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Understanding gut microbiomes as targets for improving pig gut health

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Microbiological services delivered by the pig gut microbiome

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1 Introduction

The pig gastrointestinal tract (GIT) is a complex and diverse microbial ecosystem inhabited by bacteria, viruses and archaea, as well as eukaryotes including fungi and protists, that is, the gut microbiota, which, together with their genomes are collectively referred to as the gut microbiome (Ilhan, 2018; Ramayo-Caldas et al., 2020). An increasing body of research has highlighted the fundamental role of the gut microbiome in pig health and growth (Guevarra et al., 2019; Nowland et al., 2019). This chapter will focus primarily on the role of the resident bacterial communities in the pig gut and will explore their relationships, interactions and contributions to the host. An estimated 100 trillion bacterial cells in the mammalian GIT contribute to host health, with the pig colon alone estimated to contain between 10 billion and 100 billion bacteria per gram of content (Gaskins et al., 2002; Guevarra et al., 2019; Isaacson and Kim, 2012). These microorganisms deliver microbiological services such as

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the prevention of pathogen colonisation and production of volatile fatty acids (VFAs) and vitamins from food components that are typically indigestible to the host (Holman et al., 2017). This chapter also examines bacterial quorum sensing (QS) as well as the pig gut antibiotic resistome, and its implications as a reservoir of antibiotic resistance genes (ARGs).

2 Pig gut microbiome: abundance and diversity

The co-evolution of gut microbes with pigs has allowed for a synergistic relationship to develop between the host and 500–1000 distinct bacterial species that have adapted to perform a range of beneficial functions related to modulation of pig health (Patil et al., 2019). The pig gut microbiome is highly dynamic and is determined, and subsequently influenced by several factors including age, diet and antibiotic administration, for example (Niu et al., 2015). This section will serve as an introduction to the pig gut microbiome and will discuss the microbial shifts that occur in the pig GIT from birth to slaughter and along different regions of the tract, as well as recent developments in identifying the core microbiome of pigs.

2.1 Development of intestinal microbiota over the lifetime of a pig

It has long been held that during gestation, the piglet gut is sterile and that immediately following birth, microbial colonisation begins (Guevarra et al., 2019). However, studies in mice and humans suggest that some *in utero* bacterial colonisation occurs but whether this happens in pigs is currently open to debate (Ardissone et al., 2014; Jiménez et al., 2008; Nowland et al., 2019). The nature of initial colonisation is influenced by environmental factors including the sow as well as the timing of exposure to different inocula, with repeated compared to single exposures reportedly resulting in different microbiomes (Fouhse et al., 2016).

One of the most critical periods for pigs is weaning, as around this time, the gut microbiota is most susceptible to change (Nowland et al., 2019). This period is characterised by a range of stressors for piglets including separation from the sow and littermates as well as the transition from milk to a solid cereal-based diet (Guevarra et al., 2019). These weaning stressors contribute to the disruption of the gut microbiota, termed 'dysbiosis', allowing for the proliferation of pathogenic microorganisms, thereby increasing the incidence of diseases such as diarrhoea and enteritis (Yang et al., 2019).

Sun et al. (2019) found that *Enterobacteriaceae* dominated the faecal microbiota of diarrhoetic piglets during suckling, while the *Bacteroidales* family *S24-7 group* was identified as a biomarker of diarrhoetic piglets at the early weaning stage. Furthermore, *Escherichia-Shigella* was identified as the core component of the diarrhoetic piglet microbiota, while *Prevotellacea*e

UCG-003 was the dominant genus in non-diarrhoetic piglets. Yang et al. (2019) also suggested that an alteration in the relative abundance of *Escherichia* and *Prevotella* may be associated with pre-weaning diarrhoea.

De Rodas et al. (2018) observed age-related changes in the gut microbiota of pigs from birth to market, including increasing abundances of *Clostridia* and decreasing abundances of *Gammaproteobacteria*. However, at 24 h post-weaning (21 days of age), there was a significant reduction in *Lactobacillaceae*, followed by a subsequent dramatic increase at day 33. This coincided with the introduction of solid feed and had the greatest impact on gut microbiota composition compared to age, changes in solid feed type and pig movement (De Rodas et al., 2018). Motta et al. (2019) found that the weaning period resulted in a shift from a high relative abundance of *Bacteroidaceae* and *Enterobacteriaceae* to a *Prevotellaceae*- and *Ruminococcaceae*-dominated microbiota post-weaning. Functional metagenomic analysis indicated that high concentrations of long-chain fatty acids in the sow's milk may serve as an energy source for *Enterobacteriaceae* in suckling piglets (Motta et al., 2019).

Zhao et al. (2015) found that the ratio of *Firmicutes* to *Bacteroidetes* in the faeces of older pigs (2, 3 and 6 months old) was 10-fold higher than that of piglets at 1 month old (Fig. 2). As the pigs matured, they developed a more stable microbiota, in agreement with previous findings (Nowland et al., 2019; Schmidt et al., 2011). Han et al. (2018) reported that the diversity and richness of the gut microbiota decreased with age, especially in finishing pigs. They also found compositional differences with *Bacteroidetes*, *Firmicutes* and *Proteobacteria* dominating for the first 42 days post-weaning, followed by *Bacteroidetes*, *Firmicutes* and *Spirochaetes* during the growing stage, and *Bacteroidetes*, *Firmicutes* and interestingly the archaeal phylum *Euryarchaeota* during the finishing stage. Principal coordinate analysis (PCoA) also shows distinct clustering of pig gut microbiota across development stages (Han et al., 2018; Fig. 1).

Overall, these data demonstrate distinct age-related gut microbiota composition, with microbiota maturation occurring over time and weaning leading to the most dramatic microbial shifts.

2.2 Core gut microbiome of pigs and variance between intestinal sites

The conditions of the GIT vary from proximal to distal regions and between the mucosa and lumen, resulting in differing bacterial populations (Figs 2 and 3) (Kelly et al., 2017). Zhao et al. (2015), when investigating whether faecal samples were representative of the intestinal microbiome, found that the dominant phylum in faeces was *Firmicutes*, while *Proteobacteria* predominated in the small

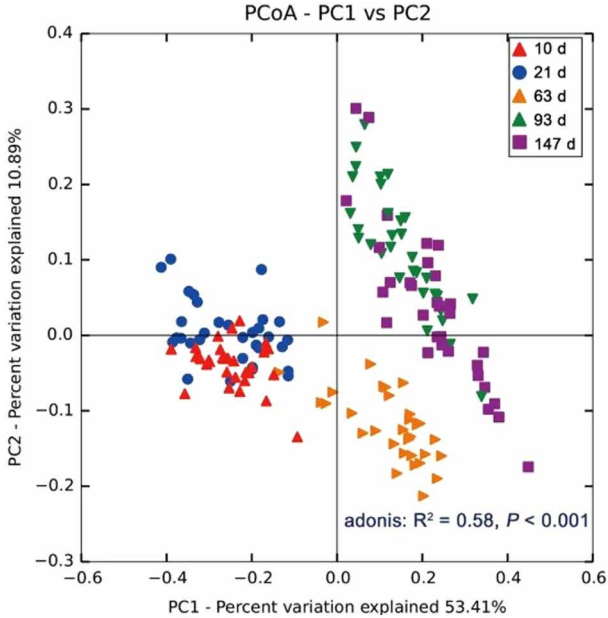

Figure 1 Principal coordinate analysis (PCoA) plot of weighted UniFrac distances displaying the diversity of faecal microbiota of commercial pigs (n = 32) at various growth stages (pigs weaned at 26 days of age). The effect of the growth stage on the microbial community was analysed using Adonis statistical tests with 999 permutations. Adapted from Han et al. (2018) distributed under terms of the Creative Commons Attribution 4.0 International License.

intestine (Fig. 2). However, as expected, the microbial composition of the large intestine was more like that of faeces, in agreement with McCormack et al. (2017).

Zhao et al. (2015) reported that *Proteobacteria* and *Firmicutes* constituted >70% and ~20% of the microbiota in the jejunum and ileum, respectively (Fig. 2). Conversely, others have reported that *Firmicutes* predominate in the small intestine, with variable proportions of *Bacteroidetes* and *Proteobacteria* (Crespo-Piazuelo et al., 2018; De Rodas et al., 2018; Quan et al., 2018). In the caecum and colon, Zhao et al. (2015) concluded that *Firmicutes* are the dominant phylum, representing >75% of the bacterial population followed by *Proteobacteria*; however, Quan et al. (2018) found that the relative abundance of *Bacteroidetes* was as high as 46% in the caecum.

An interesting concept that has emerged over the last number of years is whether a 'core' pig gut microbiome exists, independent of age, breed, origin and diet. A meta-analysis carried out by Holman et al. (2017) analysed 20 published data sets of 16S ribosomal ribonucleic acid (rRNA) gene sequences of pig gut and faecal samples, in order to determine if certain bacterial taxa prevailed, irrespective of age, gut location and so on. *Firmicutes* and

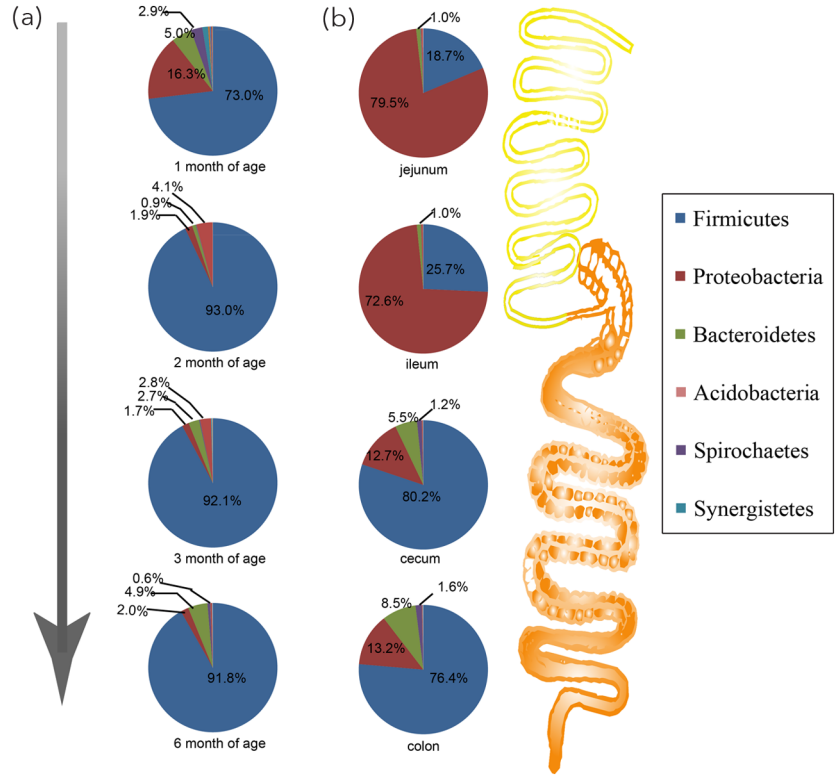

Figure 2 Microbial profile within distinct sections of the pig intestinal tract and faeces at the phylum level. (a) The faecal microbiota of pigs at 1, 2, 3 and 6 months of age. (b) Microbial profile in the small and large intestines at 6 months of age (slaughter). Adapted from Zhao et al. (2015) distributed under terms of the Creative Commons Attribution 4.0 International License.

