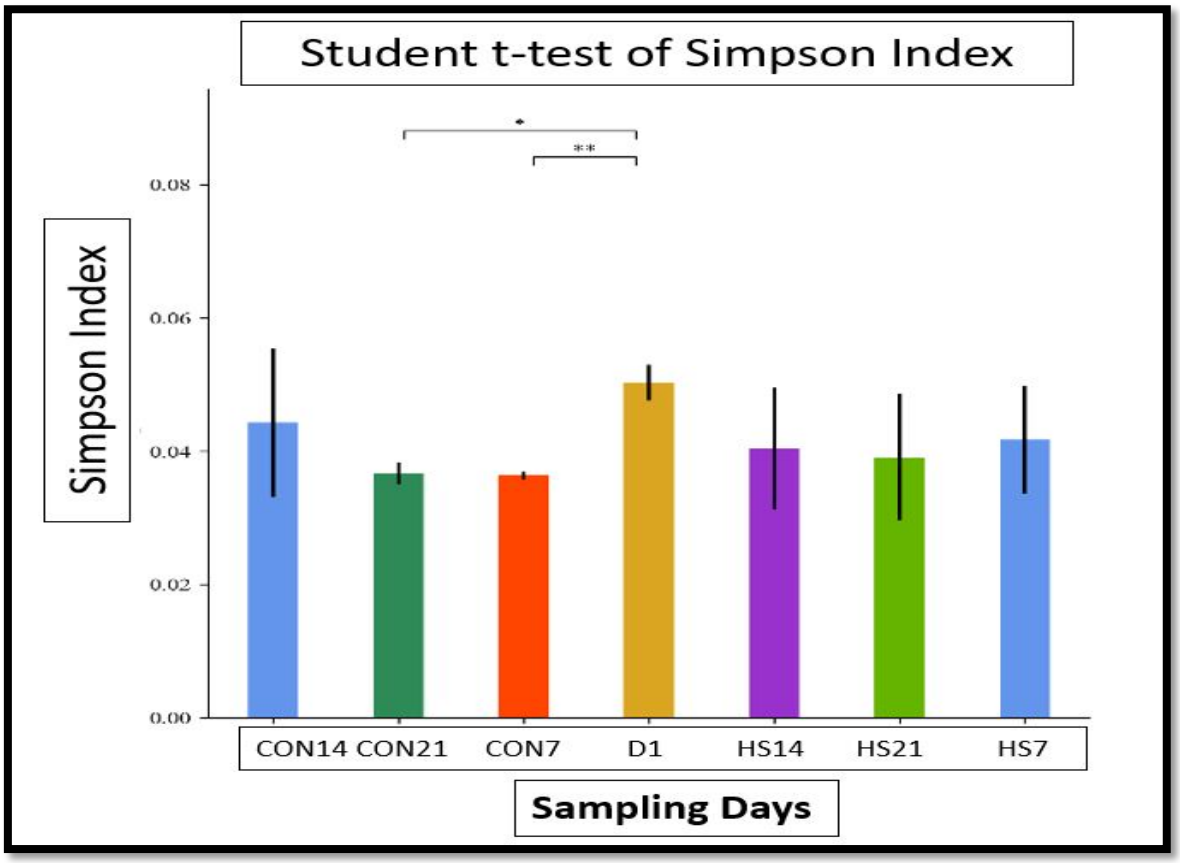

Figure 4.4.8 Simpson Index.

The type and abundance of species also markedly changed in the HS pigs. When the bacterial OTU representative sequences were classified taxonomically, four dominant phyla, namely *Firmicutes*, *Bacteroidetes*, *Proteobacteria,* and *Spirochetes*, were the prominent bacterial species, making up over 80% of the community. The abundance of *Firmicutes* and *Bacteroidetes*, the two major phyla, varied with time from day 1 to day 21 in the HS pigs. Metastats analysis, which allows a comparison of metagenomic samples (represented as counts of individual features such as organisms, genes, and functional groups) from two treatment populations (CON vs HS, in this case) and identifies those features that statistically distinguished the two populations, was carried out to identify the differentially abundant phyla within the CON and HS groups. This gives an insight into how the gut bacterial composition altered over time at the phylum level. We used weighted UniFrac distances to evaluate the beta diversity (diversity between individuals), and the HS pigs showed continuous alterations in their gut bacterial communities with the persistence of HS, as shown in the scatterplot from PCoA (Fig. 4.4.9).

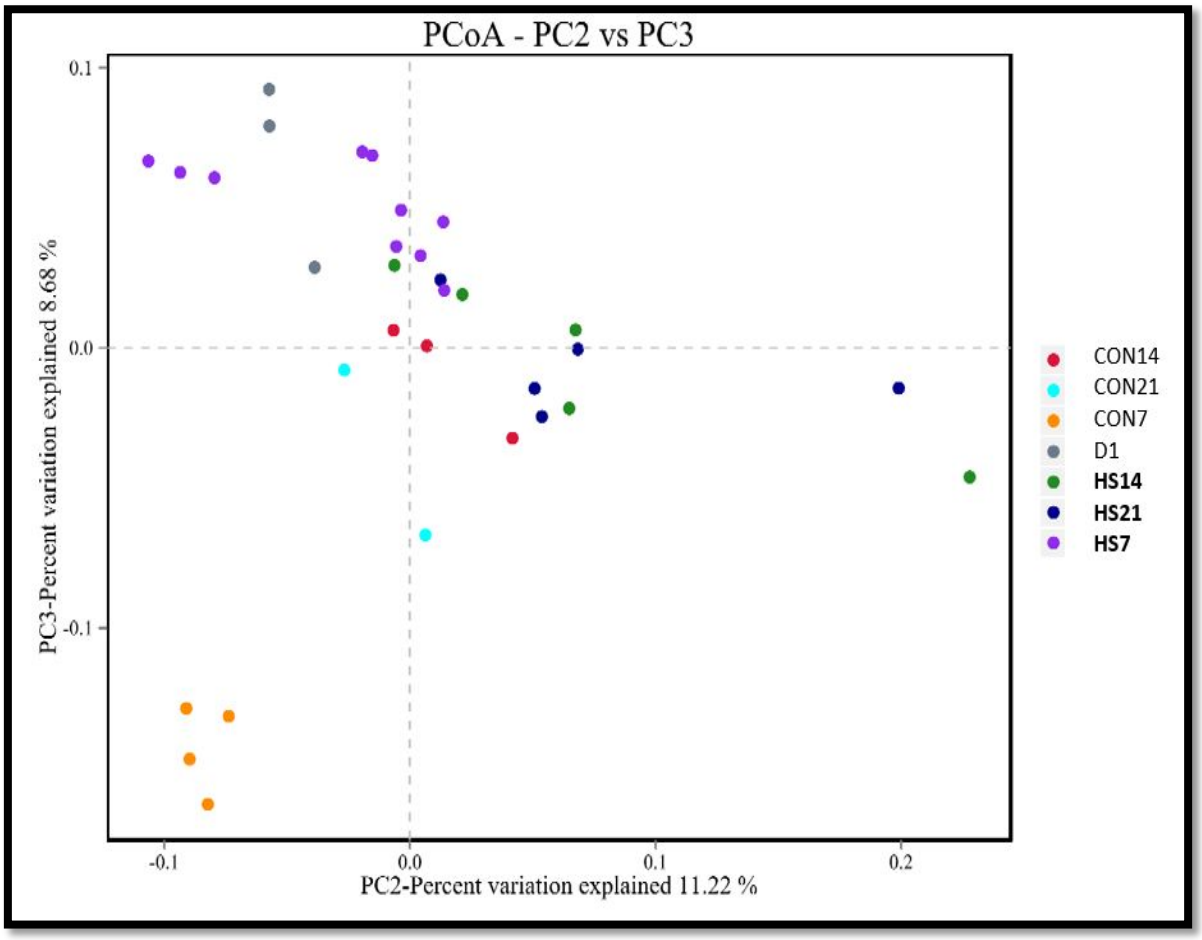

Figure 4.4.9 PCoA Scatter plot.

(PC2 and PC3 are the principal components, based on eigenvectors of correlation matrix, depicting the different linear combinations of the original data variables)

The PCoA scatter plot (Fig. 4.4.9) depicting the distances between CON and HS samples' microbiota composition on days 1, 7, 14 and 21. PCoA graphs are divided into different quadrants and objects ordinated closer to one another are more similar than those ordinated further away. In the case of this study, the CON samples tend to cluster separately from the HS samples, thus confirming there is a difference in the GIT microbiota composition between the CON and HS pig groups.

Within the *Firmicutes,* it was mainly the number of *Lactobacilli* and *Ruminococcaceae* that had reduced. Marked increases in the numbers of *Spirochaetae* were reflected in *Treponema 2.* Among the *Bacteroidetes*, the number of *Alloprevotella*, *Rikenellaceae RC9 gut group,* and *Prevotellaceae* had increased. Among the *Spirochaetes,* the numbers of Gram-negative *Treponema* had markedly increased. In the genus *Bacteroides*, the numbers declined because of a decrease in the number of *Prevotellaceae*. A QIIME analysis of four phylogenetic trees demonstrated that by day 14 the genus *Proteobacteria* in the HS pigs consisted mainly of *Campylobacter*. This microbe is postulated to be responsible for the diarrhoea in HS pigs. The LEfSe analysis showed that the relative differences in microflora between the HS and CON pigs on day 14 reflected an increase in the number of opportunistic pathogens such as *Campylobacterales*, *Veillonellaceae*, and *Megasphaera*. The KEGG

plot revealed that on day 7, HS pigs showed downregulation in functional genes responsible for signal transduction and nucleotide metabolism (Fig. 4.4.10).

Redundancy Analysis/Canonical correlation analysis based on binary-Jaccard distance showed that *Streptococcus, Prevotellaceae UCG−003* and *Ruminococcaceae UCG−002* were positively correlated with the HS treatment and *Prevotella 1, Prevotellaceae NK3B31 group, Alloprevotella, Ruminococcaceae UCG−005, Rikenellaceae RC9 gut group* and *Treponema 2,* in contrast, were negatively correlated. The *Ruminococcaceae UCG−002, Streptococcus* and *Prevotellaceae NK3B31 group* were also similarly positively correlated with temperature. In contrast, *Lactobacillus, Prevotella 1* and *Treponema 2* showed a negative correlation with temperature. The *Ruminococcaceae UCG−002, Streptococcus* and *Prevotellaceae NK3B31 group* were positively correlated with DI while *Prevotellaceae UCG−003, Lactobacillus, Prevotella 1, Treponema 2, Rikenellaceae RC9 gut group* and *Ruminococcaceae UCG−005* showed a negative correlation with DI. Binary-Jaccard distance-based redundancy analysis and the Mantel Test (Table 4.4.1) showed that HS treatment had a significant effect on microbiota ($P < 0.05$) and significantly influenced the DI ($P < 0.05$).

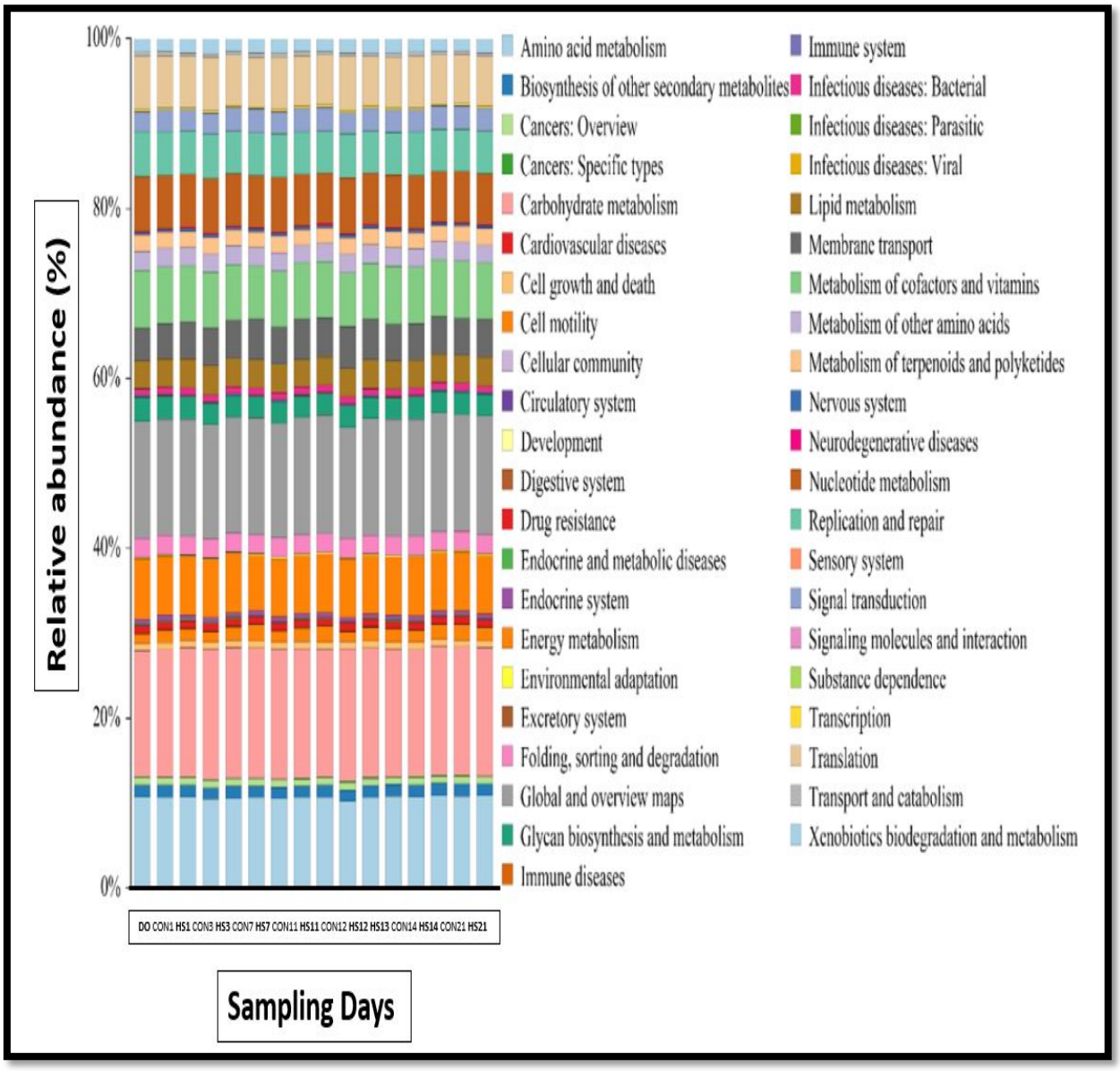

Figure 4.4.10 Differential analysis of the KEGG metabolic pathway.

Through this, it is possible to observe the differences in gene functions in the metabolic pathways of the microbial communities between the CON and HS groups. Different colours in the graph represent different metabolic pathways, which either get upregulated or downregulated depending on the presence or absence of functional genes responsible for them.

