Organic acids, Essential Oils and Permeabilising Substances as an Alternative to Antibiotic Growth Promoters and Zinc in Control of Post-Weaning Diarrhoea and Enhancement of growth Performance in Weaned Pigs.

by

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This thesis has been composed by myself and has not been accepted in any previous application for a degree. The work, of which this is a record, has been done by myself and all sources of information have been cited.

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Literature Review: Organic acids, Essential Oils and Permeabilising Substances as an Alternative to Antibiotic Growth Promoters and Zinc in Control of Post-Weaning Diarrhoea and Enhancement of growth Performance in Weaned Pigs.

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1. Introduction

Weaning piglets is usually followed by a period of diarrhoea and decreased growth performance. Post-weaning diarrhoea (PWD), associated with proliferation of certain serotypes of *Escherichia coli*, is responsible for frequent large costs for the pig producer. Traditionally in-feed antibiotic growth promoters have been used, as well as zinc oxide (ZnO) and copper sulphate ($CuSO_4$), to control PWD and optimise growth performance. However, today with the ban of in-feed antibiotic growth promoters (AGPs) in Europe, widespread antibiotic resistance and general concerns about the use of antibiotics in the pig industry, and the increase in concern of environmental accumulation of trace minerals, there is a need to find other sustainable options. Research has therefore focused on development of alternative strategies to AGPs and ZnO, with a focus on maintenance of animal health and performance.

This review will focus on some alternative nutritional strategies for the control of PWD and improvement of growth in the post-weaning period. Organic acids have for a long time been used as a food preservative and are displaying good potential as a means to aid in the control of PWD and optimisation of growth performance, due to their antimicrobial qualities. Essential oils, a phytochemical, also displays valuable antimicrobial qualities and could be another option. Together with the use of a permeabilising substance, these candidate products collectively may offer a nutritional-based solution to be able to substitute the use of AGPs in diets for pigs after weaning.

2. The gastrointestinal anatomy

The gastrointestinal tract (GIT) in the pig is a muscular tube lined with a mucus membrane consisting of mouth, pharynx, oesophagus, simple stomach, small intestine, large intestine, rectum and accessory glands such as salivary glands, liver and pancreas. It has two major functions; integration of nutrients, fluids and electrolytes and maintaining a protective barrier to prevent uncontrolled passage of toxins and infectious agents into the systemic circulation (Zhang and Xu 2003). Therefore when gastrointestinal disorders occur, one or both of these functions are compromised and may lead to growth retardation and (or) death (Zhang and Xu 2003). The functions of the GIT are protected by the barring of pathogenic compounds and this is achieved

through non-specific defence mechanisms, such as production of gastric juice, mucus coat, peristaltic movement, tight junctions, epithelial desquamation, proteolysis, bacterial competitive exclusion and the gut liver axis, as well as immunological defence mechanisms which produce secretory immunoglobulins, M-cells and lymphocytes (Halas 2009). The non-specific mechanisms are mainly influenced by the composition of the diet as well as the texture (Halas 2009).

When weaned, pigs have to deal with sudden separation from the sow and change in hierarchy with littermates as well as change in environment. The diet also changes suddenly from sow milk to a less digestible, plant-based dry diet that contains complex protein and carbohydrates as well as various anti-nutritional factors (Heo et al. 2012). As a result the feed intake immediately after weaning is severely reduced (Heo et al. 2012) and inadequate over the first few days, rendering the piglet more vulnerable to PWD as the structural and functional maintenance of the GIT is compromised (Halas 2009). Approximately 50% of the weaned piglets consume their first feed within 24 hours after weaning, whilst approximately 10% do not feed until about 48 hours after weaning (Heo et al. 2012). Immediately after cease in milk supply the structure and function of the digestive tract starts changing, namely decreasing villous height and decreasing crypt depth, which reduces the capacity of absorption (Williams 2003). The poor absorption resulting from this is often associated with the proliferation of enterotoxigenic *Escherichia coli* (ETEC) and /or fermentation of less digestible nutrients in the large intestine (Williams 2003). Under commercial conditions, both shortly before and after weaning, the piglets probably achieve less than 50% of their growth performance potential (de Lange et al. 2010).

In this review, I will briefly consider the anatomy and physiology of the small and large intestines, the microbiota colonising them and changes associated with weaning, as they are important parts of the GIT and its health and the connection between PWD, weaning and function of the GIT (Halas 2009).