Bacteroidetes were the dominant phyla, representing almost 85% of all 16S rRNA gene sequences detected across all gut locations, with *Proteobacteria* the only other phylum present at all locations. They also found a number of genera that were present in >90% of samples including (in order of decreasing relative abundance) *Prevotella, Lactobacillus*, *Clostridium*, *RC9 gut group* and *Blautia* (Fig. 3). Wang et al. (2019b) also found *Prevotella* to be the most dominant and the most diverse genus within the faecal microbiota, particularly after the introduction of solid feed to the diet at weaning. Interestingly, *Prevotella* has gained considerable attention recently as a key genus within the pig gut microbiota, having been linked with increased piglet growth rates (Mach et al., 2015).

Lactobacillus is also dominant within the core pig gut microbiome, accounting for up to 15% of 16S rRNA gene sequences in faeces, independent

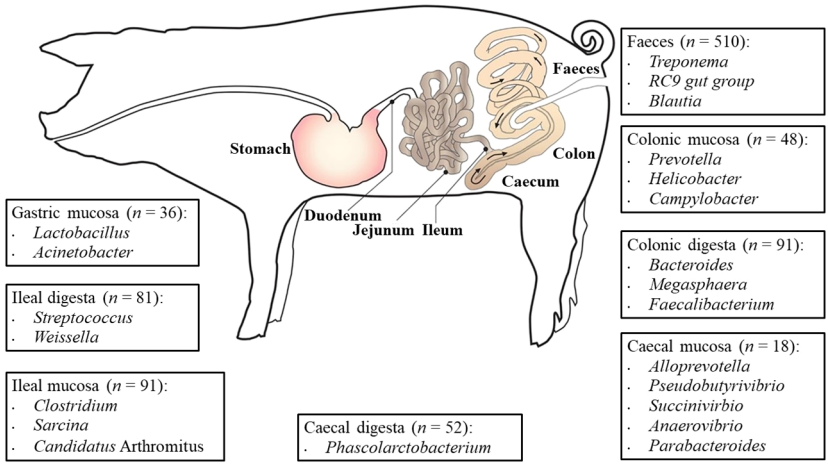

Figure 3 Diagram indicating major sections of the pig gastrointestinal tract and direction of movement of digesta in the colon. Boxes detail the differentially abundant genera in each distinct gastrointestinal section as determined by linear discriminant analysis (LDA) with effect size (LEfSe) measurements. Genera with an LDA score (log_{10}) >4.0 are displayed. Duodenum and jejunum mucosa and digesta samples were excluded from this analysis as sample numbers were insufficient. Adapted from Holman et al. (2017) distributed under the terms of the Creative Commons Attribution 4.0 International License.

of age (Niu et al., 2015), and is reportedly the dominant genus found in the stomach (Mann et al., 2014). Holman et al. (2017) also found certain genera to be differentially abundant in specific areas of the pig gut, as summarised in Fig. 3. Mucosa-associated bacterial populations are represented here, but many studies focus on the lumen contents and/or faeces, as reflected in the sample numbers indicated. This may be an oversight considering that mucosa-associated bacteria are more likely to be autochthonous than taxa found in the digesta, which may merely be passing through. Mann et al. (2014) studied the mucosa-associated microbiota of the pig GIT and found a similar composition to that reported by Holman et al. (2017).

A considerable amount of research is still required to elucidate whether a core pig gut microbiome exists. Perhaps, identifying the core functionality of the microbiota, through functional metagenomic and metabolomic studies, may provide a clearer picture, as opposed to identifying the predominant taxa alone. An additional challenge in identifying the core gut microbiome of pigs is that many studies have focussed primarily on faecal samples, as outlined above (Guevarra et al., 2018; Han et al., 2018; Kim et al., 2015a; Kubasova et al., 2018; Motta et al., 2019) (Fig. 3). Some of the reasons for this include the relatively high rearing cost and long growth cycle of pigs from birth to slaughter, when

compared to poultry, for example, and the ease of obtaining repeated faecal samples from the same pig (De Rodas et al., 2018).

There is also considerable study-to-study variation in the deoxyribonucleic acid (DNA) extraction methods used, the 16S rRNA gene hypervariable region sequenced and the sequencing platforms employed, all of which are likely to impact the reported microbial composition. In fact, Holman et al. (2017) reported that study-level effects were the strongest predictors of microbiome structure, followed by intestinal location and age, respectively. It should be noted though, that age, among other metadata categories, was also associated with study-level effects, as several studies sampled at only one timepoint.

3 Colonisation resistance

As outlined previously, early microbial colonisation of the piglet GIT plays a crucial role in establishing the resident microbiome, which subsequently influences host phenotype, nutrient utilisation and immunity (Mulder et al., 2011; Mach et al., 2015; Umu et al., 2017). One of the microbiological services provided by the pig gut microbiome is colonisation resistance. This concept refers to the ability of the commensal microbiota to act as a barrier, thereby offering intestinal protection as a result of direct competition between commensals and potential pathogens, for intestinal niches and the limited nutrients available (Iacob et al., 2019; Lawley and Walker, 2013; Spees et al., 2013). A number of mechanisms of colonisation resistance exist (Fons et al., 2000; Pickard et al., 2017). These include 'bacterial antagonism', via the production of bacteriocins and other antimicrobial compounds (Fons et al., 2000; Hu et al., 2018) which will be discussed in Section 4. Other mechanisms include competition for nutrients and/or receptor sites along the GIT, generally referred to as 'competitive exclusion', as well as microbiota-mediated upregulation of mucin secretion by goblet cells which prevents pathogen binding (Iacob et al., 2019; Liao and Nyachoti, 2017; Sicard et al., 2017; Spees et al., 2013). These mechanisms of colonisation resistance, among other beneficial pig gut microbiota-mediated microbiological services, are summarised in Fig. 4.

It should be noted that much of the research on the mechanisms of competitive exclusion to date has been carried out in murine models and refers to the human gut microbiome. However, it is reasonable to assume that similar mechanisms of competitive exclusion occur within the pig gut microbiome, considering the physiological similarities of the GIT, and that pigs are often used as a model for humans (Zhang et al., 2013).

It is widely reported that the resident gut microbiota competitively excludes pathogens by competing for nutritive sources. As outlined by Pereira and Berry (2017), in a stable, mature gut microbiome, all available nutritional niches would be expected to be occupied. Subsequently, new potential colonisers,

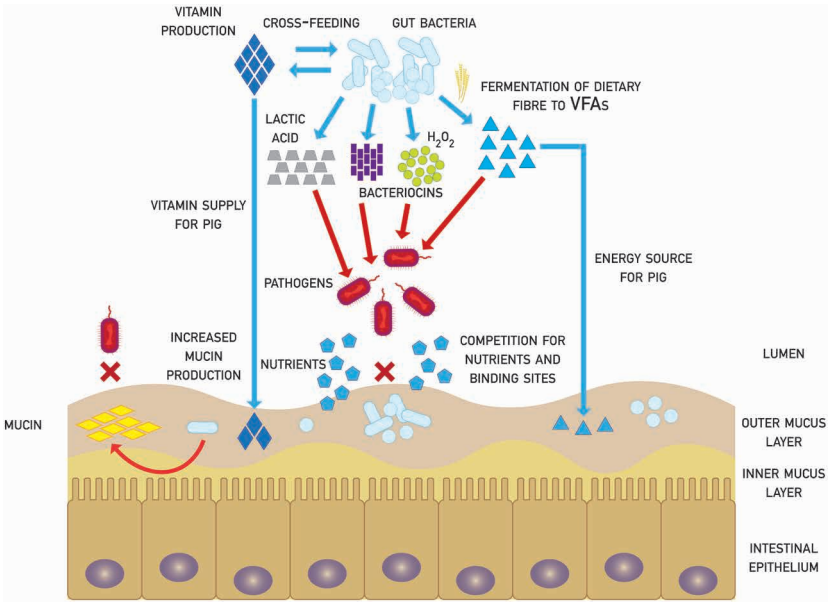

Figure 4 Schematic diagram of mechanisms of colonisation resistance and beneficial microbiological services provided by the pig gut microbiome. Straight red arrows denote inhibitory/bactericidal activity; curved red arrows denote stimulatory activity; red crosses denote inhibition of pathogen binding (Credit: Jonathan Brazil).

whether commensal or pathogenic, would have to either outcompete a resident species, colonise a new nutritional niche arising from a change in host diet or take the place of an eliminated resident species, such as in the case of dysbiosis induced through antibiotic treatment.

The metabolic pathways to which commensals have adapted are also a key factor in maintaining colonisation resistance. For instance, some strains of *Escherichia coli* have developed to utilise specific carbon sources that some commensal *E. coli* cannot metabolise. For example, in the presence of two commensal *E. coli* strains, Maltby et al. (2013) demonstrated that enterohemorrhagic *E. coli* (EHEC) failed to colonise the gut in a mouse model. They hypothesised that this occurred because the commensal strains occupy slightly different nutritional niches to each other, but both use five sugars determined to be necessary for EHEC colonisation, indicating that the commensal *E. coli* had competitively excluded EHEC via direct competition for specific sugars. In addition, one of the commensal *E. coli* strains (Nissle 1917) used in the study by Maltby et al. (2013) has been shown to out-compete *Salmonella* Typhimurium in mouse models due to superior iron uptake ability (Deriu et al., 2013). Maldonado-Gómez et al. (2016) demonstrated that a strain of *Bifidobacterium longum* was capable of colonising and persisting in the

human gut, but only in the absence of metabolically similar competitors. If present, these competitors occupied its niche and competitively excluded *B. longum*.

One of the other key mechanisms of competitive exclusion is competition for adhesion sites along the intestinal mucosa (Fons et al., 2000; Monteagudo-Mera et al., 2019) (Fig. 4). However, much of the research on the mechanisms of pathogen exclusion through competition for binding sites in pigs comes from probiotic studies (Liao and Nyachoti, 2017; Plaza-Diaz et al., 2019; van Tassell and Miller, 2011; Yang et al., 2015). The mucus layer of the mammalian GIT is known to protect against pathogen invasion by preventing colonisation and aiding in the removal of bacteria by peristalsis (Singh et al., 2018). Although the mechanisms of bacterial adhesion to the gut mucosa are not well understood, it has been proposed to be mediated by a number of surface adhesion proteins such as the mucus-binding protein MUB, fibronectin-binding protein, S-layer protein and collagen-binding protein (Monteagudo-Mera et al., 2019; Singh et al., 2018).

Enterotoxigenic *E. coli* (ETEC) and several other intestinal pathogens are known to initiate colonisation through surface adhesins, which interact with various receptors on the surface of intestinal epithelial cells in order to mediate bacterial binding (Singh et al., 2018). Resident bacterial communities and pathogens compete for these cell surface receptors for colonisation of the GIT. Competitive exclusion via inhibition of adhesion was first hypothesised by Chan et al. (1985), where human *Lactobacillus* isolates were found to inhibit the adhesion of uropathogenic bacteria to uroepithelial cells *in vitro*. They suggested that lipoteichoic acid was involved in the attachment of *Lactobacillus* to the cells but that steric hindrance most likely played a role in preventing uropathogen attachment (Chan et al., 1985; Reid et al., 1985).

Competitive exclusion cultures (CECs) have been developed for use in pigs to inhibit enteropathogen colonisation. Genovese et al. (2003) administered a caecum-derived mixed bacterial CEC to piglets twice within 24 h of birth, prior to challenge with *Salmonella* Choleraesuis 48 h after birth. These piglets shed *Salmonella* at a lower rate and had reduced *Salmonella* counts in the GIT compared to a control group, with effects persisting for up to 10 days post-weaning.