Table 4.2 Mantel Test of environmental factors in pig microbiome

Environmental factors	Mantel Test R statistic	P-value
Temperature	0.1290	0.021
Days	0.2630	0.003
Diarrhoea Index	0.4264	0.001

In the Relevance heatmap, temperature had a marked impact on *Campylobacterales*, *Treponema 2*, *Ruminococcaceae UCG-002*, *Alloprevotella* and *Lactobacillales*. Changes in *Campylobacterales*, *Alloprevotella* and *Prevotella 1* were significantly correlated with the HS treatment.

4.5 Discussion

This experiment aimed to trace the possible alteration of gut microbiota in pigs subjected to HS conditions for 21 days. To achieve this, faecal samples were collected at different times during the course of the experiment, at days 1, 7, 14 and 21, which were then analysed and compared with each other to visualise the differences in the microbiota. Many studies have reported that when pigs are subjected to persistent HS, there is an increase in morbidity and mortality (Johnson, 2014). Stress can reduce feed intake, decrease physical activity, and cause physiological, hormonal, and immunologic deficiencies, which not only reduce animal performance but also diminish the quality of animal-derived food products (Rostagno, 2009). Heat stress leads to sub-optimal growth, which in turn results in inconsistent and unacceptable market-weights in hogs. Inefficient uptake and use of nutrients mean poor sow performance. An immediate reaction to an increase in ambient temperature is a reduction in caloric intake. Pigs reduce feed intake to minimise metabolic heat production (S. C. Pearce et al., 2013). Heat stress downregulates lipolytic enzyme activities. The dampened lipolytic activity of the adipose tissue is a form of adaptation to limit *in vivo* heat generation in HS animals (Slimen et al., 2016). These results from around the world are consistent with our findings that HS pigs did not accumulate as much weight as the CON pigs (Fig. 4.4.3). The lower bodyweight gain in pigs due to HS exposure is a well-documented phenomenon (Lambert, 2009; Pearce et al., 2013; Pearce et al., 2014)

We observed that the forehead and rectal temperatures of the HS pigs were higher for the control pigs (Figs 4.4.1, 4.4.2). This increase was visible from day 1 of the experiment. (Lambert, 2009) found that HS pigs exhibit above-normal rectal temperatures and twice the normal respiration rate to that of normal pigs. Heat-stressed pigs act to divert blood from the intestines to the peripheral organs to dissipate the radiant heat. This is accompanied by vasoconstriction of the GIT, increased permeability of the intestine, hypoxia, endotoxemia, and inflammation (Renaudeau et al., 2011; Ross et al., 2015a).

In our experiment, the weights of the HS pigs did not increase as much as that of the CON pigs over the 21 days (Fig. 4.4.3). This is consistent with the fact that pigs voluntarily reduce feed intake when exposed to higher temperatures to minimise metabolic heat generation (Pearce et al., 2014; Ross et al., 2015a). The high temperature is a source of physical stress for the animals, and it triggers a series of responses within the body. Fatigue, tissue and organ damage, and departure from the homeostatic condition can occur due to this stress. If the core temperature of the body is increased,

the intracellular proteins and enzymes undergo denaturation leading to cell damage. Many proteins with a variety of functions are altered. The cellular environment becomes pro-oxidative with an accumulation of hydrogen peroxide and hydroxyl radicals (Cruzen et al., 2015; Ganesan et al., 2016).

Heat stress results in a chain of reactions within the body, especially in the GIT. Due to the diversion of blood from the internal lumen towards the periphery, hypoxia, oxidative and nitrosative stress is caused within the enterocytes. Cellular membranes and tight junctions are damaged because of this, which leads to an increase in intestinal permeability, also known as 'leaky gut.' Heat stress increases the autolysis of the ileum epithelium, and the subsequent damage caused to the intestines by HS increases the movement of high molecular weight substances and pathogens, including LPS and other bacterial components, away from the lumen (Pearce et al., 2014). This begins to happen as early as after 2 h of exposure to heat. At the same time, the shortening of the villi occurs at ~ 2 h and epithelial sloughing, autolysis, and linear lamina separation is observed at 6 h (Pearce et al., 2014). Due to changes in intestinal integrity caused by HS, there is an increase in circulating endotoxin (Armstrong et al., 2018). The increase in the OTUs of Gram-negative bacteria in our findings can be traced back to this.

The host endeavours to minimise contact between the microbiota and its tissues. This occurs in various ways, primarily with the help of a 'mucosal firewall,' a set of structural and immunological components in the GIT. The mucus is the primary shield that limits contact between the microbiota and host tissue and prevents the translocation of the commensals. All intestinal epithelial cells produce antimicrobial peptides that also limit exposure to the commensal microbiota. Accumulation of antimicrobial peptides such as 'RegIIIγ' in the mucus maintains separation between the microbiota and the host intestine, and a physical separation called the 'demilitarised zone.' Secreted IgA also plays a part in compartmentalisation. IgA specific for commensals is produced with the help of intestinal dendritic cells by B and T cells in the Peyer's patches. Commensals that translocate across the intestinal epithelial cell are engulfed and eliminated by macrophages within the lamina propria or carried alive by dendritic cells (DC). Since mucosal IgA responses are a part of innate immunity when new anti-bacterials outnumber established IgA-producing clones, the mucosal immune system can respond quickly to changing microbiota (Macpherson & Uhr, 2004).

The principle co-ordinates analysis (PCoA) results (Fig. 4.4.9) summarise and represent dissimilarities between two objects (i.e., CON vs HS pig groups), utilising the distances between them in four quadrants. It was found that the distances within the OTUs of the CON samples were narrow, meaning the data points were close to each other in the same quadrant, whereas, for the samples from HS pigs, distribution was widely separated. This suggests a deviation from the normal microbiota in HS pigs due to persistent HS treatment. When encountered with stress, the endocrine

system in the host secretes various hormones in response. These mainly include glucocorticoids and catecholamines (Möstl & Palme, 2002). The adrenal glands play a significant role in the secretion of the stress response hormones by their involvement in the SAM (sympathetic-adreno-medullar) and HPA (hypothalamic-pituitary-adrenal) axes (Nicol, 2001). Numerous studies have found that catecholamines cause the growth and virulence of many pathogenic bacteria. Stress can cause the GIT enteric nervous system (ENS) to release catecholamines, which in turn can cause a significant rise in the local hormone levels (Freestone et al., 2008). The catecholamines cause the iron to liberate lactoferrin and transferrin, the high-affinity ferric iron-binding proteins (Sandrini et al., 2010). Gram-negative bacteria are seen to grow in the presence of catecholamine hormones, as they provide the bacteria with iron (Freestone et al., 2002; Lyte & Ernst, 1992). Our results mirror this phenomenon, as *Proteobacteria* and *Spirochetes* were seen to increase in HS pigs. A few studies in other mammals (rodents) have shown that there is a bi-directional relationship between the gut microbiota and the response to various stressors applied at various time points and that this results in changes in the composition of the gut microbiota. These changes, a departure from the normal state, have implications on the host physiology (Galley et al., 2014; Golubeva et al., 2015; O'Mahony et al., 2009). The colonisation of farmed animals with enteric pathogenic *Proteobacteria* such as *Escherichia coli* O157: H7 and *Salmonella enterica* and their subsequent spreading into the human food chain is a major health concern for meat producing industries (Freestone et al., 2008).

Consistent with previous reports, we found that *Firmicutes* and *Bacteroidetes* were the most dominant phyla in the faeces of pigs(Ruth E Ley et al., 2008). Also, previously, it has been reported that the gut microbiota in obese pigs has more *Firmicutes* but fewer *Bacteroidetes* (Ley et al., 2006), significantly lower amounts of *Bacteroides*, which are essential for the degradation of carbohydrates (Manimozhiyan et al., 2011). Our results showed increases in the relative abundance of Gram-negative phyla such as *Bacteroidetes*, *Proteobacteria,* and *Spirochetes*. The genus-level cluster analysis performed using a heat map showed that the CON groups from different sampling days (1, 7, 14 and 21) had a higher similarity among themselves as compared to the HS groups from the same sampling days. The relative abundance of different phyla (Fig. 4.4.5) shows an increase in numbers of *Spirochaetae*. These *Spirochaetae* are slow-growing anaerobes that colonise the large intestine and can cause colitis/typhlitis, diarrhoea, and reduced growth and production rates in pigs (Hampson, 2018). In most of the pigs subjected to HS, there is also an abnormal increase in numbers of *Proteobacteria* (Fig. 4.4.5). Many different diseases, such as gastroenteritis, and overall dysbiosis of the gut have been associated with raised levels of *Proteobacteria,* and this generally follows the introduction of a stressor to the host animals like pigs (Carvalho et al., 2012; Indrani et al., 2012; Shin et al., 2015a).

The numerous statistical methods employed in this study suggest that a majority of the metagenomic reads of the microbiota were associated with a relatively conserved core microbiome, and HS caused fluctuation in the numbers of the individual species (from this conserved microbiome).

4.6 Conclusion

This experiment was conducted to understand the effects of persistent HS on the gut microbiota of pigs. The higher-than-normal atmospheric temperatures cause qualitative as well as quantitative changes in the OTUs of the pig gut microbiota, causing a decrease in Gram-positive species and an increase in the number of Gram-negative species, many of which have been classified as potential mammalian pathogens. There was a clearly visible loss in bodyweight gain in the HS pigs and also diarrhoea. This departure from normality can be attributed to the morphological as well as microbial composition changes that occur in the pig gut due to the HS. These experiments can help us conclude that unwarranted changes in the pig gut microbiota composition (due to HS) have a detrimental effect on the physiology of the animal, eventually leading to lower bodyweights and financial losses for the producers. These changes can form the basis for the development of therapeutic solutions in important livestock animals such as pigs, against the continuously increasing problem of high global environmental temperatures. This study provides a baseline for understanding the complexity of the pig gut microbial ecology, while also highlighting the intricate differences between normal and HS animals, which can have many adverse effects on the health of the host animals.

Chapter 5

Heat Stress Activates TLR4/NF-κB Signalling Pathway Contributing to Inflammatory Bowel Disease in Pigs

5.1 Abstract

Pathological mechanisms of gastrointestinal disorders, including IBD in pigs, are poorly understood. In this study, the induction of intestinal inflammation in HS pigs and the role of intestinal microbiota in TLR4/NF-κB signalling pathway activation are described. Six adult pigs were subjected to heat stress [35 ± 2°C; 75–85% relative humidity] and 6 control pigs to 23 ± 2°C, same relative humidity. Pigs were sacrificed on days 1, 7, 14, and 21. Colonic epithelial transcriptomic analyses were conducted. From day 7 onwards, HS pigs exhibited fever, diarrhoea; significantly lower colonic villus length, crypt depth/width, and goblet cell number; and TLR4, TRAF6, and nuclear p65 upregulation at RNA and protein level. Also, an upregulation of pro-inflammatory cytokines like IL-6, IL-8, and IL-17 was observed. Colonic microflora composition in HS pigs was different from that in control pigs. Heat stress promotes changes in gut microflora composition (as described in Chapter 4), activating the TLR4/NF-κB signalling pathway, causing IBD in pigs. This study would aid in determining management strategies for people suffering these diseases.

5.2 Introduction

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, affect > 3.1 million people in the United States alone and 2.5 million in Europe annually. Their incidence is increasing worldwide, especially in East Asia and South Asia (Dahlhamer et al., 2016; Kaplan, 2015). These diseases are characterised by chronic inflammation of the GIT and other lesions triggered by genetic predisposition and environmental factors (Ananthakrishnan et al., 2017). Various stressors contribute to GIT disorders, and hypo- and hyperthermia may aggravate IBD by hitherto unknown mechanism(s). Summer heatwaves have increased in incidence and duration in many countries with global warming (Amengual et al., 2014), and since the 1880s, the global mean temperature and relative humidity have risen by approximately 10.1% and 1.8%, respectively. Previous studies have shown that HS induces inflammation and damage to intestinal mucosa in pigs (Pearce et al., 2014), chickens (Quinteiro-Filho et al., 2010), and rats (Hall et al., 2001), hence the implication that rising temperatures may similarly affect other mammals and humans and increase the incidence of IBD.

In the mammalian innate immune system, cell membranes function as gatekeepers. In response to invasion by microbial pathogens (e.g., bacteria, viruses, and parasites), these immune cells express

proteins called TLRs, which recognise these invading pathogens and activate the body's immune response. One of these TLRs, TLR4, recognises LPS, which are present in large numbers on the cell walls of Gram-negative bacteria. When TLR4 attach to the LPS, a conformational change occurs, and intracellular Toll-interleukin receptor domains containing adaptor molecules are recruited to this site. These adaptors associate with the TLR4 cluster *via* homophilic interactions between receptor domains. Consequently, the NF-κB protein complex and mitogen-activated protein kinase (MAPK) are activated, resulting in the production of various pro-inflammatory cytokines (interleukins) (Brubaker et al., 2015; Doyle & O'Neill, 2006; Gilmore, 2006; Hayden et al., 2006; O'Neill & Bowie, 2007).

The TLR4 proteins occur in the peripheral lymphatic endothelial cells of the small intestine and on the surface of intestinal epithelial cells in IBD patients (Toiyama et al., 2006). The number of TLR4 Asp299Gly polymorphism alleles are significantly higher in IBD patients than in healthy controls (Perez-Pardo et al., 2019). The TLRs mediate NF-κB activation; participate in the inflammatory response and cause changes in interleukin levels such as IL-6 and IL-8 (Fu et al., 2016; Gao et al., 2015; Jian et al., 2015). When TLR4/MyD88/p65 is upregulated, the number of pro-inflammatory cytokines (interleukins) is relatively high in the intestinal mucosa of patients with ulcerative colitis. Previous studies have shown that expression levels of TLR4 and its alternative splicing variants are increased in pigs exposed to HS (Ju et al., 2014). However, the role of TLR4 signalling pathway activation in HS-induced IBD is as yet unknown.

A healthy intestinal microbial community is diverse, stable, resistant to minor changes, and resilient (Levy et al., 2017). Human and mouse studies have shown that gut dysbiosis or disequilibrium of the microbial community is associated with various acute and chronic inflammatory conditions, bowel diseases, metabolic syndromes, and diabetes (Brown, 2012). Gut dysbiosis and reduced gut microbial ecosystem complexity are common symptoms in patients with Crohn's disease or ulcerative colitis. Nevertheless, it is unknown whether such alterations are causes or consequences of these diseases (Manichanh et al., 2012). It has been reported that faecal microbiota transplantation (FMT) can successfully treat relapsed *Clostridium difficile* infections that are ineffective to antibiotics. Such FMT seems to also be beneficial for some patients with other gastrointestinal diseases such as IBD, and its effects may be through specific microorganisms or their active products adjusting intestinal flora to treat diseases (D'Haens & Jobin, 2019).

Pigs and humans have anatomical, physiological, and immunological similarities. Thus, studies of the immunological mechanisms in pigs could be directly relatable to humans. Moreover, pigs have similar susceptibilities and clinical manifestations in response to pathogens that cause certain human GIT disorders (Meurens et al., 2012; Zhang, 2013). Xiao et al. (2016) reported that while the homology between human and pig microbiomes is low at the gene level, it is significantly

higher at the level of KEGG orthology functions, where there is greater similarity than between human and mouse microbiomes. Furthermore, ~96% of the functional pathways described in the human gut microbiome resemble those of pigs (Xiao et al., 2016). This study, therefore, aimed to elucidate the roles of intestinal microbial composition and the TLR4/NF-κB signalling pathway in IBD development in HS pigs, with possible implications for disease management in humans.