2.1 The small intestine

The small intestine is the longest part of the GIT, consist of three regions; duodenum, jejunum and ileum and is where most nutrients are absorbed (Zhang and Xu 2003). The wall of the small intestine consists of four layers; mucosa, submucosa, muscularis and serosa, of which the mucosal layer is divided into three sublayers; muscularis mucosa, the lamina propria, and the epithelium (Zhang and Xu 2003). The epithelial lining of the small intestine has finger-like projections knows as villi, which increases the surface area for digestion and absorption (Heo et al. 2012). At the base of the villi there are tubular glands that open into the intestinal lumen called crypts, which contain epithelial stem cells required for repopulation of epithelial cells (Heo et al. 2012). For optimal function of the small intestine, long villi are desired, however due to anorexia (being the main factor) after weaning there are periods of transient villous atrophy and crypt hyperplasia (Heo et al. 2012).

2.2 The large intestine

The large intestine is divided in three compartments; the caecum, colon and rectum, and among its physiological functions are fluid and electrolyte absorption, and provision of a physical barrier to hinder microbial invasion (Heo et al. 2012). The large intestine is also the home for billions of microorganism, which utilise the food residue that is discharged from the small intestine. They convert this discharge into useful nutrients such as short chain fatty acids and vitamins, which the animal then is able to absorb and utilise (Zhang and Xu 2003). The wall of the large intestine has the same four layer structure as the small intestine and the structural organisation is similar, except for the structure of the mucosal layer (Zhang and Xu 2003). The mucosal surface is lined with crypts like the small intestines, however it lacks villi (Zhang and Xu 2003). The process of weaning provokes quite profound changes in intestinal structure, which again disrupts the functional capacity (Hopwood and Hampson 2003). It may take several weeks before full, efficient and appropriate functionality is restored (Hopwood and Hampson 2003).

2.3 The gastrointestinal microbiota

The intestinal microbiota of the piglet is established within the first 48 hours via the ingestion of maternal faeces and involves successional changes until a stable and dense population colonises the GIT (Pluske et al. 2002). This microbiota is characterized by its high population density, its extensive diversity and the complex interactions throughout the GIT (Pluske et al. 2002). After the initial establishment of the microbiota, it remains reasonably stable except when there are major changes in diet and/or environment, such as weaning (Hopwood and Hampson 2003). The GIT is colonised by two different types of bacteria. Autochthonous bacteria or indigenous bacteria, have coevolved with the host and colonise all habitats and niches in the GIT (Pluske et al. 2002). Allochthonous or non-indigenous bacteria may pass through specific microhabitats, being derived from food, water, or another gut habitat and do not colonise the GIT (Pluske et al. 2002). Pathogens can be either of these two and in general cause disease when the GIT ecosystem is disturbed in some way (Pluske et al. 2002). Therefore it is important that the predominance is of beneficial species of microorganisms over pathogenic species as it is essential for the stability of the immune system of the small intestines and the entire body (Vondruskova et al. 2010).

When the piglet is still suckling, the dominant bacteria within the stomach and small intestine usually are lactobacilli and streptococci, whereas the large intestine has a larger and more diverse selection of mainly obligate anaerobic bacteria, including *Bacteroides, Eubacterium, Bifidobacterium, Propionibacterium, Fusobacterium, and Clostridium species* (Hopwood and Hampson 2003). The metabolic activity and physical presence of a stable microbiota, reduces chance of colonisation by other bacteria, including potentially pathogenic species such as ETEC (Hopwood and Hampson 2003). The change in intestinal structure and function result in a change in mass, composition and complexity of the microbiota in the intestinal flora (Hopwood and Hampson 2003). In the small intestine a decrease in lactobacilli has been detected, whilst the total number of bacteria and especially the proportion of coliforms, *Escherichia coli* (*E. coli*) in particular, increased (Hopwood and Hampson 2003). As each limiting nutrient will support the one bacteria or strain which is best at utilising it,

the diversity of the bacterial population will be directly related to composition and number of limiting nutrients (Pluske et al. 2002).

3. *Escherichia coli* and post-weaning diarrhoea

Weaning imposes high levels of stress to the piglets, due to nutritional, social and environmental changes, as well as gut dysfunction which is associated with distinct changes in the GIT physiology, microbiology and immunology (Pluske 2013). PWD is a condition in weaned pigs which is characterized by frequent discharge of watery faeces during the first 2 weeks after weaning (Cutler and Gardner 1988), and is usually developed within 4-5 days of the change from sow milk to solid food (King 2003). Diarrhoea can be observed as a yellowish or grey fluid, lasting up to a week (Cutler and Gardner 1988) and can be one of the most frequent causes of heavy economic losses in pig herds (Vondruskova et al. 2010).