In addition to directly competing for attachment sites, there is also *in vitro* evidence to suggest that members of the commensal microbiota can promote mucin production, thereby enhancing the barrier function of the mucus layer and preventing pathogen binding (Sicard et al., 2017) (Fig. 4). For example, a well-studied commensal bacterium, *Bacteroides thetaiotaomicron* increased goblet cell differentiation and gene expression related to mucus production in a mouse model (Wrzosek et al., 2013). Although it is difficult to determine the exact mechanisms by which competitive exclusion occurs and there is a lack of

data for pigs, it is most likely through a complex combination of competitive interactions between the resident microbiota and pathogens for nutrients and binding sites along the GIT, some of which have been outlined above. The gut microbiome also confers colonisation resistance to the host via a range of other mechanisms, one of which is the production of antimicrobial substances.

4 Production of antimicrobial substances

Members of the gut microbiome secrete a wide range of antimicrobial substances capable of altering the composition of the resident microbiota, amongst other functions (Fig. 4). These bacterial metabolites may be generated either as intermediates or end products (Engevik and Versalovic, 2017) and include bacteriocins, hydrogen peroxide, lactic acid and VFAs. In fact, because of the abundance and diversity of antimicrobials produced by members of the gut microbiome, it is considered a bountiful source of novel antimicrobials for potential therapeutic applications (Garcia-Gutierrez et al., 2019).

4.1 Bacteriocins

Bacteriocins are classified as small, heat-stable peptides that are synthesised ribosomally and secreted by bacteria, with narrow- or broad-spectrum bactericidal activity against competing bacteria, to which the producer has 'immunity' (Lawley and Walker, 2013; Umu et al., 2017). Although they differ widely in terms of chemical structure and mode of action, many bacteriocins target bacterial cell membrane phosphate groups and disrupt the structural integrity of the membrane by decreasing the potential and/or the pH gradient across the membrane, forming pores that lead to cellular leakage (Engevik and Versalovic, 2017).

Many microorganisms including both Gram-positive and Gram-negative bacteria, as well as certain archaea, produce bacteriocins (Umu et al., 2017). Lactic acid bacteria (LAB) and members of the genus *Bacillus* are known to produce a large number of bacteriocins which have been better characterised than those produced by many other bacterial groups in light of their use as probiotics (Abriouel et al., 2011; Hu et al., 2018; Liao and Nyachoti, 2017; Plaza-Diaz et al., 2019). Therefore, many of the taxa found within the pig gut microbiota are capable of producing bacteriocins, and in fact, a number of bacteriocins produced by porcine gut-derived bacteria have been described in the literature (Barrett et al., 2007; Du Toit et al., 2000; Han et al., 2014; Lin et al., 2020; O'Connor et al., 2015; O'Shea et al., 2009, 2011, 2013; Robredo and Torres, 2000) (Table 1). The range of activity of these bacteriocins can be seen in Table 1, with a number of significant pig pathogens (or human pathogens carried by pigs), such as *E. coli, Salmonella* and methicillin-resistant *Staphylococcus aureus* (MRSA), amongst the targets. This highlights the

Table 1 Range of bacteriocin-producing bacteria isolated from the pig gut or pig faeces and their spectra of inhibition

Strain (bacteriocin produced)	Source of strain	Bacteria inhibited	References
Lactobacillus animalis 30a-2[1]	Pig ileal mucosa	Methicillin-resistant *Staphylococcus aureus* (16 isolates) *Bacillus cereus* *Listeria monocytogenes* *Acinetobacter baumannii* *Escherichia coli* K12 *Pseudomonas aeruginosa* including MDR[2] *Salmonella* Choleraesuis *Salmonella* Enteritidis *Salmonella* Typhimurium *Shigella flexneri* *Shigella sonnei* *Yersinia enterocolitica* MDR *Acinetobacter baumannii* Extended-spectrum β-lactamase *Escherichia coli*	(Lin et al., 2020)
Lactobacillus salivarius DPC6005 (Salivaricin P and Bactofencin A)	Pig caecum	*Enterococcus faecalis* *Enterococcus faecium* *Lactobacillus casei* *Lactobacillus helveticus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Leuconostoc* sp. *Listeria innocua* *Pediococcus pentosaceus*	(Barrett et al., 2007; O'Connor et al., 2015; O'Shea et al., 2009, 2011, 2013)
Streptococcus hyointestinalis DPC6484 (Nisin H)	Pig caecum	*Bacillus cereus* *Enterococcus faecalis* *Listeria innocua* *Listeria monocytogenes* *Staphylococcus aureus*	
Enterococcus faecium BFE 1072 (Enterocin L50A and L50B)	Pig faeces	*Lactobacillus helveticus* *Lactobacillus acidophilus* *Pediococcus pentosaceus* *Leuconostoc cremoris* *Enterococcus faecalis* *Listeria monocytogenes* *Clostridium sporogenes* *Clostridium tyrobutyricum* *Propionibacterium acidopropionici*	(Du Toit et al., 2000)

(*Continued*)

Table 1 (*Continued*)

Strain (bacteriocin produced)	Source of strain	Bacteria inhibited	References
Enterococcus faecalis AP 45[1]	Pig faeces	*Clostridium perfringens* *Enterococcus faecalis* *Lactobacillus brevis* *Lactobacillus delbruekii* *Lactobacillus plantarum* *Listeria monocytogenes*	(Han et al., 2014)
Enterococcus faecalis AP 216[1]	Pig faeces	*Clostridium perfringens* *Listeria monocytogenes*	
Lactobacillus salivarius X13[1]	Pig faeces	Methicillin-resistant *Staphylococcus aureus* *Staphylococcus epidermidis* (Methicillin-resistant and susceptible) *Micrococcus luteus* *Lactobacillus salivarius* *Lactobacillus fermentum* *Lactobacillus paracasei* *Pediococcus acidilactici* *Pediococcus pentosaceus*	(Robredo and Torres, 2000)

[1] Bacteriocin responsible for antibacterial activity has not been defined/identified.
[2] MDR – Multidrug-resistant.

potential microbiological service offered by bacteriocin-producing members of the pig gut microbiome.

It is important to note that *in vitro* production of bacteriocins by gut-derived bacteria does not necessarily imply production in the gut or that they are mediators of anti-infective activity. However, a few studies to date have demonstrated the production of bacteriocins *in vivo*. For example, Corr et al. (2007) showed in a mouse model of *Listeria* infection that the bacteriocin-producing strain *Lactobacillus salivarius* UCC118 protected the mice, while a non-bacteriocin-producing mutant did not, demonstrating that the anti-infective activity was mediated primarily by the bacteriocin. Following on from this, Riboulet-Bisson et al. (2012) showed, via administration of the wild-type alongside a mutant lacking bacteriocin production, that *Lb. salivarius* UCC118 had a 'significant but subtle' impact on the pig gut microbiota, including inhibition of potentially pathogenic Gram-negative taxa, mediated, at least partially, by bacteriocin production. A study by Hu et al. (2018) highlighted the importance of gut microbiota-derived bacteriocins in maintaining gut health in pigs. They identified two bacteriocin-producing *Lactobacillus* strains, *Lb. gasseri* LA39 and *Lb. frumenti,* as mediators of the diarrhoea resistance conferred by faecal microbiota transplantation (FMT) from diarrhoea-resistant

to susceptible piglets. Moreover, they demonstrated that the diarrhoea resistance was facilitated by the bacteriocin gassericin A, which was found to be essential for modulating diarrhoea-associated fluid absorption and secretion across the intestine through binding to Keratin 19 on the plasma membrane of the host's intestinal epithelial cells (Hu et al., 2018). The results also indicated that this plasma protein may mediate signal transduction from gassericin A to the cell, with the bacteriocin acting as a signalling molecule. There is also other evidence to show that bacteriocins may act as signalling molecules, either from one bacterium to another via QS or to host cells (Dobson et al., 2012).

It is also interesting to note that many of the pig gut microbiota-derived bacteriocin-producing strains identified to date also inhibit closely related genera/species (Table 1). This is a common finding for bacteriocin-producers, most likely due to the fact that bacteriocins are thought to confer a competitive advantage on producing strains by enabling them to colonise a particular niche. This potentially occurs in the pig GIT, with Walsh et al. (2008) concluding that one of the strains within a 5-strain *Lactobacillus/Pediococcus* probiotic mixture predominated in the ileum, possibly due to the production of salivaricin P, a bacteriocin active against *Listeria* and also against other *Lactobacillus* species (Barrett et al., 2007).

Therefore, when considering the microbiological services provided by the gut microbiota of pigs, it is not only the anti-pathogen activity of bacteriocins produced by members of the gut microbiome that is important, but also their role in aiding colonisation and their physiological activity in the gut. Overall, the findings outlined here highlight the significant contribution that bacteriocin secretion from the commensal microbiome plays in conferring colonisation resistance and promoting the health of pigs.

4.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) which is produced by many microbes, is a reactive oxygen species (ROS) capable of creating breaks in the phosphate backbone of DNA, which leads to the release of nucleotides, thereby inhibiting DNA replication (Engevik and Versalovic, 2017; Finnegan et al., 2010; Gough and Cotter, 2011). Additionally, the dissociation of H_2O_2 produces other ROS such as hydroxyl radicals which can attack the methyl group of thymine, resulting in DNA damage (Engevik and Versalovic, 2017; Li et al., 2020). There is a lack of information on H_2O_2 production by members of the pig gut microbiota and research into its role within the pig gut microbiome.

However, many bacterial taxa found within the pig gut microbiota, for example, members of the LAB, can produce H_2O_2, leading to inhibition of pathogenic bacteria that lack catalase, the enzyme responsible for the breakdown of H_2O_2 (Vieco-Saiz et al., 2019) (Fig. 4). For example, Lin et al.

(2020) isolated a strain of *Lb. animalis* from pig ileal mucosa which had antimicrobial activity against a range of pathogens including *S. aureus*. Upon addition of catalase, *Lb. animalis* lost its *S. aureus* inhibitory activity, indicating that it was mediated, at least in part, by H_2O_2. It should be noted that *S. aureus* usually produces catalase; however, protease was added to degrade any antimicrobial peptides and therefore most likely inactivated *S. aureus*-secreted catalase (Lin et al., 2020). However, whether this gut-derived *Lb. animalis* has any H_2O_2-mediated anti-pathogen activity *in vivo* remains to be investigated.

Anaerobic bacteria generally lack catalase and are therefore usually more sensitive to H_2O_2. In addition, Gram-negative bacteria are more sensitive compared to Gram-positives (Engevik and Versalovic, 2017). Bacterially produced H_2O_2 is known to act synergistically with lactic acid; the antimicrobial properties of which will be discussed in Section 4.3. Lactic acid disrupts the outer membrane of Gram-negative bacteria, rendering the cells sensitive to H_2O_2 and other antimicrobial substances (Engevik and Versalovic, 2017; Garcia-Gutierrez et al., 2019).

In addition to the inter-bacterial interactions mediated by ROS, the host gut epithelium plays a key role in influencing the microbiome via the production of antimicrobials and ROS which may act as signalling molecules in the communication between gut microbiota and the intestinal mucosa (Berstad et al., 2016). The enzyme dual oxidase 2 (Duox2) produces H_2O_2 in the GIT, and its expression is induced by the microbiome via different signalling pathways (Sommer and Bäckhed, 2015).