5.3 Materials and methods

5.3.1 Animals and management

The experimental protocols involving the management and care of pigs and mice were pre-approved by the Animal Care and Use Committee of Guangdong Ocean University, Zhanjiang, China (Permit No. 206-1108), and are described in Chapter 3 Materials & Methods (section 3.1).

5.3.2 Pig study

Twelve pigs (Luchuan sows × Duroc boars), each initially weighing 15 ± 2 kg, were housed in two animal rooms at the Animal Hospital of Guangdong Ocean University, Zhanjiang, China. The pigs were divided into two groups. The pigs were maintained for 1 week at 20 ± 2°C and RH 75–85% to acclimatise them to the environment before the experiment began. To minimise acute HS, the animal facility was gradually warmed over a 7-day period. During the 21-day trial, the 6 pigs from the CON group were subjected to 23 ± 2°C, and 6 pigs from HS group were exposed to 35 ± 2°C, both groups maintained at 75–85% RH. Throughout the study, the pigs were fed a complete commercially available feed formula in the morning, afternoon, and evening with ~6-h intervals between feedings. Drinking water was freely available throughout the experiment.

5.3.3 Sample collection

Colon tissue samples were collected from sacrificed animals (see section 3.2.1) on day 21, and immediately stored at −80°C until the subsequent cell culture studies, and transcriptomic analyses, were carried out.

5.3.4 Morphological analyses

Samples of colonic tissue were subjected to morphological analyses, including histopathological examination, and the goblet cells/unit area calculated. These procedures were adapted from (Lan, 2015; Nagao-Kitamoto et al., 2016). (See Chapter 3, Materials & Methods).

5.3.5 Western Blotting

Western Blot analysis was carried out to identify the critical TLR4/NF-κB signalling pathway proteins – TLR4, TRAF6, MyD88, and p65 – in the colonic tissue, following standard protocols. (See Chapter 3, Materials & Methods).

5.3.6 Immunohistochemistry (IHC)

Immunohistochemistry for TLR4-, MyD88-, and TRAF6-positive cells, and p65 (NF-κB) activity, was conducted to quantify the upregulation of the TLR4, TRAF6, MyD88, and p65 proteins in the colonic tissues. (See Chapter 3, Materials & Methods).

5.3.7 Transcriptomics

Transcriptomic analyses of colonic epithelial tissue, RNA extraction, and cDNA synthesis were conducted as described in previous reports (Caporaso et al., 2011; Edgar, 2010). (See Chapter 3, Materials & Methods).

5.3.8 In vitro IPEC-J2 LPS validation

The IPEC-J2 cells were subjected to LPS, and ELISA was subsequently performed as per standard published protocols (Livak & Schmittgen, 2001). (See Chapter 3, Materials & Methods).

5.3.9 Statistics

The data were subjected to *t*-tests using SPSS v. 21.0 (IBM Corp., Armonk, NY, USA). Data are expressed as means ± standard deviation. $P \leq 0.05$ indicates a significant difference. $P \leq 0.01$ indicates a highly significant difference.

5.4 Results

5.4.1 Histopathology of colonic mucosa in pigs

Figure 5.4.1 (a–c) represents the effects of HS on the structure of the colonic mucosa in pigs. Morphological observation of the colonic tissues revealed that the mucosal layer of the CON group was intact, and the shedding of epithelial cells was not apparent. In contrast, sustained HS caused epithelial cell sloughing, vasodilation, and mucosal hyperaemia in the colonic intestinal epithelium of HS pigs from day 7 onwards (Fig. 5.4.1a). The crypt depth for the HS pigs became shallower, and by day 14, it was significantly different ($P < 0.05$) from the controls (Fig. 5.4.1b). In the HS pigs, the number of goblet cells per unit area was markedly lower than in CON pigs. The difference between groups was significant by day 7 ($P < 0.05$) (Fig. 5.4.1c). The number of immune cells between the

epithelial cells was also more significant in the HS than the CON pigs. The purplish-blue stained cells are of this colour due to H&E staining and the magenta-coloured cells are due to PAS staining.

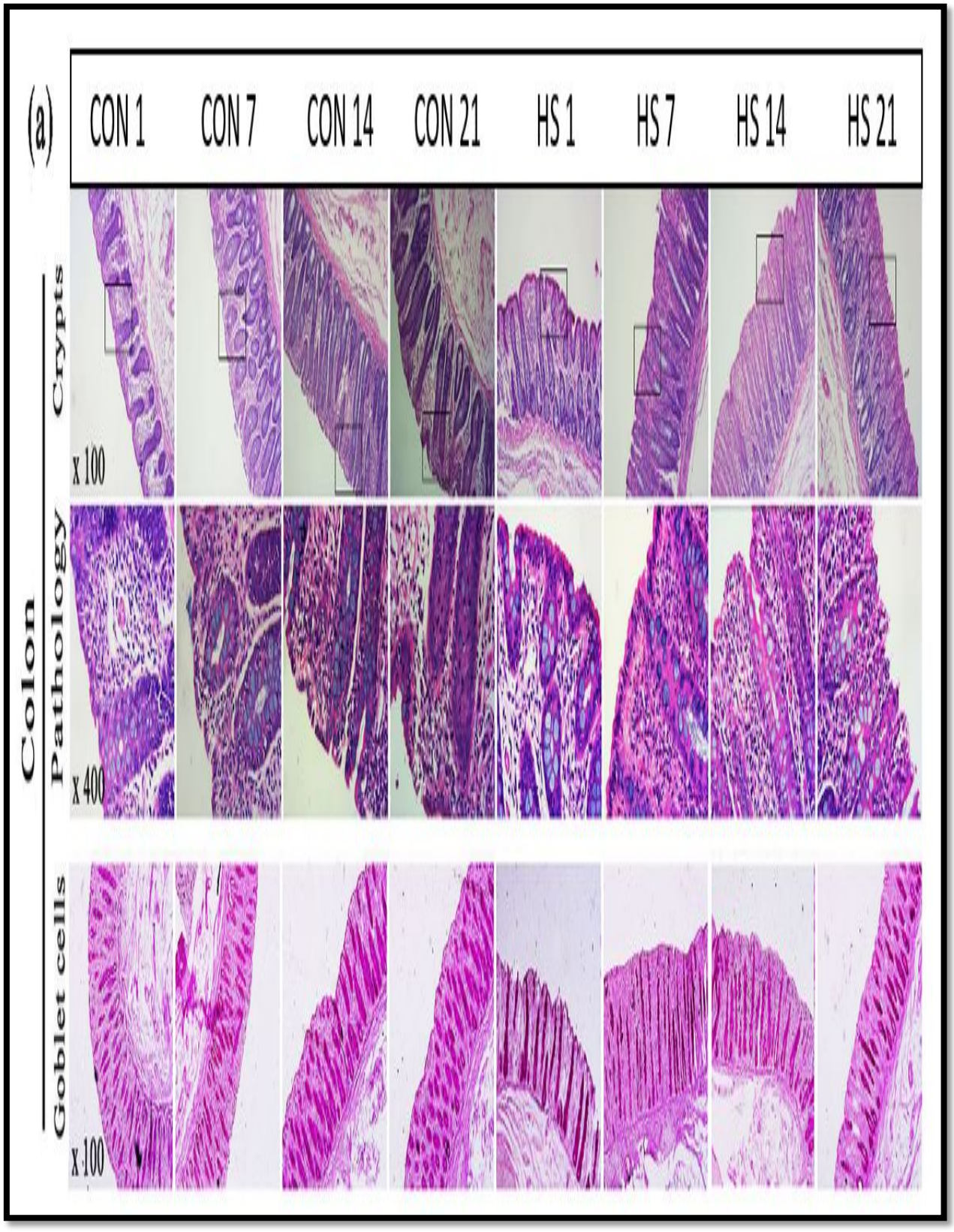

Figure 5.4.1 (a) Haematoxylin and eosin (H&E) and PAS (Periodic acid–Schiff) staining of the pig colonic mucosa.

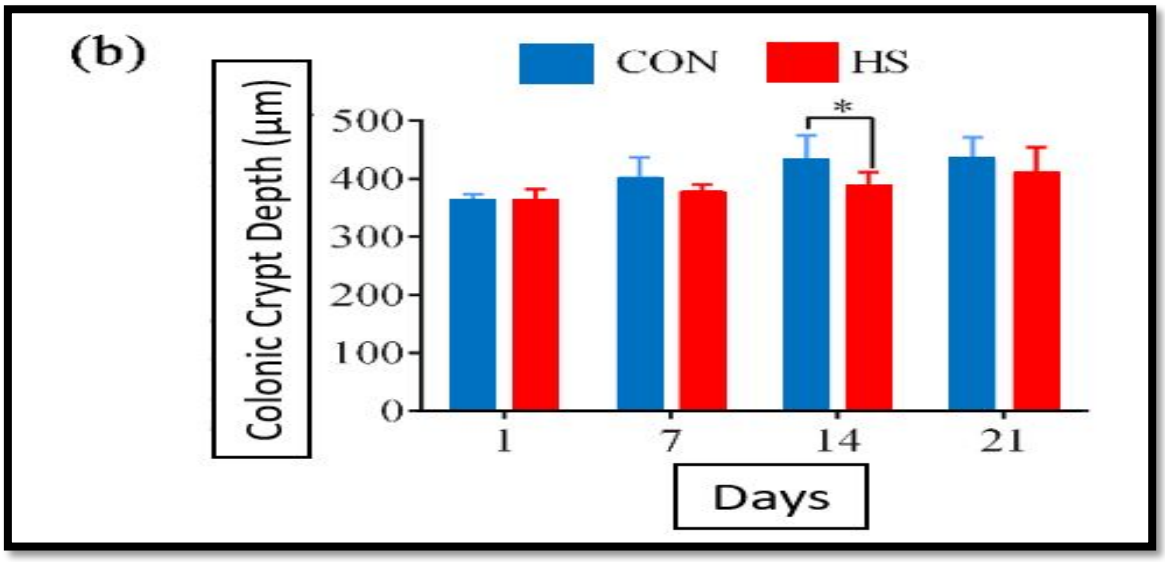

Figure 5.4.1 (b) Crypt depth of pig colonic mucosa.

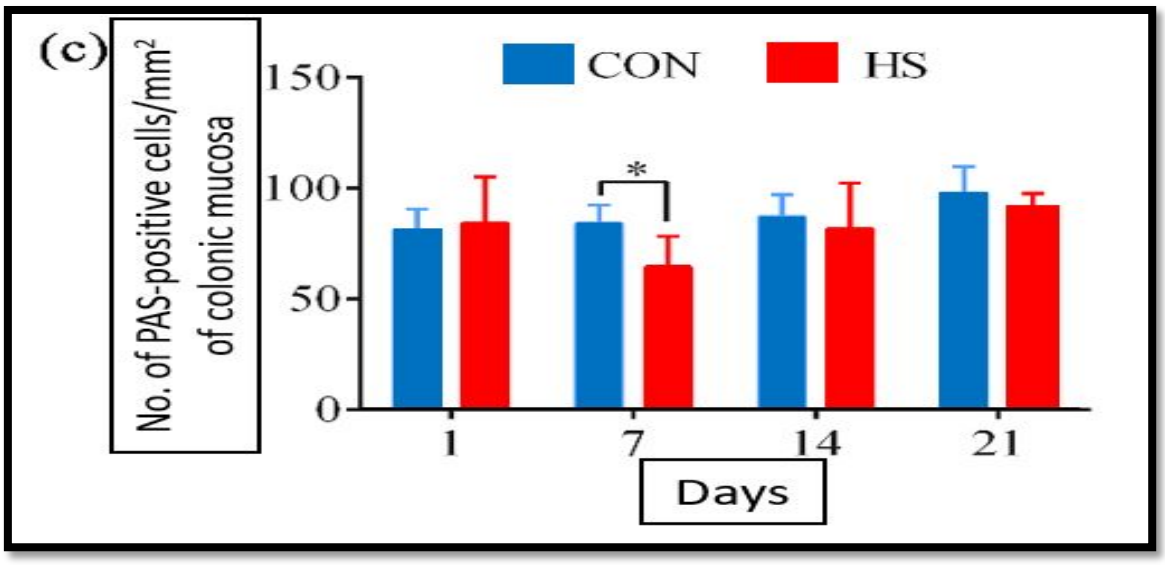

Figure 5.4.1 (c) Number of PAS-positive cells per square millimetre of colonic mucosa.

5.4.2 Transcriptome of pig colonic mucosal tissue

The superposition of gene coverage reflects whether the sequences are evenly distributed. The plot in Fig. 5.4.2a shows that the sequencing results for each sample were not biased. The PCA showed a strong similarity between samples, and the differences between groups were small (Fig. 5.4.2b). A heatmap showed that gene expression levels and their correlation coefficients between samples were high for the HS pigs (Fig. 5.4.2c). A differential expression volcanic plot (Fig. 5.4.2d) revealed that the expression levels of 14,968 genes in the HS samples on day 7 were significantly different

from those for the CON pigs. There were 7,163 upregulated and 7,705 downregulated genes in the HS pigs. On day 14, there were 15,036 differentially expressed genes, of which 7,114 were upregulated and 7,922 were downregulated (Fig. 5.4.2e). The KEGG plot disclosed that the top 20 signalling pathways of differential gene enrichment included IL-17, TLR, and cytokine–cytokine receptor interaction (Fig. 5.4.2f). Transcriptomic analysis showed that HS induces the TLR4/NF-κB signalling pathway and significantly upregulates TLR4, MyD88, NF-κB, IL-17α, and IL-8 (Fig. 5.4.2g–i).