PWD is associated with the proliferation of enterotoxigenic ß- haemolytic strains of *E. coli* in the small and large intestines of newly weaned pigs and the pigs usually show signs of diarrhoea, depression, reduced appetite, a rough hair coat, and may shiver (Halas 2009, Wellock et al. 2007). The main infection is in the small intestine, and is the site of underlying fluid and electrolyte loss into the intestinal lumen, though the bacterium is also present throughout the small and large intestine (Hopwood and Hampson 2003). E. *coli* is a member of the *Enterobacteriacea* family and is facultatively anaerobic, gram negative rod (Broeck et al. 2000). Most strains of E. *coli* in the GIT are harmless, however there are also virulent strains such as ETEC which are strongly related to enteric disease such as diarrhoea, especially in young animals (Nagy and Fekete 1999). These *E. coli* attach to glycoprotein receptors expressed on the brush border of cells lining the intestinal villi, and this attachment prevents the bacteria from being flushed through the large intestine (Pluske et al. 2002). The attachment may cause lesions and it produces enterotoxins, which it releases. This production and release of enterotoxins changes the water and electrolyte flux of the small intestine, which may lead to diarrhoea if excess fluid is not retained in large intestine and excessive excretion leads to dehydration, metabolic acidosis and possibly death (Cutler and Gardner 1988).

The receptor for *E. coli* disappears a few weeks after weaning, providing the bacteria a short amount of time to attach and proliferate (Pluske et al. 2002).

Post weaning strains produce one or more of the enterotoxins STa, STb, LT and EAST-1 (Cutler and Gardner 1988). A heat labile-toxin binds irreversibly to the mucosal cells and activates the adenyl cyclase-cyclic AMP system, inducing the secretion of chloride ions, sodium ions, bicarbonate ions and water into the lumen (Pluske et al. 2002). The heat-stable toxin (ST, with the subtypes a and b) are small cysteine-rich peptides secreted by ETEC (Fleckenstein et al. 2010), which inhibits absorption of chloride and sodium ions from the lumen into the epithelial cell via the guanyl cyclase-cyclic GMP system (Pluske et al. 2002). Intestinal secretion of chloride is also stimulated by the pathway for production of nitric oxide, where the nitric oxide stimulates the secretion of chloride via elevation of intracellular cGMP levels, where the activation of inducible nitric oxide synthase expression (an enzyme which converts L-arginine to L-citrulline to produce nitric oxide) can be activated by the proliferation of *E. coli* (Kim et al. 2012). All these activities contribute to increase in chloride secretion, reduced sodium absorption and the associated massive loss of water into the intestinal lumen (Fairbrother, Nadeau, and Gyles 2005). The steps in the development of *E. coli* PWD are illustrated in figure 1. The consequence can be diarrhoea, dehydration, reduced feed intake, reduced nutrient digestibility, reduced growth, loss of weight and even death for pigs that are affected (Halas 2009). Animals may also die without sign of PWD as enterotoxins enter the bloodstream and lead to septicaemia and/or endotoxemia(Fairbrother, Nadeau, and Gyles 2005).

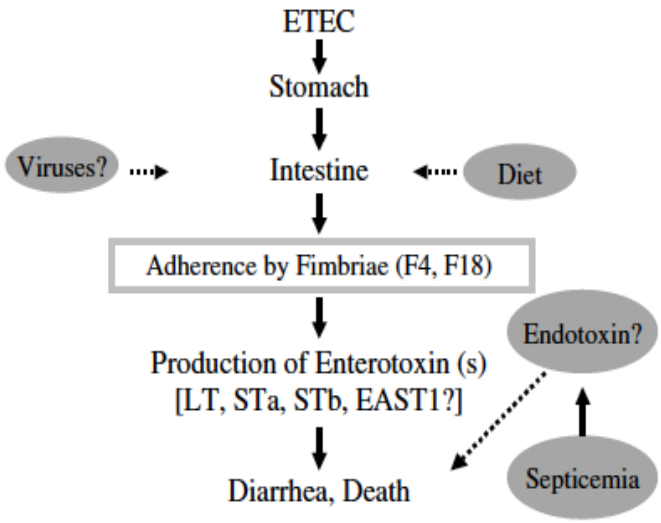

Figure 1. Overview of steps in development of *E. coli* post-weaning diarrhoea in pigs (Fairbrother, Nadeau, and Gyles 2005)