However, it should be noted that some inflammatory diseases of the GIT are associated with high levels of H_2O_2 (Basu Thakur et al., 2019; Garcia-Gutierrez et al., 2019). In addition, H_2O_2 production is not limited to beneficial commensals; pathogenic bacteria such as *Streptococcus pneumoniae* are also thought to produce H_2O_2 to inhibit competing organisms (Engevik and Versalovic, 2017). In fact, Erttmann and Gekara (2019) have shown that H_2O_2 released by *S. pneumoniae* inhibits inflammasome-dependent innate immunity, and thus may contribute to pathogen colonisation.

4.3 Lactic acid

Lactic acid is an organic acid and is a major metabolic end product of carbohydrate fermentation by LAB, the group of Gram-positive aerotolerant anaerobic bacteria named as such due to their fermentative metabolism (Tannock, 2004; Yang et al., 2015). Lactic acid bacteria are classified into three different groups: obligately homofermentative which produce lactic acid as their sole metabolite (e.g. *Lb. acidophilus*, *Lb. delbrueckii*, *Lb. salivarius*), facultatively heterofermentative (e.g. *Lb. plantarum, Enterococcus*,

Lactococcus, *Pediococcus*, *Streptococcus*) and obligately heterofermentative (e.g. *Leuconostoc*, *Weissella*), which generate less lactic acid but produce other end products, including acetic acid, formic acid, ethanol and carbon dioxide (Du Toit et al., 2001; Endo and Dicks, 2014).

Lactobacillus alone, many species of which are homofermentative, has been reported to account for up to 15% of the pig faecal bacterial community (Niu et al., 2015). Hence, a relatively large quantity of lactic acid can be assumed to be produced by the pig gut microbiota. For instance, in pigs fed a dry diet, lactic acid concentrations in the stomach are ~70 mmol kg^{-1} while pigs fed fermented liquid feed can have concentrations as high as 120 mmol kg^{-1}, with a decreasing trend observed along the GIT, in both cases (Højberg et al., 2003). Lactic acid production in the stomach of suckling and newly weaned pigs is particularly relevant. At this time, the pig has a poorly developed ability to produce gastric acid and relies on the fermentation of lactose to lactate to maintain a low pH in the stomach, which is the first line of defence against ingested pathogens (Lawlor et al., 2020).

Lactic acid is known to inhibit the growth of, and also to directly kill, pathogens (Fig. 4). Wang et al. (2015) determined, *in vitro,* that exposure to 0.5% lactic acid for 1 h was sufficient to completely inactivate the Gram-negative pathogens *Salmonella* Enteritidis and *E. coli,* while *L. monocytogenes* (Gram-positive) required 2 h of exposure. However, the lactic acid does not generally affect host epithelial cells due to the secretion of bicarbonate by the mucus layer, creating a pH gradient with a pH close to neutral (Allen and Flemström, 2005; Vieco-Saiz et al., 2019).

Apart from acidification of the gut, the antimicrobial effects of lactic acid produced by the gut microbiota are achieved through several mechanisms. Alakomi et al. (2000) demonstrated that lactic acid effectively permeabilises the outer membrane of Gram-negative bacterial cells, thereby inducing lipopolysaccharide (LPS) release and rendering the cell susceptible to antimicrobial substances including lactic acid itself. Lactic acid can also penetrate the cytoplasmic membrane of Gram-negative bacteria in its undissociated form. Once inside the cell, the higher cytosolic pH causes the acid to dissociate into lactate, releasing protons, which reduces intracellular pH, disrupting enzymatic activity, protein function and DNA structure (Stanojević-Nikolić et al., 2016; Suiryanrayna and Ramana, 2015).

In addition, in order to counteract the low pH, the cell must use adenosine triphosphate (ATP) to pump protons out of the cell, which depletes cellular energy and upon prolonged exposure to lactic acid, this can result in cell death (Suiryanrayna and Ramana, 2015). Another antimicrobial mechanism of lactic acid involves inhibition of substrate transport as a result of the aforementioned changes in membrane permeability. In addition, the changes in pH within the cell can suppress the oxidation of the co-enzyme nicotinamide adenine

dinucleotide (NADH) which is critical for the electron transport chain during cellular respiration and thus can lead to the death of the bacterium (Stanojević-Nikolić et al., 2016).

As mentioned in Section 4.2, lactic acid also acts synergistically with other antimicrobial substances including H_2O_2 and bacteriocins to inhibit the growth of pathogens (Atassi and Servin, 2010; Engevik and Versalovic, 2017). This is likely the result of the outer membrane-permeabilising activity of lactic acid which renders the cell susceptible to the antimicrobial action of H_2O_2, which is exacerbated by the pH-associated damage mediated by lactic acid. In addition to the antimicrobial properties of lactic acid, the associated reduction in gastric pH due to the high abundance of *Lactobacillus* in the pig stomach, particularly in the *Pars oesophagea*, may also increase the activity of pepsin, thereby enhancing protein utilisation (De Witte et al., 2019; McGillivery and Cranwell, 1992; Suiryanrayna and Ramana, 2015). This is particularly important in suckling and newly weaned pigs, as they have insufficient gastric acid production, as outlined above. An additional beneficial effect of lactic acid is that lactate can be converted by members of the gut microbiota, into butyrate, the beneficial properties of which will be discussed in sections 4.4 and 6 (Esquivel-Elizondo et al., 2017).

4.4 Volatile fatty acids

Short-chain fatty acids (SCFAs), particularly acetate (C2), propionate (C3) and butyrate (C4), are the major VFAs produced by the gut microbiota, and therefore will be the focus of this section (Fig. 4). They are produced primarily in the large intestine of hindgut fermenters including pigs, in which they have been estimated to contribute between 10% and 25% of basal energy requirements (Agyekum, 2016; Bergman, 1990; Nakatani et al., 2018), which will be discussed in Section 6.1. Short-chain fatty acids are carboxylic acids, generally classified as having less than six carbon atoms, produced in the gut lumen by bacterial fermentation of primarily undigested dietary carbohydrates. Short-chain fatty acid concentrations are generally highest in the proximal colon, where most fermentable substrates are available, with a decline towards the distal colon (Liu et al., 2018; Venegas et al., 2019; Yoon et al., 2018).

Butyrate is mostly produced by *Firmicutes* in the colon, while acetate and propionate are produced mainly by members of the phylum *Bacteroidetes* (Iacob et al., 2019; Venegas et al., 2019). *Clostridium*, *Blautia* and *Ruminococcus* (*Firmicutes*) typically produce butyrate from acetate through the butyryl coenzyme A (CoA): acetate CoA transferase pathway. *Prevotella* (*Bacteroidetes*) among other genera, produce acetate, and therefore act as an energy source for butyrate producers via a process known as cross-feeding (Holman et al., 2017). Additionally, as previously mentioned, butyrate can be formed from

lactate, specifically from the conversion of lactate to pyruvate through either the butyrate kinase or butyryl-CoA: acetate-CoA transferase pathways (Esquivel-Elizondo et al., 2017). Cross-feeding also occurs here, as lactate is a major end product of many of the LAB found within the pig gut, as outlined above. While SCFAs have a range of functions in the host (Sun and O'Riordan, 2014; Venegas et al., 2019) (see Section 6), this section will focus on their antimicrobial properties.

Short-chain fatty acids directly acidify the GIT, aiding in colonisation resistance (Iacob et al., 2019). Like lactic acid, the non-ionised form of SCFAs can exhibit antibacterial activity once inside the bacterial cytoplasm. Upon entry, dissociation of the acid leads to an accumulation of protons, resulting in pH reduction and subsequent disruption of the transmembrane proton motive force. Additionally, the dissociation of acids results in a build-up of SCFA anions which interferes with osmotic balance. The combination of these factors ultimately leads to disruption of critical cellular processes including ATP generation, resulting in the death of the bacterial cell (Sun and O'Riordan, 2014).

Jacobson et al. (2018) showed that the anti-*Salmonella* activity of *Bacteroides* was mediated by propionate which directly inhibited growth *in vitro* via disruption of intracellular pH. Other pig pathogens that are susceptible to the antibacterial effects of VFAs include *E. coli*, *Salmonella* spp., *Clostridium perfringens* and *Campylobacter coli* (Beier et al., 2018; Gómez-García et al., 2019). Gómez-García et al. (2019) determined the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of propionic acid and sodium butyrate against some of these pathogens (Table 2;

Table 2 Antimicrobial activity (MIC_{50}[1], MBC_{50}[2] and MBC_{50}/MIC_{50} ratio[3]) of propionic acid and sodium butyrate against pig pathogens. Adapted from Gómez-García et al. (2019) distributed under the terms of the Creative Commons Attribution 4.0 International License

		Propionic acid (ppm)	Sodium butyrate (ppm)
E. coli	MIC_{50}	1200.0	50 000.0
	MBC_{50}	9600.0	125 000.0
	MBC_{50}/MIC_{50}	8.0	2.5
Salmonella spp.	MIC_{50}	1200.0	125 000.0
	MBC_{50}	2400.0	125 000.0
	MBC_{50}/MIC_{50}	2.0	1.0
C. perfringens	MIC_{50}	2400.0	31 250.0
	MBC_{50}	2400.0	62 500.0
	MBC_{50}/MIC_{50}	1.0	2.0

[1]MIC_{50} = lowest concentration that inhibited the growth of 50% of the strains of each bacterial species tested; [2]MBC_{50} = Median of the MBC (MBC was the lowest concentration which killed 99.9% or more of the bacteria in the original inoculum (less than five colonies)). [3]MBC_{50}/MIC_{50} ratio = [3]MBC_{50}/MIC_{50}.

the lower the values the more active the compound). Propionic acid had a more pronounced inhibitory and bactericidal effect on all tested pathogens compared to sodium butyrate; however, both acids were inhibitory as well as bactericidal. Gómez-García et al. (2019) reported MIC_{50} values of 1200 ppm and 125 000 ppm for propionic acid and sodium butyrate, respectively, against *Salmonella* (Table 2), which compares well to the MIC of 3750 ppm reported for butyric, propionic and valeric acids against poultry-derived *Salmonella* (Lamas et al., 2019).

Interestingly, SCFAs are also known to help maintain the integrity of intestinal epithelial tight junctions. By decreasing intestinal permeability in this way, they aid in the prevention of bacterial translocation across the gut barrier, thereby preventing infection (Kelly et al., 2015). Overall, the findings outlined here indicate that the production of certain VFAs by the pig gut microbiota may have a pronounced impact on colonisation resistance via antimicrobial activity against pathogens.

5 Production of enzymes

The pig gut microbiome contributes to host metabolism by providing a plethora of enzymes that the host does not produce. Many of these enzymes are essential for the digestion of complex polysaccharides (Mohammed and Guda, 2015). This section will focus primarily on the enzymatic capacity of the pig gut microbiota for dietary fibre digestion. In commercial pig production, dietary carbohydrates account for 60–70% of total energy intake (Bach Knudsen et al., 2012). Specific microbial taxa have developed specialised enzyme-catalysed metabolic pathways for nutrient digestion and energy harvest from these host-indigestible polysaccharides, thereby providing an indispensable service to the host (Wang et al., 2019a). The majority of these dietary fibres, such as resistant starch, arabinoxylan and β-glucan are fermented in the proximal colon, leading to the production of SCFAs which are used as an energy source by the pig, in addition to having a range of benefits for host health (Tiwari et al., 2019) (see Sections 4.4 and 6; Fig. 4).