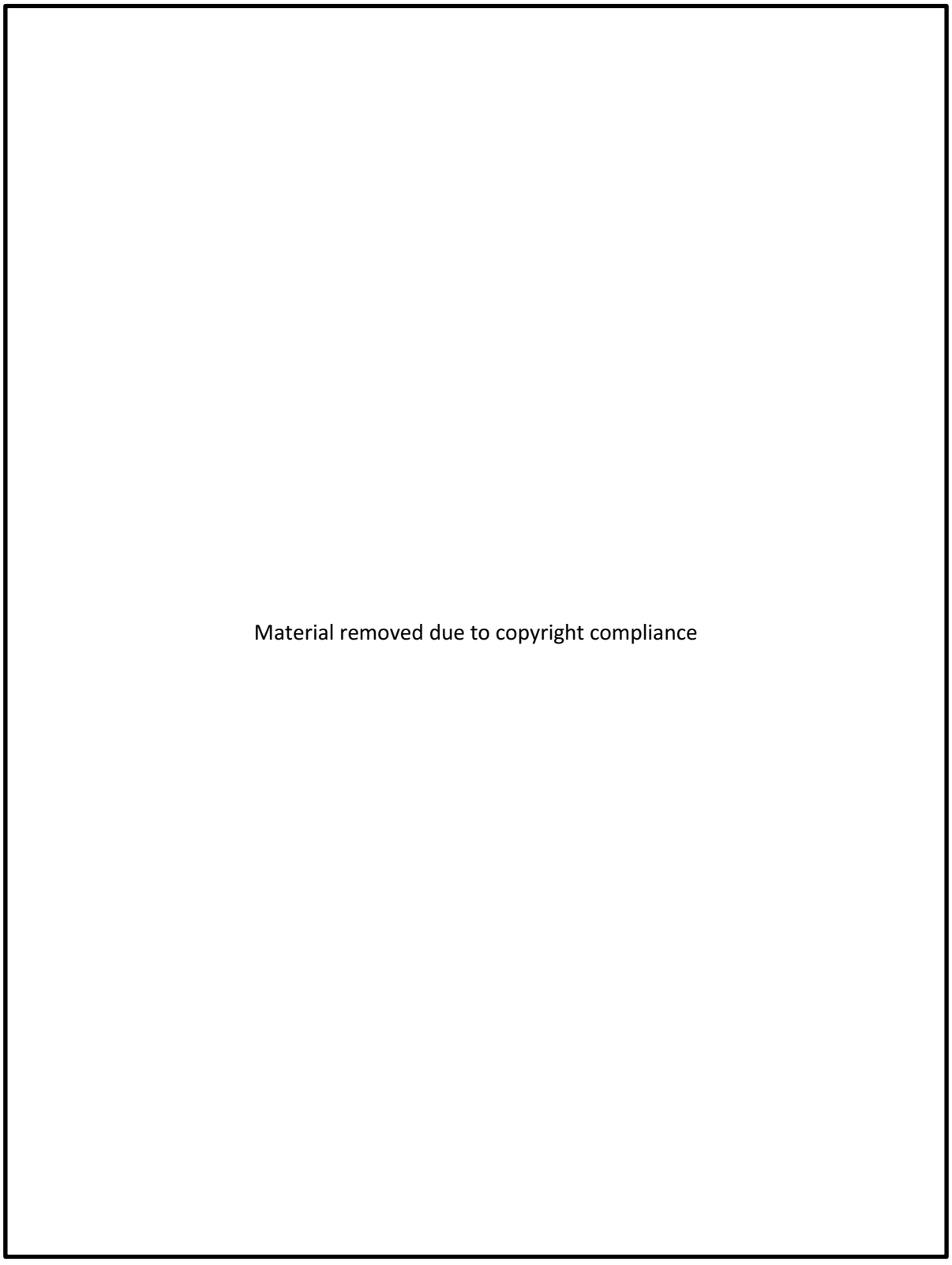

Figure 5.4.2 Effect of HS on pig colonic mucosal transcriptome.

Gene coverage analysis, in the abscissa: 0 represents the 5' end of the gene, 100 represents the 3' end of the gene, and the ordinate is the sum of the number of sequences in the corresponding interval on the horizontal axis position of all genes; (b) PCA analysis of colonic samples, CON and HS; (c) Heat map analysis between CON and HS (d) Differential expression volcanic plot (CON vs. HS7); (e) Differential expression volcanic plot (CON vs. HS14); (f) KEGG enrichment analysis (CON vs. HS7); (g)–(i) Effects of HS on the expression of crucial genes in TLR4/NF-κB signalling pathway.

5.4.3 TLR4/NF-κB signalling pathway in pig colonic mucosa

In the colonic tissue of HS pigs, TLR4 was significantly upregulated on day 1 and peaked by day 7 ($P \leq 0.05$), at which time its expression level was 21.43% higher than on day 1 (Fig. 5.4.3a, b). There was no significant difference in MyD88 expression (Fig. 5.4.3a, d). For the HS group, TRAF6 was significantly upregulated on day 1 and peaked by day 7 ($P \leq 0.05$), at which time its expression level was 12% higher than on day 1 (Fig. 5.4.3a, d). There were no changes in the expression levels of p65 in the nucleus or extranuclear cells of the control group. Nevertheless, in the HS pigs, nuclear p65 expression was markedly increased relative to the CON group. The p65 expression was highest on day 1 and gradually decreased after that (Fig. 5.4.3a, e, f). For the CON group, colonic mucosal cells whose nuclei were expressing p65 were mainly distributed near the side of the intestinal epithelial cells, and the expression levels did not significantly differ ($P > 0.05$) across time points. For the HS pigs, p65 expression in the nuclei of the rectal and caecal mucosal cells near the intestinal lumen increased in the early stages and decreased after that (Fig. 5.4.3g). In HS pigs, the relative expression of IL-6 mRNA was significantly elevated ($P < 0.01$) by day 7 but declined ($P < 0.05$) after that (Fig. 5.4.3h). The relative expression of IL-8 mRNA was lower in the HS group than the CON group on days 1 ($P < 0.001$), 7 ($P < 0.001$), 14 ($P < 0.05$), and 21 ($P < 0.001$) (Fig. 5.4.3i). For the HS pigs, IL-17 mRNA was significantly upregulated on days 7 ($P < 0.001$) and 14 ($P < 0.001$) (Fig. 5.4.3j).

The faecal LPS concentrations in the HS pigs were significantly higher on days 1, 7, and 14 than in the CON (Fig. 5.4.3k). Western Blotting revealed that, in HS pigs, TLR4 expression was markedly elevated following treatment of IPEC-J2 cells with 10 μg/ml LPS for 3 h (Fig. 5.4.3k). The nuclear p65 markers were also significantly upregulated ($P < 0.001$) (Fig. 5.4.3m).

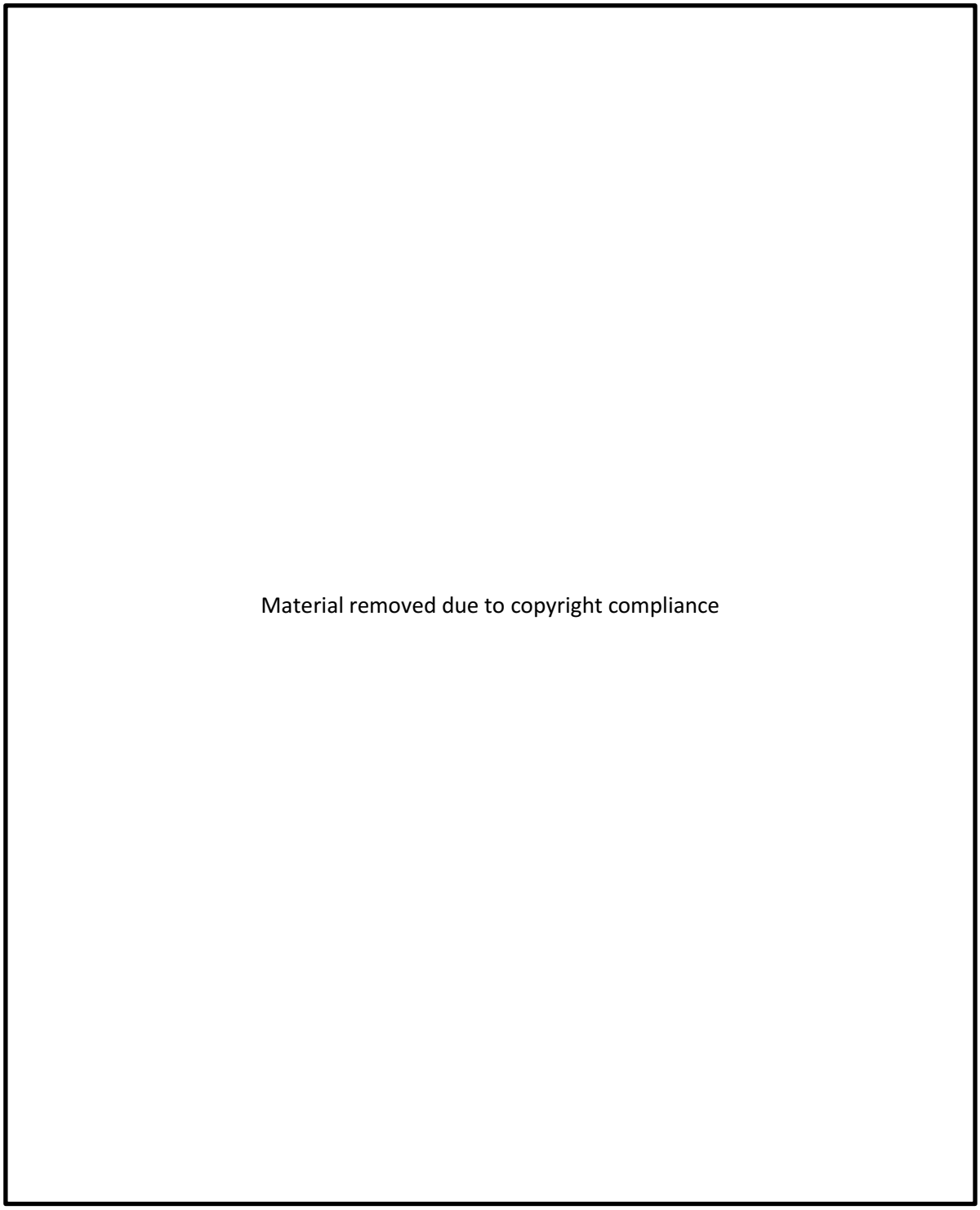

Figure 5.4.3 Expression of TLR4/NF-κB/p65 signalling pathway proteins in pig colonic mucosal tissue.

(a) Protein profiles; (b) TLR4; (c) p65 in cytoplasm; (d) MyD88; (e) p65 in nucleolus; (f) TRAF6; (g) p65-positive cell distribution in colonic mucosa (DAPI fluorescent nuclear dye); (h) IL-6 mRNA expression; (i) IL-8 mRNA expression; (j) IL-17 mRNA expression; (k) LPS concentration; (l) LPS induction of TLR4 in IPEC-J2; (m) p65-positive cell distribution in IPEC-J2 (DAPI fluorescent nuclear dye).

5.5 Discussion

Apart from a genetic predisposition, environmental stressors and the host immune responses to intestinal microflora are essential factors in IBD pathogenesis, including for Crohn's disease and ulcerative colitis. The role of stress-induced gut mucosal pathophysiology, mainly HS, has not been fully elucidated. However, recent evidence has indicated that chronic stress may be implicated in disease development (Bartosz et al., 2016). In our study, a chronic HS-induced IBD model was established. Gram-negative intestinal bacteria play an essential role in the intestinal inflammatory response mediated by the TLR4/NF-κB signalling pathway. In pigs, HS diverts blood from the internal organs to the periphery to maximise radiant heat dissipation. This response caused vasoconstriction in the GIT (Lambert, 2009), hypoxia in the intestinal epithelium because of the reduced blood supply, resulting in a reduction in nutrient flow. It compromised intestinal integrity and function (Yan et al., 2006). One study found that exposure to 37°C for 4 h can damage the intestinal mucosa of growing pigs. On exposure to 37°C for 6 h, a large number of intestinal epithelial cells were sloughed, and there was severe sub-mucosal congestion and oedema (Pearce et al., 2014). The HS pigs in the present study, similarly, showed thinner intestinal walls, shorter intestinal villi, and shallower crypts than the controls. Other stressors, such as psychosocial events in humans (Bartosz et al., 2016), cold stress (Manser et al., 2017), and restraint stress in mice (Koh et al., 2015), have been shown to induce strong inflammatory responses in the gut. Therefore, HS-induced vasoconstriction and hypoxia in the GIT do not fully explain IBD in pigs. Hence, we postulate that another mechanism may be involved in this pathogenesis.

The GIT is tightly controlled by the immune and neuroendocrine systems. Stress mitigation significantly improved gut homeostasis in pigs (Hayes et al., 2014b; Kayama, 2012). Destruction of the epithelial layer and thinning of the protective mucus layer may create conditions conducive to bacterial invasion of the mucosae and the blood vessels (Arnal & Lallès, 2016; Cox et al., 1991; Johansson & Hansson, 2014; Rashidul et al., 2005). Bacterial products such as LPS are recognised by the intestinal epithelial cells (IEC) and TLR4. The TLR4 upregulation in the gut enhances the ability of the host body to recognise antigens.

Nevertheless, sustained stress may trigger an inflammatory response (Alemu et al., 2018). Pearce et al. (2013) showed that HS and associated reduced feed intake compromised intestinal lining integrity and increased endotoxin permeability in pigs. Here, TLR4 expression was upregulated in HS pigs not only at the transcript level but also at the protein level, suggesting that the TLRs signalling pathway plays an important role in intestinal inflammation induced by HS (Pearce et al., 2013).

The nuclear transcription factor p65 induces inflammatory genes after it is translocated to the nucleolus. When rats were maintained at 42°C, their p65 activity was significantly increased relative

to a control group exposed to an ambient temperature of 25 ± 3 °C, and their IL-1B concentration was also dramatically upregulated (Arnal & Lallès, 2016). Two hours of exposure to HS in pigs can significantly increase blood TNF-α levels and inhibit lysozyme production. Numerous macrophages accumulate at the inflammation site and produce the pro-inflammatory cytokines IL-6 and IL-8, which mobilise lymphocytes and other immunocytes in the inflammatory process (Ishihara & Hirano, 2002; Kim et al., 2004). The upregulation of pro-inflammatory cytokines triggers an acute-phase reaction presenting with fever, anorexia, and hormonal and metabolic changes (Kick et al., 2011). A significant upregulation of IL-17 in the HS pig colon was also observed in my study. All the factors mentioned above contribute to IBD development and progress, suggesting that the TLR4/TRAF6/NF-κB signalling pathway plays a vital role in HS-induced IBD pathogenesis.

There are numerous microorganisms in the GIT, and the epithelial cell mucosa comprises the first-line protective barrier (Bloemendaal et al., 2016; Johansson & Hansson, 2014). Diarrhoea increased in intensity in HS pigs by day 14 when the number of opportunistic Gram-negative bacterial pathogens such as *Campylobacterales*, *Veillonellaceae,* and *Megasphaera* increased in the intestinal microflora. Oxidative stress is associated with host heat or cold stress (Sha et al., 2015) and may generate reactive oxygen species (ROS). The bacteria residing on the colonic mucosa have a relatively greater oxygen tolerance (Albenberg et al., 2014). This might favour the proliferation of aerotolerant phyla in the gut, such as *Actinobacteria* and *Proteobacteria*.

In contrast, gut microbiota can directly or indirectly contribute to ROS production *via* the mucosal cells (Tian, 2017). *Helicobacter pylori* generate ROS and also induce neutrophils to produce them (Handa et al., 2010). *Helicobacter pylori* also enhances nitric and nitrous oxide production by activating macrophages (Petrilli, 2017). As there is an interaction between inflammation and microflora, further studies are required to ascertain whether the observed intestinal microflora dysbiosis in the HS pigs is triggered by inflammation.

5.6 Conclusions

The present study showed that HS-induced intestinal dysbiosis disrupted gut microflora composition, increased the number of opportunistic pathogenic Gram-negative bacteria, activated the TLR4/NF-κB signalling pathway, and promoted pro-inflammatory cytokine production. This process triggered an inflammatory response and compromised the intestinal barrier integrity and function. This study has enhanced our understanding of stress-induced IBD, and the increase in diarrhoea in pigs subjected to prolonged HS, but further studies are required to ascertain the mechanism in its entirety.