Evidence of how the gut microbiome provides a service to the host via the production of enzymes comes from studies comparing the microbiota of suckling versus weaned pigs. It has been widely reported that the transition from sow's milk to solid feed promotes an increase in the relative abundance of plant polysaccharide-degrading *Prevotellaceae* and *Ruminococcaceae*, with a concomitant decrease in the abundance of milk glycan-degrading *Bacteroidaceae* and *Enterobacteriaceae* (Chen et al., 2017a; Frese et al., 2015; Motta et al., 2019; Wang et al., 2019a). This diet-associated adaptation of gut microbial enzymatic activity is also evidenced by a study that utilised 16S rRNA gene sequencing and whole-metagenome shotgun sequencing

to examine compositional and functional differences within the faecal microbiome of nursing versus weaned piglets (Guevarra et al., 2018). Through functional annotation of sequence reads, they found that genes mapped to the metabolism of carbohydrates such as xylose and mannose, as well as genes for L-rhamnose utilisation were more prevalent within the gut microbiome of weaned piglets, associated with increased relative abundances of *Lactobacillus* and *Prevotella*. This was expected as these sugars are the end products of non-starch polysaccharide (NSP) hydrolysis and are present in solid feed ingredients in post-weaning diets such as soybean meal and cereals. Conversely, the microbiome of the nursing piglets was enriched in genes associated with lactose and galactose utilisation (lactose and galactose being two of the main sugars present in sows' milk), along with an increased relative abundance of *Bacteroides* (Guevarra et al., 2018).

The degradation of simple and complex carbohydrates is generally catalysed by three broad enzyme classes: glycoside hydrolases (GHs), carbohydrate esterases (CEs) and polysaccharide lyases (PLs), collectively known as carbohydrate-active enzymes (CAZymes). These CAZymes are further categorised into families and sub-families in the CAZy database (Bhattacharya et al., 2015). Wang et al. (2019a) used *de novo* metagenomic binning to reconstruct 360 high-quality genomes as a metagenomic reference for the pig gut microbiome. This metagenomic reference was used against the CAZy database to predict carbohydrate metabolism within the faecal microbiome of pigs, fed six experimental diets from weaning to 21 days post-weaning. This study provided many insights into the enzymatic capacity of the pig gut microbiome in relation to carbohydrate metabolism. It showed that the microbial communities responsible for degrading starch, fructans and lactose in the post-weaning piglet are substantially different from those within the human microbiome. *Firmicutes* and *Bacteroidetes* were found to use different starch-degrading systems. *Firmicutes* used an extracellular 1,4-alpha-glucan branching enzyme (*GlgB*) and pullulanase (*Amy12*), with the majority carrying only the *GlgB* gene. *Bacteroidetes,* on the other hand, harboured multiple genes for extracellular and periplasmic starch degradation (Wang et al., 2019a). *Firmicutes* and *Bacteroidetes* also harboured distinct enzymes for fructan hydrolysis, with the former using intracellular β-fructofuranosidase and extracellular fructansucrases and the latter, fructan, by β-2,6-endo-fructanases. Most of the bacterial genomes encoding lactose degradation within the pig gut microbiome (the majority of which are *Firmicutes* including *Lactobacillus*, *Subdoligranulum* and *Ruminococcus*) hydrolyse lactose by intracellular GH2 β-galactosidase or GH42 β-galactosidase (Wang et al., 2019a).

These findings highlight the diversity of the enzymatic repertoire of the pig gut microbiome and its key role in nutrient utilisation in pigs. Other metagenomic studies of the pig gut microbiome have revealed interesting feed

efficiency (FE)-associated findings, linked with the enzymatic and metabolic capacity of the pig gut microbiome. For example, unsurprisingly, Quan et al. (2020) reported that the pig caecum and colon had higher polysaccharide-metabolising capacity compared to the ileum. Additionally, taxa that were more abundant in the caecum of highly feed efficient pigs had a greater abundance of genes associated with polysaccharide and protein metabolism pathways, in agreement with the findings of Tan et al. (2017).

McCormack et al. (2017) found that some of the more abundant predicted pathways in the ilea of low residual feed intake (RFI) (highly feed efficient) pigs were related to the biosynthesis of amino acids. In a more recent study, they found that most of the enriched predicted pathways in the feedefficient pigs were associated with core metabolism, including carbohydrate and nucleotide metabolism (McCormack et al., 2019).

In summary, the pig gut microbiome provides the host with an indispensable contribution to the metabolism of dietary constituents, in particular fibre, providing an abundance of critical enzymes that are not expressed by the host. Members of the gut microbial community have developed specialised enzyme-catalysed metabolic pathways that are critical for the promotion and maintenance of host health and productivity.

6 Benefits of volatile fatty acids (apart from antimicrobial activity)

As detailed in Section 5, dietary fibre in the pig GIT is resistant to degradation by endogenous host enzymes but can be partially or completely fermented by the hindgut microbiota to produce VFAs that play an important role in colonisation resistance (Fig. 4). They are also a key energy source for the host and are involved in the regulation of host metabolism, immune modulation and cell proliferation (Mohammed and Guda, 2015; Wang et al., 2018 Zhao et al., 2020). These services will be discussed here.

6.1 Contribution to host metabolism: energy source for colonocytes

The majority of SCFAs are produced in the large intestine where they are absorbed and used as an energy source for the pig, with an estimated 95% of those produced by the luminal microbiota absorbed by the mucosa and the remaining 5% excreted in the faeces (den Besten et al., 2013b; Nakatani et al., 2018). Absorption of SCFAs across the apical membrane of colonocytes occurs via two main mechanisms: passive diffusion of the undissociated acid and SCFA transporter-mediated active transport of the dissociated form. Short-chain fatty acid transporters include hydrogen-coupled monocarboxylate transporter isoform 1 (MCT1) and sodium-coupled monocarboxylate transporter 1 (SMCT1) (Engevik and Versalovic, 2017; Liu et al., 2018).

Despite being the least abundant of the three aforementioned main SCFAs, butyrate is the primary energy source for colonocytes, with as much as 90% of butyrate metabolised by these cells (Bedford and Gong, 2018; Rowland et al., 2018; Venegas et al., 2019). Colonocytes have a higher affinity for butyrate compared to acetate and propionate. A large proportion of butyrate is metabolised through the oxidation pathway resulting in the production of acetyl co-enzyme A (CoA) following several intermediate steps. Measurements in isolated colonocytes have shown that they obtain up to 70% of their energy supply from SCFA oxidation (Astbury and Corfe, 2012; den Besten et al., 2013b).

Donohoe et al. (2011) demonstrated *in vitro* that the colonocytes of germ-free mice exhibited an energy-deficient state characterised by decreased expression of metabolic enzymes involved in the tricarboxylic acid (TCA) cycle, resulting in decreased oxidative phosphorylation and ATP levels. Upon introduction of the butyrate-producing strain, *Butyrivibrio fibrisolvens*, mitochondrial respiration was restored, preventing autophagy, indicating that microbially derived butyrate acted as a direct energy source for colonocytes (Donohoe et al., 2011).

den Besten et al. (2013a) found that mice infused with labelled SCFAs utilised 62% of propionate as a substrate for gluconeogenesis, with glucose synthesis from propionate accounting for almost 70% of total glucose production, with acetate and butyrate acting as substrates for palmitate and cholesterol in the liver (den Besten et al., 2013a; LeBlanc et al., 2017). Although these data were not generated in pigs, they indicate that VFAs produced by the pig gut microbiota, particularly acetate, propionate and butyrate, play an intrinsic role in host metabolism, particularly as an energy source for colonocytes.

6.2 Other beneficial effects on gut health

Volatile fatty acids also exhibit a wide range of additional intestinal health-enhancing properties in the pig gut. Literature regarding the role of acetate and propionate in pigs is less abundant compared to butyrate, for which there is a broad range of research focussing on its impacts in the GIT. These impacts include gut health-promoting properties such as anti-inflammatory and antioxidant roles and improved intestinal morphology and immunomodulatory capacity, many of which are related to regulatory effects on host gene expression (Bedford and Gong, 2018; Tugnoli et al., 2020; Xiong et al., 2016). Propionate is also an important signalling molecule in the pig gut, with intestinal propionate production identified as a possible microbial signalling route linked to superior growth and feed efficiency (FE) in pigs (Gardiner et al., 2020).

Butyrate and to a lesser extent, propionate, are also known to function as epigenetic substances, acting as histone deacetylase (HDAC) inhibitors and hence may modulate disease and immune homeostasis, altering the expression

of many genes with diverse functions, including cell proliferation, apoptosis and differentiation (Li et al., 2018; Marks et al., 2000; Vinolo et al., 2011). HDACs remove the acetyl groups from histones which results in condensed and transcriptionally inactive chromatin. However, HDAC inhibitors suppress this activity and can result in hyper-acetylation of histones which is thought to increase the accessibility of the transcriptional machinery to promote gene transcription, and therefore may have a profound impact on gene expression (Bedford and Gong, 2018; Koh et al., 2016).

Due to the offensive odour of butyrate and its potential absorption in the upper GIT, alternative forms, such as sodium butyrate and butyrate glycerides are often fed to pigs (Bedford and Gong, 2018). Feng et al. (2018) found that a sodium butyrate-supplemented diet alleviated diarrhoea symptoms and decreased intestinal permeability in early-weaned piglets without impacting growth. From experiments with the Caco-2 epithelial cell line, the mechanism was suggested to be due to the upregulation of tight junction proteins, including claudin-3 and occludin (Feng et al., 2018).

Many other studies in pigs have reported similar improvements in gut barrier function and intestinal health as a result of butyrate supplementation (Wang et al., 2018; Zhong et al., 2019). Diao et al. (2019) showed that intra-gastric administration of a mixture of acetate, propionate and butyrate increased SCFA concentrations in both sera and digesta, and increased expression of occludin and claudin-1 genes in the duodenum and ileum, indicating improved barrier function. Moreover, intestinal morphology was also improved, with increased villus height observed in the jejunum and ileum, and increased villus height to crypt depth ratio found in the duodenum and jejunum, and this was associated with an increase in nutrient digestibility.

In summary, bacterially derived VFAs, particularly butyrate, acetate and propionate contribute significantly to host metabolism, with butyrate serving as the primary energy source for colonocytes in the pig gut, as well as performing numerous health-promoting functions from regulation of gene expression and gut tissue development to immune modulation and disease prevention. The production of VFAs by the pig gut microbiome exemplifies the mutualistic relationship that exists between the resident gut microbiota and the host; commensals thrive on substrates provided by the host, while the host benefits from a range of microbially derived regulatory, metabolic and immunomodulatory services.

7 Production of vitamins

Vitamins are essential organic micronutrients that are critical for cellular function, primarily required as co-enzymes for nutrient metabolism, most of which the host itself cannot synthesise. Pig diets are, therefore, always supplemented with

vitamin premixes, although many vitamins are synthesised endogenously by the pig gut microbiome, and therefore, may not need to be supplemented in the diet (Engevik and Versalovic, 2017; Gaudré and Quiniou, 2009; NRC, 2012) (Fig. 4). Bacterially synthesised vitamins of note include fat-soluble vitamin K and water-soluble B-group vitamins including biotin (B_7, B_8 or H), cobalamin (B_{12}), folate (B_{11}, B_9 or M), niacin (B_3), pantothenate (B_5), pyridoxine (B_6), riboflavin (B_2) and thiamine (B_1) (Engevik and Versalovic, 2017; Rowland et al., 2018). This section will review the services that the pig gut microbiome provides to the host via endogenous production of vitamins.

7.1 Production of vitamin K

Vitamin K is a general term used for a group of fat-soluble compounds that are essential for the conversion of inactive blood clotting factors into biologically active compounds. It may also play a role in calcium metabolism, which requires vitamin K-dependent proteins (Akbari and Rasouli-Ghahroudi, 2018; National Research Council, 2012). In plants, vitamin K exists as phylloquinone (vitamin K_1), while bacteria synthesise a family of compounds known as menaquinones (vitamin K_2) which act as electron carriers during cellular respiration (Dairi, 2009; Hiratsuka et al., 2008; NRC, 2012). Synthetic forms of menadione (vitamin K_3) are often used as vitamin K supplements in pig feed (European Food Safety Authority, 2014). Vitamins synthesised by the gut microbial community are mostly absorbed in the colon, with dietary vitamins being absorbed primarily in the small intestine (LeBlanc et al., 2013).

Rowland et al. (2018) reviewed several studies examining vitamin K deficiency in animal models including a study by Gustafsson et al. (1962) in which inoculation of germ-free vitamin K-deficient rats with either *E. coli* or a presumptive *Micrococcus* strain, both isolated from healthy rats, was found to reverse the deficiency within 48 h, indicating that the microbiota played a key role in vitamin K production. Interestingly, Frick et al. (1967) found that humans receiving low vitamin K diets did not develop vitamin deficiency; however, treatment with a broad-spectrum antibiotic decreased plasma prothrombin levels, indicating that the gut microbial community plays an important role in supplementing low dietary vitamin K intake.

However, despite the role that gut bacteria play in synthesising menaquinone, there is evidence from germ-free rat studies to suggest that menaquinone synthesis is not fully dependent on the gut microbiota (Ravcheev and Thiele, 2016). Furthermore, a recent metagenomic analysis of vitamin synthesis pathways of the human gut microbiome revealed that the number of taxa encoding menaquinone biosynthetic pathways was fewer compared to those encoding B-group vitamins (Das et al., 2019). The authors suggested that the host may have only a limited dependence on microbially derived menaquinone.

However, to our knowledge, there has been little research characterising vitamin K production within the pig gut microbiome but considering the similarities between the pig and human intestinal microbiome, some of the findings from humans can perhaps be extrapolated to pigs. Menaquinone-producing microorganisms that have been described in the human gut have been identified primarily by thin-layer chromatography (TLC). Ramotar et al. (1984) found that many species of *Bacteroides* produced menaquinone, as well as *E. coli*, *K. pneumoniae*, *Propionibacterium*, *Eubacterium* and *Veillonella*. Cooke et al. (2006) analysed lipid extracts of bacteria isolated from the human neonatal GIT and found that *Enterobacter agglomerans*, *Serratia marcescens* and *Enterococcus faecium* produced various forms of menaquinone. Certain LAB such as *Lactococcus lactis* and *Leuconostoc lactis* have also been found to be high-producers of menaquinone (Morishita et al., 1999). The wide range of menaquinone-producing species isolated from the human GIT, which are also found in pigs, implies that the pig gut microbiota could be an abundant source of vitamin K. However, further research is needed to determine the extent to which the pig gut microbiota contributes to host vitamin K utilisation.

7.2 B-group vitamins

B-group vitamins act as important co-factors for a range of biological processes, including the metabolism of lipids and carbohydrates and synthesis of nucleic acids. Most B-group vitamins are either not synthesised by the host or are synthesised in insufficient amounts, and therefore, must be obtained from the diet (Magnúsdóttir et al., 2015; Yoshii et al., 2019). Moreover, the intestinal microbiome is now also recognised as an important source of B vitamins. However, not all bacteria produce B vitamins, and many also require dietary or bacterially derived B-group vitamins and therefore, competition may occur between the host and the intestinal microbiota for these essential nutrients (Yoshii et al., 2019).

The majority of B-group vitamins are directly involved in energy metabolism; the biologically active forms of the vitamins act as co-factors for key enzymes catalysing various reactions in the Krebs cycle, as outlined in Fig. 5. Thiamine (B_1), in its active form thiamine diphosphate (TPP), aids in the cleavage of pyruvate, the main product of glycolysis. Riboflavin (B_2) is phosphorylated into flavin adenine dinucleotide (FAD) which acts as a proton acceptor and catalyses the decarboxylation of pyruvate to acetyl-CoA and the conversion of α-ketoglutarate to succinyl-CoA. Nicotinamide adenine dinucleotide (NAD) is the active form of Niacin (B_3) and acts as an electron acceptor for several important enzymatic steps of the cycle, while pantothenic acid (B_5) is required for the synthesis of CoA required for multiple steps. Lastly, cobalamin (B_{12}) and biotin (B_7) both function as enzyme co-factors for the

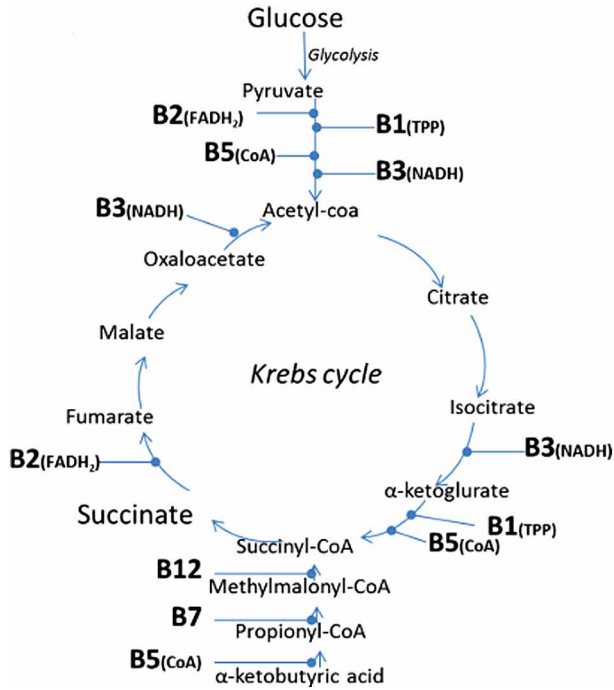

Figure 5 Diagram representing some of the key roles of bacterially-synthesised B-group vitamins (B1 – thiamine, B2 – riboflavin, B3 – niacin, B5 – pantothenic acid, B7 – biotin, and B12 – cobalamin) in energy metabolism. Abbreviations in brackets refer to active forms of the co-factors necessary for each enzymatic step: $FADH_2$ (flavin adenine dinucleotide); CoA (acetyl coenzyme A); TPP (thiamine pyrophosphate); NADH (nicotinamide adenine dinucleotide). Adapted from LeBlanc et al. (2017) distributed under terms of the Creative Commons Attribution 4.0 International License.

catabolism of fatty acids and some amino acids in the Krebs cycle (LeBlanc et al., 2017).

With regard to the capacity of the pig gut microbiome to produce B vitamins, Crespo-Piazuelo et al. (2018) found that the pathways related to the metabolism of co-factors and vitamins, including folate, vitamin B_6 and vitamin B_2 were most abundant in the proximal colon. McCormack et al. (2017) found that the relative abundance of pathways associated with thiamine (vitamin B_1) metabolism was higher in the caecal digesta of high RFI (less feed efficient) pigs than in low RFI (highly feed efficient) pigs, albeit relative abundances of most of the predicted pathways were low (0.001–0.99%). Conversely, Quan et al. (2020) found pathways associated with the metabolism of co-factors and vitamins to be more abundant in pigs with better FE.

Although, to our knowledge, there is no information on the microbes within the pig gut, which are responsible for the synthesis of B-group vitamins,

some evidence exists for humans. Magnúsdóttir et al. (2015) mined the genomes of 256 common human gut microbiome inhabitants for B-group vitamin biosynthesis pathways. Overall, between 40% and 65% of the genomes analysed were predicted to harbour all necessary pathways for the production of the eight analysed vitamins. The proportion of each bacterial phylum predicted to synthesise each vitamin is shown in Table 3. Vitamins predicted to be the most abundant, in terms of the presence of the necessary genes, were vitamin B_3, with 166 predicted producers and vitamin B_5, with 162 predicted producers. For vitamins B_3 and B_7, the vast majority of *Bacteroidetes*, *Fusobacteria* and *Proteobacteria* possessed the genes encoding the necessary synthesis pathways, with *Firmicutes* and *Actinobacteria* generally having a lower propensity for B-group vitamin biosynthesis. Regarding vitamin B_{12}, all *Fusobacteria* were predicted to be producers, with proportions of producers in the other four phyla ranging from ~10% to 50%. However, it should be noted that fewer *Fusobacteria* genomes were analysed compared to the other phyla. Excluding vitamin B_{12}, in excess of 90% of *Bacteroidetes* genomes were predicted to produce the other seven analysed B-group vitamins (Magnúsdóttir et al., 2015). Due to similarities between the human and pig gut microbiota, similar findings for pigs would be expected.

An interesting outcome of the study was the identification of organisms with vitamin biosynthesis pathways that were complementary to other microbes, indicating that some bacteria synthesise B-group vitamins that are directly utilised by neighbouring commensals in a symbiotic relationship, that is, cross-feeding (Fig. 4). Interestingly, for four of the analysed B-group vitamins, the gut microbiome was estimated to have the capacity to contribute more than a quarter of the recommended dietary requirements, without taking into consideration microbial utilisation. However, these estimations were based on intracellular vitamin concentrations of organisms cultured *in vitro* and hence do not necessarily reflect what is happening in the GIT where substrates may be less abundant (LeBlanc et al., 2017). Nonetheless, these results indicate that the gut microbiome is an important source of these micronutrients in humans (Magnúsdóttir et al., 2015; Rowland et al., 2018) but also in the pig gut (Crespo-Piazuelo et al., 2018; McCormack et al., 2017; Quan et al., 2020). However, further research is required to investigate the extent of B vitamin production by bacteria within the pig gut microbiota.

Overall, despite the lack of studies in pigs, human studies suggest that the pig gut microbiota is likely a valuable source of vitamins, particularly vitamin K and B-group vitamins, for both the host and the gut microbial community itself, and that dysbiosis may significantly impact vitamin requirements of the host. In addition to nutritional functions, many vitamins have also been implicated in the development and function of host immunity with a link between vitamin intermediates derived from commensal bacteria and immune cells that directly

Table 3 Proportion of bacterial phyla (%) predicted to synthesise eight B group vitamins via PubSEED subsystem gene function annotation of 256 human gut microbiome organism genomes. Adapted from data published in Magnúsdóttir et al. (2015) distributed under terms of the Creative Commons Attribution 4.0 International License

		Proportion (%) of bacterial phyla predicted to synthesise B-group vitamins				
Vitamin	Annotated subsystems analysed	*Bacteroidetes* (*n* = 51 genomes)	*Fusobacteria* (*n* = 14 genomes)	*Proteobacteria* (*n* = 38 genomes)	*Firmicutes* (*n* = 130 genomes)	*Actinobacteria* (*n* = 23 genomes)
Biotin (B7 or H)	'Biotin biosynthesis'	96	100	84	5	ND
Cobalamin (B12)	'Co-enzyme B12 biosynthesis'	51	100	26	43	9
Folate (B11, B9 or M)	'Folate Biosynthesis'	92	79	71	14	26
Niacin (B3)	'NAD and NADH cofactor biosynthesis global'	98	86	76	38	65
Pantothenate (B5)	'Co-enzyme A Biosynthesis'	100	ND[1]	95	32	13
Pyrodoxine (B6)	'Pyridoxin (Vitamin B6) Biosynthesis'	94	21	92	25	87
Riboflavin (B2)	'Riboflavin, FMN, and FAD metabolism Extended'	100	100	95	50	9
Thiamine (B1)	'Thiamin biosynthesis'	98	100	74	28	65

[1] ND: Not detected.

recognise these intermediates (Caballero and Pamer, 2015; LeBlanc et al., 2017; Yoshii et al., 2019). However, further research is needed into the importance of the pig gut microbiome as a source of vitamins.