Chapter 6

Heat-Stress-Associated Dysbiosis in Porcine Microbiota When Transplanted Can Alter Mice Colonic Microbiota

6.1 Abstract

Environmental stresses play an important role in inducing gastrointestinal disorders, including IBD, but the pathological mechanisms are still poorly understood. In this study, the effect of FMT from heat-stressed pigs (HF) and control pigs (CF) on the gut microbiota and intestinal pathology of mice was studied (Microbiome analyses of the pig faeces are reported in Chapter 4). The mice were transplanted with faeces collected from pigs on days 1, 7, 14, and 21 following exposure to HS (as described in Chapter 4). Pseudo-germ-free mice were orally gavaged with faecal bacteria from HS and control pigs to examine potential effects on the gut microbiome of mice. The HF mice did not gain weight, similarly to that observed in HS pigs. As described in Chapter 4, from day 7 onwards, HS pigs exhibited fever, diarrhoea, and a significant reduction in colonic villus length, crypt depth/width and goblet cell number. The results showed that gut microflora, intestinal pathology, and immune system changes in mice somewhat mimicked the changes observed in pigs even though the mice were not subjected to HS. However, the molecular mechanisms involved require further investigation. This study enhanced our understanding of HS-induced microbiome changes and related inflammatory bowel disease pathology in mammals subjected to prolonged HS. This study will provide useful information about inter-species FMT and the effect it has on the recipient species.

6.2 Introduction

Inflammatory bowel diseases affect >3.1 million people in the United States and 2.5 million in Europe annually, and its incidence is increasing worldwide, especially in East- and South-Asia (Dahlhamer et al., 2016). These diseases of the GIT are characterised by chronic intestinal inflammation and other lesions triggered by genetic predisposition and environmental factors (Ananthakrishnan et al., 2017). Various stressors, such as emotional distress, hyperthermia and sometimes hypothermia, perturb the gut–brain axis and induce inflammation and GIT disorders such as Crohn's disease and ulcerative colitis. Nevertheless, the pathophysiological mechanism of this process is unknown. As summer temperatures and relative humidity increase with global warming, the incidence and severity of illnesses related to thermal stress are expected to rise (Amengual et al., 2014). Heat stress has been found to induce IBD-associated changes such as intestinal mucosal inflammation and damage in pigs (Pearce et al., 2013), chickens (Quinteiro-Filho et al., 2010) and rats (Hall et al., 2001). Other stressors such as psycho-social events and hyperthermia may also promote IBD (Ho et al., 2019).

Understanding the stress-associated mechanisms of IBD and the increased susceptibility of livestock to all such diseases is now more important than ever because most emerging animal diseases, including Covid-19, have proven to be zoonotic, being transferred to humans, with associated threats to public health.

The mammalian GIT hosts ~1014 microbes, comprising 500–1,000 unique species, forming synergistic mutualisms with the host (Ley et al., 2005; Savage, 1977; Xu & Gordon, 2003). Co-evolution of gut microbes with their hosts has resulted in specialist roles for different microbes, including digestion, nutrient utilisation, toxin removal, protection from pathogens, and regulation of the endocrine and immune systems. A healthy intestinal microbe community is diverse, stable, resistant to minor changes and resilient (Levy et al., 2017). Human and mouse studies have shown that gut dysbiosis (i.e. disequilibrium of the microbial community) is associated with a range of acute and chronic inflammatory conditions, bowel diseases, metabolic syndromes, and diabetes (Brown, 2012). Gut dysbiosis and reduced complexity of the gut microbial ecosystem are common symptoms in patients with Crohn's disease or ulcerative colitis. It is unknown whether these alterations are causes or consequences of these diseases (Manichanh et al., 2012).

Faecal microbial transplantation (FMT) has been successfully used to treat *Clostridium difficile* infections that did not respond to antibiotics. Likewise, FMT has also been beneficial for some patients with diseases of the GIT such as IBD and inflammatory bowel syndrome, and its effects may be through specific microbes or active products that can modulate intestinal flora. However, its exact mechanism of action is still unclear (D'Haens & Jobin, 2019). The aim of this study was to elucidate the role of intestinal microbial composition in IBD occurrence in HS pigs. To accomplish this, FMT from pigs to mice was conducted to examine the inter-species effects of the modified microbiota.

6.3 Materials and Methods

6.3.1 Animals and management

The experimental protocols for management and care of pigs and mice were approved by the Animal Care and Use Committee of Guangdong Ocean University, Zhanjiang, China (Permit No. 206-1108).

6.3.2 Pig study

A heat stress (HS) study was conducted on pigs, as explained in Chapter 4. The faeces collected from both HS and control (CON) pigs from this experiment were stored immediately under liquid N_2 and later at −80°C until used for FMT.

6.3.3 Mouse study

One hundred and thirty weaned pseudo-germ-free mice (SPF grade), treated with a broad-spectrum antibiotic mixture (0.5 g/litre vancomycin, 1 g/litre neomycin sulphate, 1 g/litre metronidazole and 1 g/litre ampicillin) for 5 days, were used in this study (Rakoff-Nahoum & Medzhitov, 2008; Tiantian, 2018; Wang et al., 2018). All mice were maintained on a 12 h/12 h light-dark cycle, with free access to water in an autoclaved cage with circulating air (Exhaust Ventilated Closed-System Cage Rack, Guchuang Education Laboratory Animal Cage Co., China). Ten mice were controls and received only chow and water. The other mice (*n* = 120) were divided into four groups of 30 each. They were subjected to FMT with pig faeces from HS and CON groups collected on days 1, 7, 14, and 21, respectively. Each group was further subdivided into three treatment groups of 10 mice each. They were administered intragastric infusions of either PBS (PBS group), a 0.5-ml mixture of CON pig faeces homogenised in PBS (CON faeces; CF group), or an 0.5-ml mix of HS pig faeces homogenised in PBS (HS faeces; HF group). All mice were sacrificed by manual cervical dislocation following anaesthesia, 7 days after the intragastric infusion of faeces.

6.3.4 Sample collection

All the mice were weighed three times, once before treatment with antibiotics, once after administration of faeces, and finally just before sacrifice. After FMT administration, mice (*n* = 30 per collection day) were sacrificed on day 7. Mouse colonic faeces and colon tissue samples were collected and immediately stored under liquid N_2 at −80°C until the subsequent analyses.

6.3.5 Morphological observations

Colonic tissue was fixed in buffered formalin (10% v/v) and stained with H&E stain for histopathological examination. Image-Pro Plus v.6.0 was used to measure villus height, crypt depth, and width (Nagao-Kitamoto et al., 2016). Hydrated colonic tissue sections were treated with amylase at 37°C for 1 h, rinsed under running water for 10 min, and stained with periodic acid solution at room temperature (25°C) for 7 min according to the instructions for the Glycogen D-PAS Staining Kit. The tissue sections were rinsed with tap water, immersed in Schiff's reagent in the dark for 15 min, and rinsed with tap water for 10 min to remove the stain. The sections were dehydrated with an alcohol concentration gradient (75%, 85%, 95%, and then 100%), cleared of alcohol with xylene and sealed with neutral gum. Image-Pro Plus v. 6.0 was used to calculate the colonic mucosa goblet cells per unit area (Lan, 2015).

6.3.6 Microbial genomic sequencing

Total genomic DNA was extracted from the mice faecal samples with a QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA concentration and purity were

evaluated on 1% agarose gels. The quantity of DNA was determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) after it was zeroed with sample solvents, and the DNA was diluted to 1 ng/μL with sterile water. The V3–V4 distinct regions of the 16S rRNA genes were amplified with specific barcoded primers (Caporaso et al., 2010; Edgar, 2010). The PCR reactions were performed in triplicate in a total volume of 25 μL consisting of 1 μL of each of the primers (5 μM), 10 μL of 10 ng DNA template, 4 μL of 1×FastPfu buffer, 1 μL of 2.5 mM dNTPs, 0.4 μL of FastPfu polymerase, and 7.6 μL of nuclease-free water. The PCR program was as follows: initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 50 s, 55°C for 30 s, 72°C for 50 s, and a final extension at 72°C for 6 min. The PCR products were purified with an AxyPrep DNA Gel Extraction Kit (Axygen Scientific, USA). Amplicons from all samples were sent to a commercial company for sequencing on an Illumina HiSeq 2500 platform (Illumina, USA).

6.3.7 Microbial genomic analyses

Species classification information corresponding to each OTU was obtained by comparing the representative OTU sequence with the microbial reference database. After OTU rarefying [sample size < minimum of samples (McMurdie & Holmes, 2014)], sample community compositions were calculated at phylum, class, order, family, genus, and species levels and generated in the next-generation microbiome bioinformatics platform QIIME (v. 1.8.0). GraphPad Prism v.6.0c (GraphPad Software, USA), R v.3.0.3, Metastats, and STAMP (Statistical Analysis of Metagenomic Profiles) were used for the statistical analyses. The weighted UniFrac distances among groups were compared statistically by analysis of similarities in the 'vegan' package of R v.3.0.3. Mothur software was used to analyse the alpha diversity of each sample employing the Shannon and Simpson indexes. The QIIME software was used for beta diversity analysis to compare species diversity/similarity between different samples.

A binary algorithm was used to calculate the sample distance. The rarefied/rarefaction richness index (R) was used to draw Shannon Index curves, rarefaction curves, a rank abundance curve, species accumulation curves, principal coordinate analysis (PCoA) plots and a heatmap of sample distances. In the univariate analysis of gut microbiota and predicted KEGG metabolic pathways for each group, one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test was performed to compare the alpha diversities among the groups. Metastats identified differentially abundant phyla genera, classes, and species in the groups. Significant differences between groups were identified by the LEfSe method. The PICRUSt software was used to compare the species composition information obtained by 16S sequencing data, to speculate about the functional gene composition in the samples, to analyse the functional differences between different samples or groups (Parks et al., 2014). At the genus level, the G-TEST and Fisher-test methods were used to determine differences in

species abundance between samples. Significant differences between samples and pairwise *t*-test differences between groups were identified with the *P*-value threshold set at 0.05. Random forest analysis was used to test the importance of species. RDA/CCA, db-RDA and Mantel test analysis in the vegan package of R was used to analyse and map the relationships between treatment temperature, time and Diarrhoeal Index and the changes to microflora.

6.4 Results

At the start of the experiment, all mice were similar in weight (~15 g). When weighed immediately after faeces administration the mice were found to have not gained weight after the antibiotic treatment, since these acted to kill the native microbiota in their GIT. Administration of pig faeces had a subsequent effect on the bodyweight of the mice. It was found that the HF mice, recipients of HS pig faeces, had gained less weight over the period of testing (7 days) than the other sets of mice PBS and CF, which received PBS only or faecal microbiota from CON pigs, respectively.

6.4.1 Effect of FMT on the colonic microbiome in mice

At the phylum level, compared with the PBS group, *Firmicutes* were increased in all FMT mice groups (CF & HF, on all sampling days), except for the CF group on day 21. *Bacteroidetes* were increased by days 7 and 14 in the CF group and by day 21 in the HF group. *Verrucomicrobia* were decreased in all FMT groups. *Proteobacteria* were decreased in both CF and HF groups at all sampling days except for the CF group on day 7. When compared with the CF group at each respective sampling date, *Firmicutes* were decreased by days 1, 7 and 14 in the HF groups, but significantly increased (by >78%) by day 21. *Bacteroidetes* and *Proteobacteria* were increased at day 1 but had decreased by days 7 and 14. The changes in *Verrucomicrobia* were the opposite to that observed in *Bacteroidetes* (Fig. 4.6.1 A). At Order level, *Bacteroidales* had increased on days 7, 14 and 21 in the CF groups but decreased on days 1, 7 and 14 in the HF groups and also on day 1 of the CF group, compared with the PBS group. *Lactobacillales* were increased in both FMT groups (CF & HF) at all sampling days, except for the CF group on day 21. *Verrucomicrobiales* had decreased at all sampling days in both FMT groups, and *Erysipelotrichales* had increased on days 1,7 and 21 in CF mice and on days 1 and 21 in the HF mice. *Clostridiales* significantly increased (by 15.2%) on day 7 in the CF group compared with the PBS group. Compared with the CF mice, in the HF groups *Bacteroidales* had decreased and *Verrucomicrobiales* was significantly lower (25.03%, 29.64% respectively) on days 7 and 14. *Lactobacillales* and *Erysipelotrichales* increased by days 1 and 7 and decreased on days 14 and 21 in the HF groups, while *Clostridiales* were in contrast to *Lactobacillales* (Fig. 6.4.1 B). At genus level, compared with the PBS group, *Lactobacilli* were increased in all FMT (CF & HF) groups on all days except for the CF group on day 21. The *uncultured bacterium f Bacteroidales S24-7 group* were increased on days 7, 14 and 21 in the CF group, but decreased on day 14 in the HF group.

Akkermansia decreased in all FMT (CF & HF) groups and *Turicibacter* increased in the CF group except on day 14 but had decreased on days 7 and 14 in the HF group. The *Rikenellaceae RC9 gut group* increased on days 7 and 14 in the CF mice and in the HF group on day 21. In contrast, the *uncultured bacterium f Bacteroidales S24-7 group* increased on days 1 and 7 in the HF mice but decreased on days 14 and 21. *Akkermansia* were significantly increased (by ~ 25% and 29% respectively) on days 7 and 14 in HF mice but on days 1 and 21 were significantly decreased. *Turicibacter* had increased on days 1, 7 and 21 in the CF mice and the HF group on day 21 (Fig. 6.4.1 C).