8 Quorum sensing and manipulation

Gut microbial community structure is regulated by QS, a system of communication between bacterial cells, which relies on the production, secretion and sensing of chemical signals called auto-inducers (Jimenez and Sperandio, 2019; Xavier, 2018). It allows bacteria to sense the population density and synchronise different behaviours and expression of genes (Krzyżek, 2019), with these QS-mediated effects more efficient at high cell densities, such as those found within the GIT (Xavier, 2018). Quorum sensing is known to be involved in a range of bacterial activities including virulence factor production, toxin production and secretion, sporulation, biofilm formation and enzyme secretion (Jimenez and Sperandio, 2019; Krzyżek, 2019). Therefore, bacterial behaviours within the gut microbiome regulated by QS can be either beneficial or detrimental.

Commensals utilise QS to ensure gut homeostasis, as signalling molecules are involved in many of their vital processes including metabolism-related gene expression, cell division and DNA repair; hence, the production of auto-inducers can be seen as a microbiological service (Iacob et al., 2019; Xavier, 2018). Quorum sensing in the pig gut is less studied than in humans; however, Yang et al. (2018) recently isolated and characterised, for the first time, an N-acyl-homoserine lactone (AHL)-producing bacterium, *Aeromonas hydrophila* strain YZ2, from pig intestinal scrapings (AHLs are auto-inducers that mediate QS in Gram-negative bacteria).

In vitro research also suggests that pig pathogens, such as *S.* Typhimurium, ETEC and Shiga toxin-producing *E. coli* (STEC), use QS to mediate pathogenicity (Smith et al., 2011; van Parys et al., 2011; Yang et al., 2014; Zhu et al., 2011). This section will explore both the beneficial and detrimental roles of QS within the pig gut microbiome and how they may be manipulated. We will focus primarily on auto-inducer-2 (AI-2), as it is one of the most widely studied QS signalling molecules, primarily because it is synthesised and recognised by a wide range of bacteria and is involved in inter-species signalling.

8.1 Control of pathogenesis and biofilm formation

The diverse microbial communities within the mammalian gut consist of both planktonic and free-living bacteria as well as exopolysaccharide-coated biofilms which allow bacteria to thrive in microhabitats and nutritional niches. An example of a gut microbial biofilm can be seen in Fig. 6. Biofilms provide protection from antimicrobial substances and enzymes, and facilitate QS and

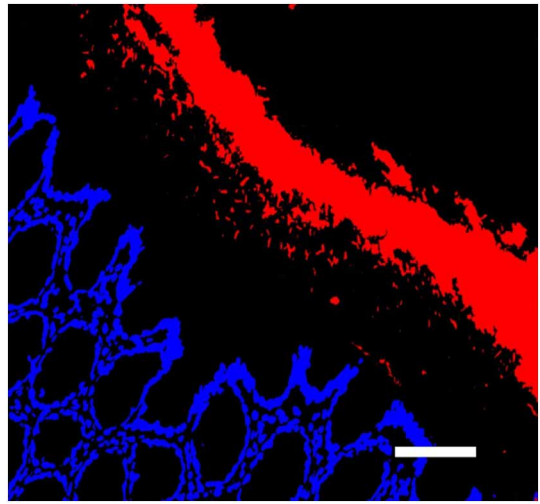

Figure 6 Example of a biofilm formed by the commensal colonic microbiota (red) of a healthy rat, separated from the epithelial surface (blue) by the intestinal mucus barrier (not stained). Scale bar = 50 μm. Adapted from Buret et al. (2019) distributed under terms of the Creative Commons Attribution 4.0 International License.

horizontal gene transfer (HGT) (Buret et al., 2019; Macfarlane and Dillon, 2007). Hence, they can be beneficial to gut commensals. However, biofilm formation involving pathogens is often associated with chronic infections, owing to their propensity to acquire and confer antibiotic resistance within the population (Jensen et al., 2017).

The role of QS in pathogenesis including expression of virulence factors, production and secretion of toxins, as well as biofilm formation, has led to the concept of anti-QS therapy, also referred to as quorum quenching (QQ), as a means of controlling pathogen proliferation. However, in a recent review, Krzyżek (2019) highlighted the need for caution with such therapies, as the same targeted signalling molecules are involved in many vital processes of commensal microbes as outlined above, and therefore disruption of signalling may result in a disturbance of microbiota homeostasis (Krzyżek, 2019). Nonetheless, several studies have investigated the potential of QQ therapy for the disruption of pathogenesis, with some research also performed on the endogenous QQ potential of the resident gut microbiota, albeit very few QQ studies have been performed in pigs.

An *in vivo* feeding trial carried out by Kim et al. (2018) investigated the QQ effects of supplementing weaned pigs with a probiotic pig gut-derived *Lb. acidophilus* strain, shown *in vitro* to reduce AI-2 production and biofilm formation by *E. coli* O157:H7, albeit this EHEC is not a pig pathogen. Using traditional culturing, the authors found reduced coliform counts in the faeces,

although it is difficult to attribute this to QQ activity of the administered strain as the pigs were not challenged with EHEC. Increased lactobacilli were also observed in the faeces and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis showed a difference in the 16S rRNA gene products after administering the *Lb. acidophilus* strain, most of which were identified as uncultured clones, *Lactobacillus* or *Bifidobacterium*. The authors concluded that bacteria with QQ properties can beneficially modulate the composition of the pig gut microbiota.

In conclusion, although data for pigs is scarce, QS potentially plays a dichotomous role in the pig GIT. First, it can serve as a mechanism for maintaining gut health by mediating gene expression related to metabolism, cell division, DNA repair and biofilm formation in commensals, although this has not been specifically shown for pigs. Conversely, QS is also a key mechanism in facilitating pathogenesis through the control of sporulation, biofilm formation and the production of virulence factors, with the latter shown for pig pathogens. Hence, there is potential to manipulate QS within the pig gut microbiome with the use of anti-QS or QQ treatments.

9 Antibiotic resistance genes

Antimicrobial resistance is a natural phenomenon that microbes have developed in order to survive in the presence of antimicrobial-producing competitors (Zeineldin et al., 2019a). Resistance to antibiotics, a broad group of naturally, as well as chemically, synthesised antimicrobial agents, is a concern due to their widespread use for the treatment and prevention of infections in both humans and animals (Sultan et al., 2018). Antibiotics have long been used in pig production for therapeutic and sub-therapeutic purposes. However, there is now widespread agreement that these practices contribute to the selection of antibiotic-resistant bacteria by transfer of the associated ARGs between populations leading to both public health and environmental concerns (Liu et al., 2019; Zeineldin et al., 2019a).

The ARG profile or 'antibiotic resistome' of the pig gut microbiome has been well characterised through high-throughput metagenomic sequencing (Hu et al., 2017). It has been shown to harbour a highly genetically diverse microbial community that facilitates HGT of ARGs between and within resident commensal organisms and pathogens (Sengupta et al., 2013; Zeineldin et al., 2019a). Focussing on ARGs, this section will discuss some of the undesirable microbiological services offered by the pig gut microbiome, namely its ability to act as a reservoir of ARGs and the transfer of these between commensal microbes and pathogens. We will also outline ways in which ARGs may offer a beneficial service to the host and possible ways in which ARG-harbouring bacteria may be excluded from the gut microbiome.

9.1 The gut microbiome as a reservoir of antibiotic resistance genes

Antibiotic administration has significant impacts on the pig gut microbiota and subsequently, on the associated antibiotic resistome (Zeineldin et al., 2019). When an antibiotic is administered, susceptible microbial populations are eliminated, with only those harbouring resistance remaining. This selective pressure gives resistant organisms an evolutionary advantage, and ultimately allows them to evolve, divide and confer their antibiotic resistance (Zeineldin et al., 2019a). However, it should be noted that antibiotic use is not the sole driver of antibiotic resistance, as several studies have shown that the antibiotic resistome is established prior to and/or in the absence of antibiotic exposure (Joyce et al., 2019; Knöppel et al., 2017; Wright, 2007; Zeineldin et al., 2019). Joyce et al. (2019) identified 56 core (present in all samples) and 201 accessory ARGs, within healthy pigs without selective antibiotic pressure, suggesting highly diverse antibiotic resistomes. Sets of ARGs suggested by Bengtsson-Palme (2018) also correlated well with those identified by Joyce et al. (2019).

A metagenomic study by Ghanbari et al. (2019) found that 41 ARGs were enriched within the faecal microbiome of weaned pigs administered therapeutic levels of in-feed oxytetracycline for 7 days (followed by 14 days on a standard diet) compared to the control group, fed a standard diet for 21 days. Increases in the relative abundances of the genera *Escherichia* and *Prevotella* were identified 7 days post-antibiotic treatment, which may be attributed to their propensity to carry ARGs such as *tetQ*, which may, in turn, be transferred to other susceptible bacteria within the GIT. Looft et al. (2012) also reported an increase in *E. coli* abundance in weaned pigs 14 days after administering a diet supplemented with chlortetracycline, sulfamethazine and penicillin.

Another interesting finding of the study by Ghanbari et al. (2019) was that, in addition to enrichment of tetracycline resistance genes, some ARGs unrelated to oxytetracyline were also enriched. This is in agreement with the findings of Looft et al. (2012) who proposed that this may be due to the co-occurrence of ARGs on mobile genetic elements (MGEs) such as plasmids and integrons. The majority of the ARGs found to be enriched by Ghanbari et al. (2019) were located on MGEs carrying at least two other resistance genes. This co-occurrence of ARGs on MGEs may facilitate HGT of ARG clusters to other commensals but also human pathogens such as *E. coli* (see Section 9.2), thereby explaining the importance of the pig gut microbiome as a reservoir of ARGs.

One drawback of metagenomic studies is that the abundance of certain genes does not necessarily reflect their expression. Wang et al. (2020) performed a metatranscriptomic study of 330 ARGs identified within the gut microbiome of pigs, humans and chickens relating to 21 classes of antibiotics. This revealed that 56.6% of the ARGs were expressed in pigs suggesting that

a substantial proportion of ARGs are transcriptionally inactive. Additionally, the authors found that the β-lactam, tetracycline and aminoglycoside ARG transcripts were primarily a result of ARG acquisition.

Antibiotic resistance genes may also have other roles in the pig gut microbiome, influencing FE, for example. In a metagenomic analysis of different intestinal regions of pigs with contrasting FE, Quan et al. (2020) found that *macB* was the most abundant ARG, attributed primarily to *Prevotella* and *Treponema* in the poorly and highly feed efficient pigs, respectively. The authors found that the *macB* gene may affect the energy metabolism of the microbiota and could be involved in regulating community composition, thereby affecting host FE (Quan et al., 2020). *Prevotella*, which was highly enriched in the caecum of pigs with poor FE, and to which *macB* abundance was linked, is associated with NSP degradation (Flint and Bayer, 2008; Wu et al., 2011). Nonetheless, it has also been suggested to be antagonistic towards some microbiota members such as *Bacteroides*, which also ferment dietary fibre but have been associated additionally with protein degradation (Chen et al., 2017b; Ley, 2016).

The authors, therefore, suggested that excessive *Prevotella* abundances may impede the development of an efficient nutrient-utilising microbiota, thereby decreasing FE (Quan et al., 2020). However, likewise, the abundance of *macB* attributed to *Treponema* in highly feed efficient pigs may implicate members of this genus as having a positive effect on FE, suggesting that some bacteria that harbour ARGs may provide a beneficial microbiological service to the host. *Treponema* has previously been associated with improved FE in pigs and has been positively correlated with digestibility and negatively correlated with fatness (Gardiner et al., 2020; He et al., 2016; McCormack et al., 2017; Yang et al., 2016; Niu et al., 2015; Yang et al., 2017). However, more extensive research is required to elucidate the potentially beneficial roles of *macB* and ARGs in general within the pig gut microbiome.

9.2 Transfer of ARGs between commensals and pathogens

Many studies have investigated the movement of ARGs between commensal and pathogenic bacteria in pigs and the farm environment, focussing on *E. coli,* as the pig gut harbours many commensal *E. coli* (Mazurek et al., 2018; Pérez Gaudio et al., 2018). A study by Reid et al. (2017) highlighted the role of commensal *E. coli* in the pig gut as contributors to the mobilisation of ARGs and the conferring of antibiotic resistance. A collection of 103 *E. coli* isolates from the faeces of healthy pigs were all found to carry class 1 integrons, genetic elements capable of integrating and expressing ARGs, with 97% of the strains found to be MDR. Moreover, most isolates carried virulence genes associated with human infection.

Pérez Gaudio et al. (2018) performed a conjugation assay to investigate HGT via class 1 integrons from a pig-derived antibiotic-resistant commensal *E. coli* to pathogenic STEC O157:H7. Following 4 h of co-culture, the STEC had acquired the class 1 integron, and presumably ARGs; however, antibiotic resistance was not investigated following the transfer. Nonetheless, the study demonstrates that commensal *E. coli* may serve as an important source of ARG transfer to pathogens in a short period of time.

Blake et al. (2003) performed a similar study where MDR commensal *E. coli* and a *Salmonella* isolate from the pig ileum were assessed for their ability to confer antibiotic resistance to antibiotic-susceptible pathogenic *E. coli* strains and a *Salmonella* Poona isolate under simulated ileal conditions. A bovine-derived pathogenic *E. coli* O157 strain dominated and persisted in the system as well as an antibiotic-resistant sub-population of this strain, which had obtained ARGs from a 'donor', co-inoculated resistant commensal *E. coli*. This, and the studies outlined above, demonstrate the ability of commensal bacteria to confer antibiotic resistance to pathogenic bacteria within the pig GIT.

9.3 Targeting the pig gut microbiome to reduce antibiotic resistance

Research on the exclusion or re-sensitisation of ARG-harbouring bacteria is mounting but is still in its infancy. Earlier, we discussed colonisation resistance via competitive exclusion as a means of inhibiting pathogen colonisation. Kim et al. (2005) performed the first study to examine the ability of a pig-derived mucosal CEC, previously shown to exclude *Salmonella* in pigs (Fedorka-Cray et al., 1999) to reduce antibiotic resistance in commensal *E. coli* in piglets. However, they found that resistance of *E. coli* to tetracycline and streptomycin was higher in the CEC-treated group, although streptomycin resistance returned to baseline at weaning. The authors indicated that the tetracycline resistance was most likely influenced by a combination of resistant *E. coli* from the sows, the environment and the CEC, all of which were found to harbour tetracycline resistance. Although mechanisms of transfer such as MGEs were not investigated, these results highlight a safety concern regarding the administration of CECs and their potential to confer resistance to the commensal gut microbiota. Consequently, guidance from the European Food Safety Authority (EFSA) requires comprehensive characterisation of microbial feed additives to avoid adding to the gut antibiotic resistome and to decrease the risk of transfer of antibiotic resistance (EFSA, 2018).

A more recent study in rabbits by Achard et al. (2019) yielded more promising results. They evaluated the effect of oral delivery of a faecal suspension, or faecal pellets added to nests (both derived from three different

antibiotic-naive does) on the antibiotic resistome of kits from antibiotic-exposed dams. The three different faecal inocula differed widely in their impact on the microbiome and associated antibiotic resistome, with one inoculum reducing the proportion of resistant *Enterobacteriaceae* from 93% to 9% and reducing the relative abundance of eight ARGs. Conversely, the least effective inoculum had no impact on ARGs or the microbiota composition. Interestingly, the authors found that exposure to faecal pellets was more effective than oral inoculation. This suggested that coprophagy, the behaviour of consuming faeces, is important in the transmission of microbes and associated ARGs to offspring. Coprophagy has been widely reported in pigs and recently, piglets that were deprived of maternal faeces for seven days after birth, showed poorer immune function and growth performance (Aviles-Rosa et al., 2019). Further studies are required to replicate these findings in pigs and to elucidate the mechanism and components of the inocula responsible for the competitive exclusion of ARG-harbouring microbes.

Pigs are known to be reservoirs of several species of staphylococci including *Staphylococcus suis* and *S. aureus*; the former is an important pig pathogen and an emerging zoonotic pathogen, while antibiotic-resistant strains of the latter, namely MRSA are considered a serious public health threat. A potential means of increasing antibiotic susceptibility of MDR-bacteria is to target bacterial QS. For example, hamamelitannin (HAM) is a QQ molecule that affects the susceptibility of *S. aureus* biofilms to antibiotics by suppressing cell wall synthesis and extracellular DNA release, two mechanisms facilitating vancomycin resistance in *S. aureus*. There is also *in vitro* evidence to suggest that HAM increases the susceptibility of *S. aureus* to other classes of antibiotics (Brackman et al., 2016).

Several other technologies have also shown promise in tackling antibiotic resistance. One is the revolutionary genome-editing tool: the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9) system. This is a natural prokaryotic defence mechanism that acts as a nuclease and can be guided to cleave any target DNA (Goren et al., 2017). Kim et al. (2015b) applied the CRISPR/Cas9 system to kill extended-spectrum β-lactamase (ESBL)-producing *E. coli* which are generally MDR and harbour plasmid-encoded ARGs that are transferred via HGT. However, the frequency of mutations on ESBL gene sequences meant that finding a target for one mutant would be therapeutically impractical. For this reason, the authors used a highly conserved sequence in ESBL mutants as a CRISPR/Cas9 target and successfully cleaved the ESBL plasmid of a clinical isolate, restoring susceptibility to both ampicillin and ceftazidime; the latter was not specifically targeted but was disarmed because it was encoded on the same plasmid. This technology has the potential to be an effective method for combatting plasmid-carrying MDR bacteria (Kim et al., 2015b).

There are, nonetheless, significant challenges with applying such technologies to complex microbial ecosystems such as the pig gut where individual species or strains may contain lineages with highly diverse antibiotic resistomes, carrying a variety of different plasmids and MGEs. Another challenge of using genome editing tools such as CRISPR/Cas9 is the risk of undesirable knock-on effects within the microbial community. For example, like the microbiota perturbations that occur following antibiotic administration, removal of a particular strain from the ecosystem may promote the proliferation of other potentially pathogenic species. The consequences of antibiotic resistance manipulation with CRISPR/Cas9 have not been well studied to date, and must be considered for any potential therapeutic applications (Pursey et al., 2018).

10 Conclusion

The resident pig gut microbial community, dominated by the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, is provided with a hospitable habitat that provides protection and a continuous supply of nutrients. The gut microbiota, in turn, provides a plethora of beneficial services to the host, including conferring colonisation resistance through competitive exclusion and the production of antimicrobial substances, production of enzymes, metabolism of dietary fibre and the production of VFAs and vitamins (Fig. 4). Quorum sensing can also be considered a beneficial service offered by the gut microbiome, as it can act as a mechanism for maintaining gut health by mediating the expression of genes controlling essential functions in commensals. However, the pig gut microbiome can also deliver negative microbiological services; for example, it can act as a reservoir of ARGs which can be transferred to pathogens and disseminated to other animals, humans, food and the environment.

Recently, the concept of a 'core' pig gut microbiome, independent of age, origin, breed and diet, has emerged. This provides insights into the most prevalent genera colonising different sections of the GIT, which may act as potential markers of gut health. As pig gut microbiome data becomes more abundant and as advances in functional metagenomics continue to provide valuable insights into the role of gut microbes, there is huge potential to identify microbial targets and mechanisms that can be exploited to improve gut health. The focus should be on enhancing the beneficial services offered by the pig gut microbiome, while reducing/eliminating services with negative impacts.

Specific approaches could include the administration of probiotic microorganisms as a means of implanting microbes that can offer beneficial services within the gut microbiome or alternatively, prebiotics or other feed additives which can increase the numbers of microbes already providing benefits. In terms of reducing/eliminating negative gut microbiome-related

services, there is potential to manipulate QS within the pig gut microbiome with the use of anti-QS treatments, as QS facilitates pathogenesis in gut microbes, as well as benefitting commensals. Using microbiota-derived CECs for the exclusion of ARGs or technologies such as CRISPR/Cas to restore antibiotic susceptibility in MDR bacteria are other options. Some of these approaches are already being exploited by commercial pig producers, while the more novel strategies are only at the research stage and safety and efficacy must be demonstrated before they can be adopted commercially.

11 Where to look for further information

For further information on the pig gut microbiome, we direct readers towards two recent collections of published papers. The first is a special issue series published in 2019 in the Journal of Animal Science and Technology, 'Pig gut microbiota: Challenges and opportunities to improve the pig health' (https://www.biomedcentral.com/collections/PGM). The second is a special issue of the journal Microorganisms on 'Gut Microbial Ecology in Pigs - Impact on the Gut and Beyond', published in 2020. The Microbiology and Microbiome section of the Journal of Animal Science also often publishes relevant papers in the pig gut microbiome area.

There are a number of ongoing research projects in the pig gut microbiome area that readers should keep an eye on. For example, the Horizon 2020 Marie Skłodowska-Curie Innovative Training Network 'Training and Research for Sustainable Solutions to Support and Sustain Gut Health and Reduce Losses in Monogastric Livestock', in short 'MonoGutHealth' recently funded by the EU Commission (https://monoguthealth.eu/). This project will explore the efficiency of innovative feeding strategies prior to birth and/or during the early neonatal periods to improve the development of the GIT and its microbiome and has a number of work-packages which are centred on the gut microbiome of pigs.

In addition, some regularly held conferences that cover the pig gut microbiome include:

- International Symposium on Digestive Physiology of Pigs, which is held every 3 years in various locations around the world. This symposium is considered 'the most important global scientific event in the fields of pig nutrition and gut physiology'. Information on the next meeting, which is due to be held in 2022 can be found at https://dpp2022.com/.
- International Symposium on Gut Microbiology. This biennal meeting is recognised as 'one of the most important meetings in the animal and human gut microbiology research areas', and usually has a considerable amount of content in the pig gut microbiome area. The 12th symposium is

being held this year and information can be found at https://gutmicrobiology-2021.symposium.inrae.fr/.

Readers can also obtain further resources and keep up to date on developments in the area by visiting the authors' institute websites:

- https://www.teagasc.ie/animals/pigs/.
- https://www.wit.ie/about_wit/contact_us/staff_directory/gillian_gardiner.

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