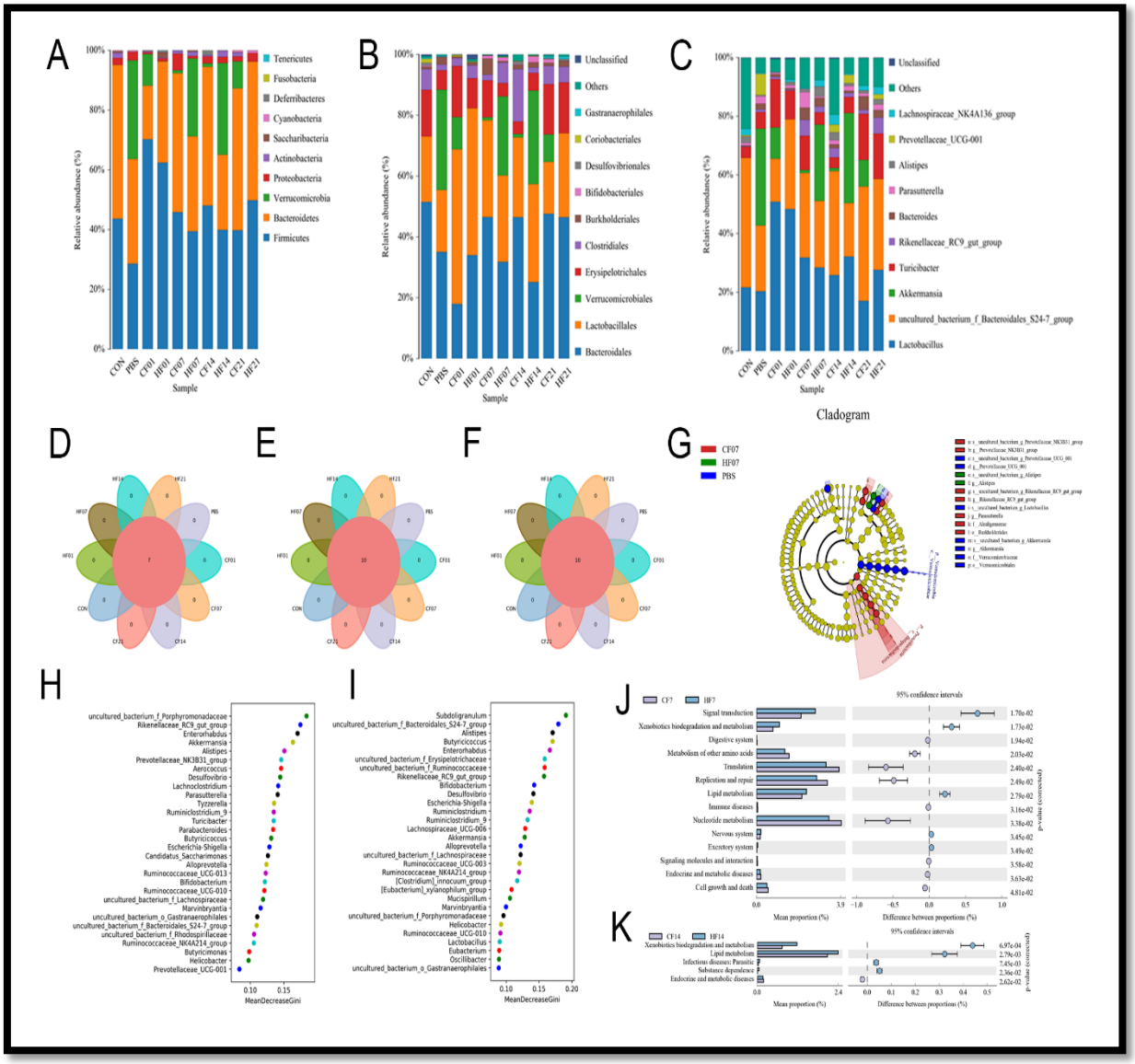

Figure 6.4.1 Effect of FMT on the microbiota composition in mice GIT.

(A) Intestinal flora structure by phylum; (B) Intestinal flora structure by class; (C) Intestinal flora structure by genus; (D) Venn analysis by phylum; (E) Venn analysis by class; (F) Venn analysis by genus; (G) LEfSe (Line Discriminant Analysis Effect Size) analysis on day 7; (H) Random forest distribution analysis on day 7; (I) Random forest distribution analysis on day 14; (J) KEGG functional difference prediction (CF7 vs HF7); (K) KEGG functional difference prediction (CF14 vs H1F4). CF & HF refer to infusion with faeces from control and HS animals, respectively; 1, 7, 14, and 21 refer to

sampling days of pig faeces and mouse transplantation. Only the results with Line Discriminant Analysis (LDA) significant threshold of >4 were shown.

Venn analyses showed that there are no differences at the phylum (Fig. 6.4.1 D), Order (Fig. 6.4.1 E) or genus levels (Fig. 6.4.1 F) between samples. The LEfSe analysis (LDA significant threshold of >4) showed that the relative differences in microflora between the PBS, CF, and HF mice on day 7 reflected increases in *Akkermansia, Prevotellaceae UCG-001* in the PBS group, increases in *Prevotellaceae NK3B31 group, Parasutterella, Alcaligenaceae* and *Burkholderialea* in the CF group, and increased *Alistipes* in the HF group (Fig. 6.4.1 G). Random forest analysis of microflora on day 7 showed that the top five dominant genera were *uncultured bacterium f Porphyromonadaceae, Rikenellaceae RC9 gut group*, *Enterorhabdus*, *Akkermansia* and *Alistipes* (Fig. 6.4.1 H), while on day 14, they were *Subdoligranulum, uncultured bacterium f Bacteroidales S24-7 group, Alistipes, Butyricicoccus* and *Enterorhabdus* (Fig. 6.4.1 I). The KEGG plot revealed relative differences in lipid metabolism and xenobiotic biodegradation and increases in signal transduction in HF mice sampled on days 7 and 14 (Fig. 6.4.1 J & K).

6.4.2 Mouse colonic histopathology following FMT

The following figure (Fig. 6.4.2) represents the effects of FMT from pigs on the histopathology of colonic mucosa in recipient mice.

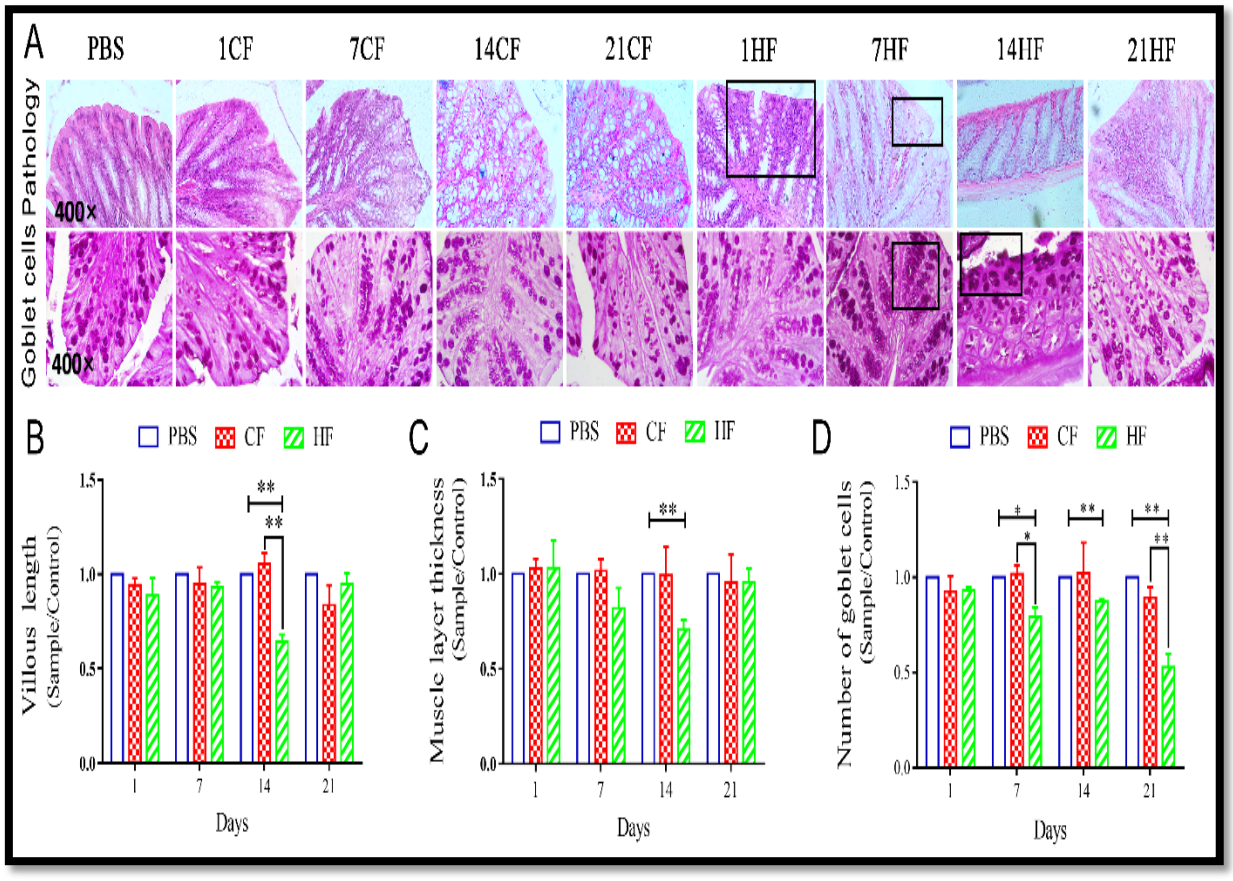

Figure 6.4.2 Histopathology of colonic mucosa of mice following pig faecal microbiota transplantation.

(A) H&E and PAS staining; (B) Colon villus length; (C) Colon muscle layer thickness; (D) Number of PAS-positive goblet cells per square millimetre of colonic mucosa.

* indicates significance at $P < 0.05$ and ** at $P < 0.01$ compared with the control groups (CF and PBS-only). Following FMT, on days 1, 7 and 21, lymphocyte infiltration and tissue gaps were observed in the colonic mucosa of the HF mice that had received faeces from HS pigs (Fig. 6.4 2 A). Goblet cell and mucosal epithelial shedding were comparatively less in the HF group on day 14 (Fig. 6.4.2 A, D). Compared with the CF and PBS controls, the colonic villi were significantly shorter ($P < 0.05$) (Fig. 6.4.2 B) and the intestinal muscle layer markedly thinner in the HF mice on day 14 ($P < 0.05$) (Fig. 6.4.2 C).

6.4.3 Effect of FMT on microbial diversity in mice

The Shannon Index (Fig. 6.4.3 A) and Simpson Index (Fig. 6.4.3 B) analyses showed that alpha diversity significantly changed ($P < 0.1$) in mice after FMT. The rarefaction curves showed that sample sequencing was sufficient to obtain meaningful results (Fig. 6.4.3 C). The Shannon Index plot indicated that for each sample, the curve was flat. Thus, there was enough sequencing data, and the number of OTU species did not increase with sequencing quantity (Fig. 6.4.3 D).

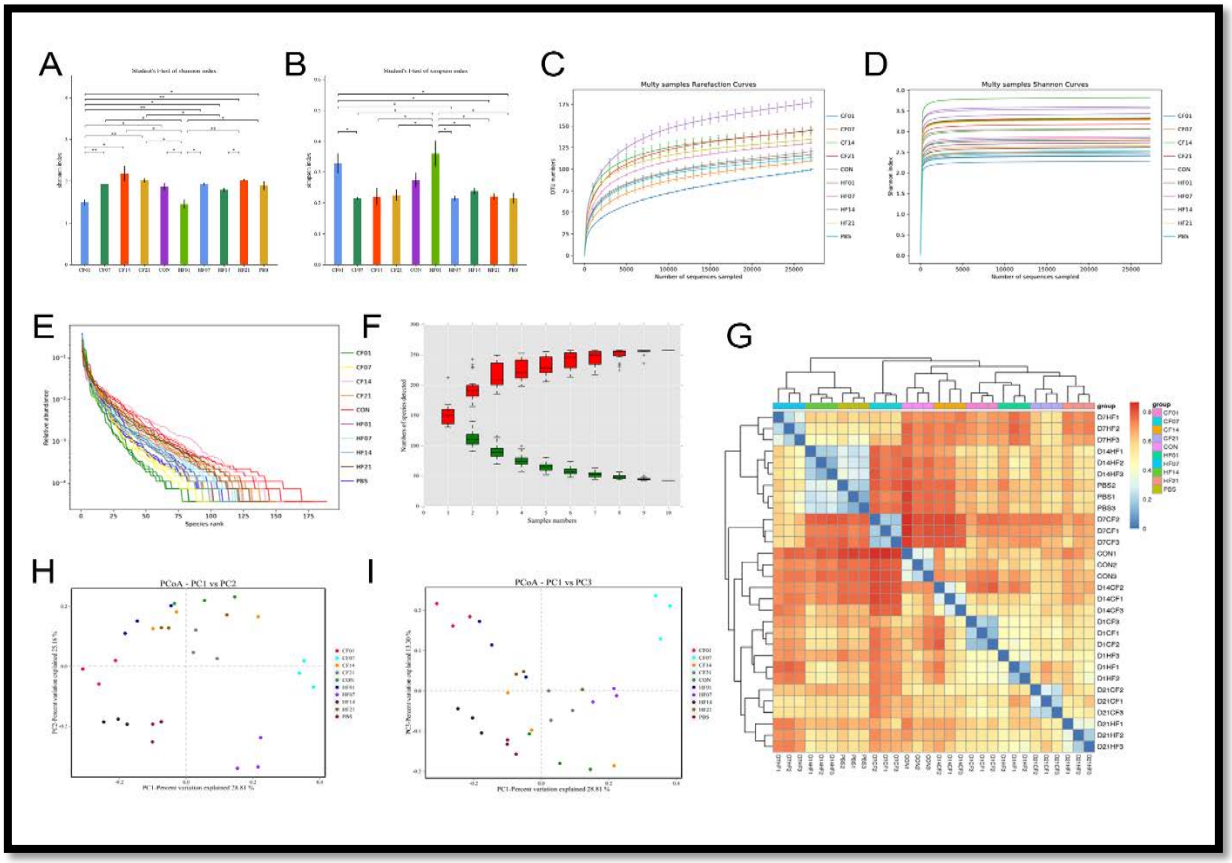

Figure 6.4.3 Effects of HS on the colonic microbiome in mice.

(A) Shannon Index plot; (B) Simpson Index plot; (C) Multi rarefaction curves; (D) Multi Shannon curves; (E) Rank abundance curve; (F) Species accumulation curves; (G) Heatmap of sample distances; (H) & (I) PCoA
* indicates significance at $P < 0.05$ and ** at $P < 0.01$ compared with the PBS-only control group.

The rank abundance curves were higher in the control mice (FMT with PBS only) compared to CF (FMT with faeces from CON pigs) and HF (FMT with faeces from HS pigs) groups, with the results of the PBS-only group sitting between the CF and HF groups on microbial species richness and uniformity of species composition (Fig. 6.4.3 E & F). Each sample had numerous OTU and was species rich. Most species were detected in all the samples (Fig. 6.4.3 F). The heatmap of sample distances showed that there was close clustering of species within each group (Fig. 6.4.3 G). The PCoA indicated that the microflora samples from the control mice (PBS-only) were very similar and equidistant. In contrast, those of the FMT groups (CF & HF) had relatively greater similarity (Fig. 6.4.3 H & I).

6.4.4 Correlation analysis of FMT on microbiota composition in mice

The RDA/CCA analysis based on binary-Jaccard distance showed that *Lactobacillus, Akkermansia, Turicibacter, Rikenellaceae RC9 gut group* and *Bacteroides* were positively correlated with FMT administration, whereas *Parasutterella*, *Faecalibaculum* and *Prevotellaceae UCG-001* were negatively correlated with FMT. The *Lactobacillus, Akkermansia, Faecalibaculum* and *Prevotellaceae UCG-001* bacteria were positively correlated with FMT of pig faeces from different HS sampling days, whereas *Turicibacter, Rikenellaceae RC9 gut group* and *Bacteroides* were negatively correlated with days after FMT (Fig. 6.4.4 A). Binary-Jaccard distance-based redundancy analysis (Fig. 6.4.4 B) showed that FMT treatment significantly affected microbiota formation ($P < 0.05$). In the Relevance heatmap, the *uncultured bacterium f Bacteroidales S24-7 group, Alistipes, Parasutterella* and *Bifidobacterium* were markedly affected by FMT.

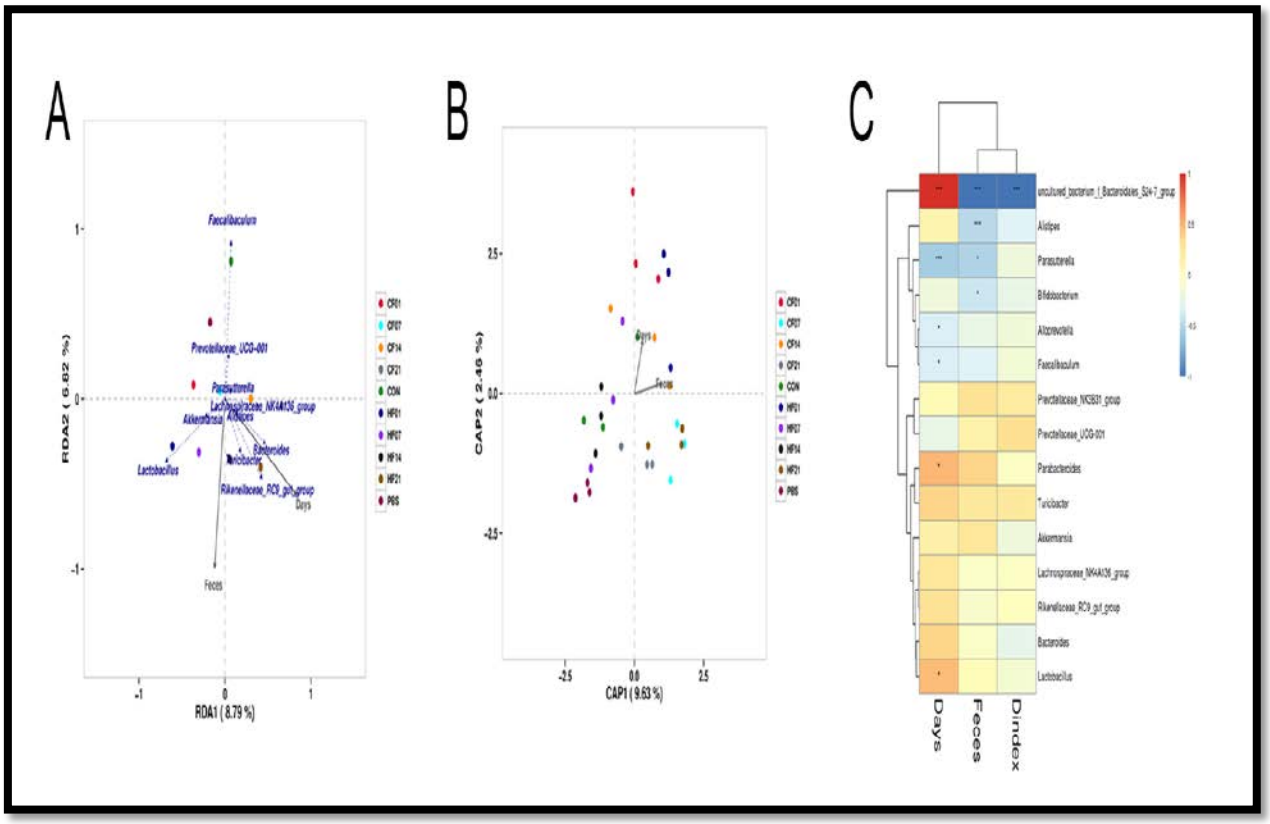

Figure 6.4.4 Correlation analysis of environmental factors on microbial species composition in mice. (A) RDA/CCA analysis; (B) Distance-based redundancy analysis; (C) Relevance heatmap.
* indicates significance at $P < 0.05$ and ** at $P < 0.01$ compared with the control (PBS only) group.

6.5 Discussion

It is now known that some environmental factors are important determinants in how a host immune system responds to shifts in intestinal microflora in the pathogenesis of IBD, including Crohn's disease and UC (Tabib, 2020; Zheng et al., 2020); but the role of HS in inducing gut mucosal pathophysiology has not been fully elucidated. In pigs subjected to HS, blood is diverted to the periphery of the body to maximise the dissipation of radiant heat and blood supply to the GIT is reduced via vasoconstriction (Lambert, 2009). This results in hypoxia in the intestinal epithelium and reduced nutrient flow, which compromises intestinal integrity and function (Yan et al., 2017). It appears that HS-induced vasoconstriction and hypoxia in the GIT do not fully explain IBD in pigs and we believe that another mechanism may be involved in the pathogenesis of this disease. There are numerous microorganisms in the GIT, and the epithelial cell mucosa comprise the first-line protective barrier (Bloemendaal et al., 2016; Johansson & Hansson, 2014). Diarrhoea increased in intensity in HS pigs by day 14 when the number of opportunistic bacterial pathogens such as *Campylobacterales*, *Veillonellaceae*, and *Megasphaera* increased in the intestinal microflora. Interestingly, *Clostridium Sensu Stricto-1* were decreased on exposure to HS. Heat or cold stress can induce oxidative stress in the host (Li et al., 2015) resulting in the generation of reactive oxygen species (ROS). The bacteria

residing on the colonic mucosa have a relatively greater oxygen tolerance (Albenberg et al., 2014) and favour the proliferation of aerotolerant phyla in the gut such as *Actinobacteria* and *Proteobacteria*. Also, gut microbiota can directly or indirectly contribute to ROS production *via* the mucosal cells (Tian et al., 2017). *Helicobacter pylori* both generates ROS and induces neutrophils to produce ROS (Handa et al., 2010). This *H. pylori* enhances nitric and nitrous oxide production by activating macrophages and induces severe inflammation of the gut epithelium (Petrilli, 2017).

Our results showed that the mice treated with HS pig faeces gained less bodyweight as compared to mice treated with PBS or CON pig faeces. This supplements my theory that certain species within the microbiota affect bodyweight gain in the host animal. Pigs subjected to HS also did not gain as much weight as CON pigs and this property has been probably transferred *via* the faecal matter to the recipient mice. The composition of the intestinal microbes in HF mice changed after FMT with pig faeces. Correlation analysis showed that changes in microbial composition were more significant as the HS treatment progressed. The FMT alters bacterial composition and establishes trans-kingdom equilibrium between gut fungi, viruses, and bacteria to produce homeostasis among gut microbes. Also, FMT is not a 'one size fits all' therapy and further studies are required to identify the specific components of the intestinal microbiome that affect patients with different gut-related syndromes (Ng et al., 2020).

Relative to the untreated mice, the FMT-treated HF mice exhibited fewer goblet cells, shorter intestinal villi, thinner muscle layers and an inflammatory response in their intestinal linings. Similarly, following exposure of Cherry-Valley ducks to 32°C for 3 weeks, He and group observed a change in gut microbial composition along with associated intestinal injury and fat deposition, and also a reduction in growth rate in the birds (He et al., 2019). Changes indicated by our LEfSe analysis and KEGG plotting revealed relative differences in lipid metabolism and xenobiotic biodegradation, as well as increases in signal transduction, in FMT mice, along with changes in microbial composition on days 7 and 14. Another group also reported damage to the intestinal mucosa in mice with IBD (Breitrück et al., 2013). Similarly, it was also reported that gut microbiota influenced certain traits in pigs and transferred their phenotypes to mice receiving pig FMT (Diao et al., 2016).

Gut microbes also influence epithelial cell morphology and renewal rates, intestinal nutrient digestion, and absorption, and affect the gut barrier (Diao et al., 2016; Pearce et al., 2014). In mice fed with mixed antibiotics, the distribution of microbes changed to create a consistent microbial structure in the mouse colon. In pseudo-sterile mice, the diversity of intestinal microbes is less and hence the number of OTUs is low. Interestingly, the construction of our pseudo-sterile mouse model resulted in an increase in *Akkermansia* in the colon. This suggests that feeding mixed antibiotics significantly affects the number of *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia* in the mice colon.

However, *Akkermansia* were less following FMT on days 7 and 14 in HF mice transplanted with HS pig faeces compared with the mice given FMT from CON pigs, in our study, indicative of the negative impact on mice receiving FMT from HS pigs. The number of opportunistic bacterial pathogens such as *Turicibacter* were increased on day 14 in the HF groups, which also suggests potential harm to mice receiving FMT from HS pigs. Correlation analysis also showed that changes in microbial composition in mice were significant following FMT from HS pigs and that FMT induced a negative impact on mice as early as day 7.

6.6 Conclusion

This study has shown that FMT can induce similar effects in the recipient species as seen in the donor. Heat stress had induced intestinal dysbiosis, disrupted gut microflora composition, and increased the number of opportunistic pathogenic Gram-negative bacteria in HS pigs. We successfully reproduced similar conditions in healthy, previously untreated mice following FMT from these HS pigs. This work has enhanced our understanding of stress-induced IBD, and the increased diarrhoea observed in mammals subjected to prolonged HS. Building on this, inter-species FMT and its effects can be utilised for development of therapeutics, not just in livestock animals, but also in humans.

Chapter 7

General Discussion, Conclusion and Future Research

7.1 General discussion

Global warming, and the ensuing climate change is causing high ambient temperatures around the world, in both tropical and temperate regions. Many climate models have predicted an increase in average worldwide ambient temperatures and more frequent and intense heat events in the future (Bernabucci et al., 2010; U.S. Environmental Protection Agency, 2015). Today, climate change is considered the most severe long-term challenge confronting farmers and livestock owners since it negatively impacts livestock production and the health of both people and animals. The Intergovernmental Panel on Climate Change predicts that by the year 2100, global surface temperatures will rise by 1.8–4.0°C (Shukla, 2019). Any such increase in temperatures by 1.5–2.5°C can risk extinction for approximately 20–30% of livestock and animal species around the world (Aggarwal, 2013; CDC, 2006), and untold temperature-induced increases in the human diseases burden.

In the swine industry particularly, HS has therefore become a critical stress factor for animal health (Upadhyay, 2011). Abnormally high temperatures affect thermoregulation and induce suboptimal animal performance and welfare, which results in economic losses and reduced food security (Johnson, 2018). Consequently, in addition to growth performance, it has become imperative to understand how HS affects animal nutrition and immunity.

Stress is a broad term and is described as the cumulative detrimental effect of a variety of factors on the health and performance of animals. Stress is defined as the magnitude of forces, external to the body, that together displace its systems from their resting or ground state (Aggarwal, 2013). Heat stress occurs in animals when there is an imbalance between production of heat within the body and its dissipation. Thermoregulation, a balance between heat gain and heat loss, is how an animal maintains its body temperature. Climatic stressors, coupled with environmental, nutritional, physical, social, or physiological stressors, are likely to diminish the welfare and performance of animals (Baumgard & Rhoads, 2013). Of these stressors, HS is one of the most critical, especially in tropical regions, where temperatures are already high. The endeavour by homeotherms to stabilise body temperature within reasonably narrow limits is essential to control biochemical reactions and physiological processes associated with healthy metabolism (Aggarwal, 2013).The general homeostatic responses to thermal stress in mammals include a reduction in faecal and urinary water losses, reduction in feed intake and production, and increased sweating, respiratory rates, and heart

rates. In response to stress, mammals set physical, biochemical, and physiological processes into play to try and counteract the negative effects of such HS and maintain thermal equilibrium (Pearce et al., 2013). Adaptation to HS requires the physiological integration of many organs and systems, namely, endocrine, cardiorespiratory, and immune systems (Kick et al., 2011). In pig production, previous research has also shown that HS causes death in sows in their perinatal period and in piglets, because pigs lack functional sweat glands and typically have thick layers of subcutaneous adipose tissue, thus causing immense financial losses to the pig industry (Cervantes et al., 2016). In this experiment, we subjected pigs to HS conditions similar to the raised temperatures predicted with global warming, to understand how HS affects the composition of the microbiota in their GIT.

The magnitude to which an animal can tolerate elevated ambient temperatures is defined by the animal species and its physiological state. Among livestock species, goats were found to be most tolerant of elevated temperatures (Silanikove, 2000). Likewise, pregnant or lactating ruminants are more susceptible to higher temperatures than non-pregnant and non-lactating ones (Silanikove, 2000). Also, animals chosen for their higher production potential are comparatively less tolerant of HS than animals with low production potential (Najar et al., 2011).

Previous researchers have shown that animals reduce their feed intake under both acute and chronic HS conditions (Lu et al., 2007; Pearce et al., 2014). Heat stress causes the upregulation and secretion of two adipokines, leptin and adiponectin, along with increased expression of their receptors (Bernabucci et al., 2010; Morera et al., 2012). Leptin stimulates the hypothalamic axis causing reduced feed intake, whereas adiponectin regulates the feeding behaviour through peripheral and central mechanisms, acting as ‘a starvation signal’ (Hoyda et al., 2011; Rabe et al., 2008). This form of caloric restriction allows hyperthermic animals to reduce metabolic heat generation. However, the full effect of HS on growth performances cannot be explained solely by reduced feed intake. Heat stress also reduces RNA content, proteolytic rates, and muscle protein turnover (Temim et al., 2000). Based on our findings, HS also negatively affects the composition of the gut microbiota. All these factors need to be studied individually and holistically to thoroughly understand the effect HS has on the body of an animal. This study found evidence of reduced feed intake in pigs put under HS, with the bodyweight gains in HS pigs being lower than in CON pigs.

Poor welfare occurs when an animal has difficulty managing a stressor and cannot adequately cope. It has been well-documented that HS negatively impacts well-being as animals try to maintain the response to thermal strain. A thermal strain refers to internal displacement in the body from its resting basal state and will result in an internal adjustment to achieve homeostasis in the face of the external stressor. This response differs based on previous exposure and tolerance level, genetics, lifecycle phase, and production stage. When exposed to the same heat load, tropically adapted

animals may display a decreased strain response when compared to their temperately adapted counterparts (Johnson et al., 2012; Renaudeau, 2005).

Livestock are regularly exposed to various stressors, including changes in diet and temperature, at weaning, and by infection. An increase or decrease in the magnitudes of these stress factors can influence animal health negatively, resulting in lower productivity and worsening health conditions. Such stressors induce systemic or local inflammatory responses coincident with neuro-endocrine alteration, challenging homeostasis. Since the GIT function is tightly controlled by a reciprocal circuit made of the immune system and neuroendocrine system (Hayes et al., 2014a; Kayama & Takeda, 2012), reducing stress would substantially improve the gut homeostatic balance of livestock. Gut microbiome and associated immune functions are undoubtedly crucial factors that are responsible for growth performance and health of animals. This PhD research investigated the impact of HS on the gut microbiome and immune status of pigs and whether transfer of pig FMT to mice could reciprocate similar changes in the gut microbiome and immune system of the mice.

7.2 Effect of HS on pigs

Pigs are subjected to numerous types of stresses that affect their production, reproduction, and health. Recently, environmental-induced HS has become a significant cause of concern due to its damaging impacts on animals, especially in highly productive animals such as pigs. In tropical, subtropical, and arid regions of the world, high ambient temperatures are now the primary factor endangering pig production (Slimen, 2016).

Pigs, when exposed to temperatures higher-than-normal, exhibit antagonistic effects such as increased respiration rate, higher rectal and forehead temperatures, decreased food intake, poor carcass quality, and reduced meat quality (Huo et al., 2019). Our studies also showed that pigs subjected to similar HS for 3 weeks reduced their feed intake over time and exhibited lower bodyweight gains as compared to the control pigs - which were maintained at normal ambient temperature. One of the reasons for this is that pigs have a very low tolerance level for thermal stress.

7.2.1 Effect of HS on pig microbiome

The GIT of mature pigs has quite a stable microbiota, consisting mostly of beneficial species like lactobacilli, but also potentially pathogenic bacteria, such as *E. coli* (Gaskins, 2001). Over the past decade, the scientific community has come to realise that GIT health in pigs is mainly influenced by the composition of their gut microbial community and the diverse end-products its differing member microbes produce for the benefit of the host (Rist et al., 2013).

In pigs, the GIT microbiota primarily has Gram-positive bacteria, such as the aerotolerant *Streptococcus*, micro-aerobe or obligate anaerobes like *Lactobacillus*, *Bifidobacterium*, the obligate anaerobes *Peptostreptococcus*, *Clostridium* and *Ruminococcus*, and facultative anaerobe *Escherichia*, but also has obligate anaerobe Gram-negative bacteria such as *Fusobacterium*, *Bacteroides*, *Selenomonas*, *Butyrivibrio* and *Prevotella* (Gaskins, 2001). The relative proportions of species and absolute quantity of bacteria vary substantially along the digestive tract (Savage, 1977). The proximal part of the GIT has least density of bacteria, with the distal part being most dense (Jensen, 2001). As opposed to the human GIT, the pig GIT also contains many indigenous bacteria, mainly lactobacilli, within the stomach and small intestine (Jensen & Jørgensen, 1994). Overall, there are four microhabitats colonised by the commensal microbiota: the gastrointestinal lumen, the unstirred mucus layer, the deep mucus in the crypts, and the surface of the intestinal epithelial cells (Pluske et al., 2018).

Heat stress is a complex and highly stressful event in a pig's life; numerous reports have found evidence that HS causes an abrupt taxonomic and functional shift in the intestinal microbiota of the pigs (Mayorga et al., 2019; Ross et al., 2015b). One of the major associations the GIT microbiota has with the host is its influence on bodyweight. It has been found in rodents and humans that a certain composition of microbiota affects development of obesity (Davis, 2016; Ridaura et al., 2013; Turnbaugh et al., 2008). This was mirrored in my results from this project; pigs subjected to HS had lower bodyweight gains as compared to CON pigs.

Healthy, normal-weight mammals tend to have a higher *Firmicutes*/*Bacteroidetes* ratio than their leaner, low-weight counterparts (Ley et al., 2006). Our study found that the numbers of *Firmicutes* had decreased and *Bacteroidetes* increased over time in HS pigs, meaning this ratio had been disrupted causing a decrease in bodyweight gain. A recent study reported a positive correlation between *Proteobacteria* and fat intake. It showed that an increase in *Proteobacteria* was a risk factor for human health, including dysbiosis, and hence abnormal growth of *Proteobacteria* could be a cause for imbalance in the gut microbial community and be a future disease risk (Méndez-Salazar et al., 2018; Shin et al., 2015b). This PhD study also found an increase in the numbers of these Gram-negative *Proteobacteria*.

The phylum *Spirochaetes* comprises a large group of motile bacteria, widespread in the environment so highly prevalent disease-causing agents (Gupta et al., 2013). They have a characteristic feature called the endoflagella, a special flagella that folds back into the cell and remains within the periplasm (Li et al., 2008). The phylum *Spirochaetes* has four important genera – *Treponema*, *Borrelia*, *Leptospira*, and *Brachyspira* – whose species cause many globally prevalent illnesses (Gupta

et al., 2013). As seen in the sequencing results of this study, the number of *Spirochaetes* bacteria in the GIT of HS pigs increased with HS, making the animals more prone to future infections.

A 2008 study found that higher temperatures, even up to 32°C, can reduce the average daily feed intake by 32%, the average daily gain by 39%, bodyweight by 10% and gain:feed ratio by 16 (White et al., 2008). These results are similar to our findings that HS pigs were seen to reduce their feed intake and have a reduction in bodyweight gain over the HS period of 21 days.

7.2.2 Effect of HS on pig GIT immunity

Intestinal mucosa acts principally as a barrier between the inner part of the body and the external environment. In livestock animals, large quantities of various food ingredients, and also antigens, are digested and absorbed daily, which are generally immunologically tolerable at intestinal mucosa level. The protective mucosal immune responses rapidly eliminate most of the pathogens entering the body. To do this, the gut immune system has to distinguish between harmless antigens and the harmful ones (Wilson et al., 1996). The swine intestinal immune system is immature at birth and develops during the perinatal period and then reaches the adult stage between the first 5–7 weeks of life. Toll-like receptors are a functionally important class of membrane and cytosolic receptors with the primary role of recognising pathogens for innate immune modulation. Induction of adaptive immune responses begins with the processing and presentation of antigens by specialised antigen-presenting cells (Lee et al., 2014).

The most over-represented biological processes are generally related to the immune system. A few previous studies have associated immune function genes with mammalian response to HS. It was found that the heat response cascade in animals involved three mechanisms, beginning with heat shock proteins, followed by expression of interferon-inducible genes, and finishing with small non-specific stress responses of specific cell lines (Daniel et al., 2006). One study observed that mice that were intolerant to heat had a greater expression of inflammatory cytokines after exposure to HS compared to mice that were heat tolerant (Islam et al., 2013). Similarly, in chickens, white blood cell counts and antibody production were detected to have changed as a result of HS (Mashaly et al., 2004). Heat stress disrupts normal folding of newly synthesised proteins, which, hence, are not recognised as native proteins and are targets for degradation. The glutathione transferase pathway breaks down molecules that it recognises as potential toxins or foreign material, and it was shown that genes in this pathway are upregulated during HS (Stallings et al., 2014). In a similar experiment , it was found that during short-term HS there was an increase in the relative abundance of the NF-κB activator inhibitory κB kinase-α, and also in the relative abundance of phosphorylated NF-κB, in nuclear fractions (Shanthi Ganesan et al., 2016). These results agree with what we observed with

nuclear p65 upregulation at RNA and protein level, and upregulation of the pro-inflammatory cytokines IL-6, IL-8, and IL-17.

Table 7.2.1 gives an idea of how HS impacts the immune system in the gut. In our research, it was found that HS caused epithelial cell sloughing, vasodilation, and mucosal hyperemia in the colonic intestinal epithelium of HS pigs. Also, the levels of TLR4 and TRAF6 were significantly upregulated in HS pigs on day 1 of the experiment. These results are similar to those reported by (Huang, 2017) in broiler chickens, and in Bama miniature pigs (Ju et al., 2014).

Table 7.1 Impact of HS on gut immunity

Stressor	**Conditions**	**Sample**	**Changes**	**Reference**
Heat	35°C for 24 h	Ileum	GLUT2 (1.5×), HSP70 (2×) and HIF-1α (1.5×) – upregulated Myeloperoxidase activity – increased by 4 U/mg	(Pearce et al., 2013)
		Ileum and colon	The permeability measured by using TER and FITC-dextran transport – increased	
		Serum	Endotoxin – increased by 3×	
	37°C for 6 h	Ileum	Mucin 2 – increased by 0.35 ng/ml at 6 h post-HS Villi height – decreased by 181 µm at 6 h post-HS	(Pearce et al., 2014)
		Colon	HSP70 – increased 2× at 2 h post-HS	
		Serum	LBP – decreased at 2 h post-HS Endotoxin showed the tendency for a linear increase over time	

In our research, it was evident that the faecal LPS concentrations in the HS pigs on days 1, 7, and 14 were significantly higher than in the controls. Western Blot analysis revealed that TLR4 expression was markedly elevated in HS pigs after treatment of IPEC-J2 cells with 10 µg/ml LPS for 3 h. These

results are identical to the findings of one study in which growing pigs were exposed to acute HS for 24 h (Pearce et al., 2014). When Webel and colleagues challenged pigs with intraperitoneal LPS injections, they observed an increase in plasma urea nitrogen levels in association with increased circulating tumour necrosis factor (TNF)-α and IL-6 (Webel et al., 1997), similar to what we observed on day 7 in HS pigs.

7.3 Faecal microbiota transplantation

The first known documentation of the use of human donor faeces as a therapeutic agent was recorded in the Chinese *Handbook of Emergency Medicine* by Ge Hong in 340 B.C., in which he prescribes ingestion of faeces from babies (called 'yellow soup') for a variety of diseases (Zhang et al., 2012). Lewin reported in his book that '… consumption of fresh, warm camel faeces has been recommended by Bedouins as a remedy for bacterial dysentery; German soldiers confirmed its efficacy in Africa during World War II' (Lewin, 1999). The first modern use of FMT was in 1958 by Eiseman and his group for the treatment of pseudomembranous enterocolitis, presumably due to *C. difficile* infection (Khoruts & Sadowsky, 2011). Since then, FMT has gained popularity due to its efficacy, low-cost applicability, and ease of use in treatment (Nieuwdorp, 2014).

Many studies have linked altered microbiota to a variety of disease conditions suggesting that it may be a novel diagnostic and therapeutic target. Van Nood and group, in 2013, found FMT to be superior to antibiotics for antibiotic-associated *C. difficile* infections. Many randomised clinical trials with FMT have been undertaken for IBD, diabetes mellitus, and non-alcoholic steatosis hepatitis (van Nood et al., 2013). Although causality has not yet been proven for the involvement of intestinal microbiota in most of these conditions, FMT promises to be an exciting tool for diagnostic and therapeutic leads.

In 1989, physician Bennet treated himself with several donor faecal enemas and was able to induce long-term remission of his IBD flares (Brinkman & Brinkman, 1989). From then, many case reports have been published with a range of therapeutic effects in patients with Crohn's disease and ulcerative colitis (Conceição-Neto et al., 2018; Rezapour et al., 2017). The use of FMT in patients with IBD should be viewed with caution since adverse effects such as transient fever, abdominal pains, bloating, and no or little clinical improvement have been reported in some cases (Smits et al., 2013).

The intestinal tract of animals is swiftly colonised, right after birth, by a heterogeneous group of micro-organisms, called the intestinal microbiota. Previously, the relationship between the host and the intestinal microbiota was termed as commensalism or parasitism, but recent research has revealed that their relationship is mutualism. Since the 1990s, high-throughput sequencing technology, culture-independent analysis techniques have been developed, which have revealed to scientists that intestinal microbiota affects various physiological traits in host animals, including the

immune function (Ivanov & Littman, 2011), age (Yatsunenko et al., 2012), brain development and behaviour (Heijtz et al., 2011), obesity (Turnbaugh et al., 2008), and hypertension (Li et al., 2017). Although studies of the intestinal microbiota–host interaction are ongoing, very few studies have been performed in livestock like pigs, cattle, and chickens, and even among these, basic studies related to the host physiology are scarce. Our study is one of the pioneers to acknowledge that changes in microbiota affect the immune system as well as the overall physiology of pigs.

Many previous studies have focussed on FMT within the same species (e.g., mice to mice) mostly on changes in physical characteristics. A recent study showed that FMT was able to reverse gut dysbiosis in mice induced by antibiotics and chemotherapy (Le Bastard et al., 2018). Some studies have documented the effects of FMT within pigs also (Lin et al., 2018; McCormack et al., 2018; Niederwerder et al., 2018), including how FMT can be utilised as an effective strategy to improve the health of pigs after a disease, or to improve feed efficiency. Similarly, many studies have been undertaken with mouse models to study human diseases. One study successfully managed to transplant human microbiota into germ-free mice and preserve the taxonomical and functional features of the human donor microbiota (Ridaura et al., 2013). Building upon a similar premise, we attempted to transplant faecal microbiota from HS pigs into germ-free mice. Changes related to those found in the intestine of the HS pig donor were also found in the corresponding recipient mice. The microbiota transplanted from the pigs activated the immune system of the mice and induced similar upregulation of pro-inflammatory cytokines. This has boosted our knowledge about inter-species FMT and the resulting consequences of the same.

7.4 Conclusions

Climate change is real and it cannot be denied anymore. The resulting rise in atmospheric temperatures around the world is affecting not just humans but also animals, even more so with consequent overall reduction in meat production for human consumption. Numerous previous studies, including this study, have consolidated the fact that the stress caused by these higher-than-normal temperatures is harmful to the health of animals and is fast becoming a significant cause of bad health, loss in productivity and, in extreme conditions, increased mortality. The proper functioning of different systems within the body of the animals is hampered, resulting in a diseased state and can culminate in death.

This study was undertaken to ascertain as to how thermal stress due to an increase in atmospheric temperature negatively impacts on the health of pigs, a vital livestock species. Pork is a cheap and popular source of protein for humans, and also pigs are very similar genetically to humans. This enables scientists to visualise and experiment on pigs to understand human diseases and, maybe in future, to find a remedy for them.

As our research progressed, the initial hypothesis that higher temperatures modify the composition of intestinal microbiota was found to be true. Genetic sequencing studies showed that the number of potentially pathogenic Gram-negative micro-organisms increased, and the rate of bodyweight gain in HS pigs was less when compared to the control set of pigs. The increase in number of pathogens within the pig gut triggered a response from the immune system, activating the NF-κB signalling pathway, causing inflammation (increase in pro-inflammatory cytokine numbers) and subsequently IBD in most cases. Analysis of histological samples collected during sacrifice also confirmed our hypothesis about the detrimental effects HS has on the intestinal cell lining, colonic villus length, crypt depth/width, and goblet cell numbers.

In a subsequent part of the project, an attempt was made to confirm the findings of the effect of HS on microbiota, in another mammalian species, the mouse. The faecal matter from the pigs used in the first stages of the project was transplanted orally into SPF mice, who were made devoid of any native microbiota by an initial treatment with an antibiotic mixture. This enabled us to test the effect of transferring the modified microbiota (caused by HS) from pigs into previously unaffected host bodies and observe the changes that occurred in the recipient FMT mice as compared to normal healthy mice. In accordance with our hypothesis, the modified microbiota from the affected pigs caused similar changes in the mice, including having a major influence on their immune system and causing IBD.

7.5 Future research

The unfortunate reality of the world currently is that we, as a human race, have not yet been able to mitigate or overturn climate change successfully. Moreover, it has been predicted that the increased use of fossil fuels, plastics, and other pollutants contributing to the greenhouse effect will continue for the foreseeable future. Due to this, scientists have been forced to devise methods and strategies to keep humans and animals safe from these rising temperatures. The present research is one of the first studies in this field to investigate the effect of HS on the health of pigs in terms of their microbiome composition and immune function changes. We now know the impact an altered microbiota can have on the production, welfare, and health of pigs. Through this research work, we are also beginning to understand how the microbiota influences different body systems and the physiological and immunological pathways in the body of animals. This work will open up vistas that will allow future researchers to develop novel therapeutics to mitigate the ill-effects of higher temperatures. Once the causality for IBD and other such intestinal disorders is established, cures can be sought for these age-old health issues that have troubled the swine industry, and human health, for decades. An attempt can hence be made in the future to transplant microbiota from pigs into

humans or vice-versa to study human diseases and develop diagnostic and therapeutic solutions for mammalian diseases.

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