The environment is the most likely source of pathogenic *E. coli* strains for weaned piglets (Zimmerman et al. 2012) and intestinal infections caused by E. *coli* are contagious and transmitted to other pigs via contaminated feed, handlers, drinking water and by aerosol and the infection occurs by the oral route (Zimmerman et al. 2012). *E. coli* appears commonly in the faeces of pigs in increased numbers in the first week after weaning in both healthy and diarrheic pigs, while the numbers are higher in diarrhoeic pigs (Pluske et al. 2002). Immunity from to one strain of pathogenic *E. coli* does not protect from other strains, and many strains show resistance to multiple antibiotics (Hopwood and Hampson 2003).

4. Dietary methods to control PWD

Traditionally dietary antibiotics and (or) mineral compounds such as ZnO and $CuSO_4$ have been used to control PWD (Gong et al. 2008, Pluske 2013), however due to concerns of antibiotic resistant strains of bacteria, the European Union for instance has banned the use of in-feed antibiotic growth promoters in livestock diets in 2006 (Casewell et al. 2003, Riemensperger et al. 2012), and there is also a pressure to minimise or eliminate the use of in-feed antibiotics in livestock diets in other parts of the world (Lusk, Norwood, and Pruitt 2006). There are also environmental concerns for accumulation of minerals such as zinc (Pluske 2013). Currently the pork industry has no sustainable way of reducing susceptibility to gastrointestinal pathogens, such as strains of *E. coli* causing diarrhoea and production loss in the post-weaning period. This situation is exacerbated by widespread antibiotic resistance against common enteric pathogens in pigs (Smith et al. 2010) and increasing consumer concerns related to the use of antibiotics in pig production (Lusk, Norwood, and Pruitt 2006).

4.1 Dietary antibiotics

Antibiotics were discovered more than 60 years ago and have since then been widely used in the livestock industry (Cromwell 2002). They are used therapeutically, treating animals when clinical symptoms occur, or prophylactically, treating groups of animals in advance of clinical symptoms, for example in connection with weaning (Wegener 2003). Antibiotics are also used to improve growth performance, increasing growth rates and feed efficiency and the majority of these are given in feed (Wegener 2003).

These are termed antibiotic growth promotants (AGPs) or growth promoting antibiotics. Traditionally these were used sub-therapeutical in-feed, as a growth stimulant and to treat gastrointestinal infection (Bhandari et al. 2008). In-feed antibiotics enhance animal growth by reducing microbial use of nutrients as well as microbial production of ammonia (Partanen et al. 2001). This was a widely used health strategy before the ban in Europe in 2006 (Vondruskova et al. 2010)

4.2 Antimicrobial mineral compounds

Copper and zinc are essential trace minerals and the basal requirement is approximately 10 and 100 ppm, respectively, with weaner pig diets supplemented with these (Tokach et al. 2003). ZnO and $CuSO_4$ are often included in weaner diets to control PWD and enhance growth performance (Heo et al. 2012). Zinc oxide is widely accepted in the pig industry in weaner diets due the proven effects on decreasing PWD and increasing performance (Kim et al. 2012). High doses of zinc oxide, 2500 – 3000 ppm mg Zn/kg feed, have been added to feed as a preventative measure against PWD (Vondruskova et al. 2010), as well as a growth promoter (Tokach et al. 2003). Zinc is a significant nutritional factor as it regulates the metabolism of amino acids and protein, as well as contributing to stabilisation of sensitive intestinal mucosa, diversity and functions of bacterial microflora, inhibits growth of some pathogens and enhances the immune response against infections (Vondruskova et al. 2010). It is also associated with insulin, and hence plays a important role in lipid, protein and carbohydrate metabolism in the pig (Pluske et al. 2002). Zinc is a component of several enzymes, is involved in their action and is consequently essential to biochemical processes in the body (Vondruskova et al. 2010) Therefore a deficiency can cause growth retardation and depletion of overall enzyme activity in tissues (Heo et al. 2012).

Højberg et al. (2005) demonstrated that 2500 mg/kg of ZnO did not inhibit the growth of pathogenic gram-negative bacteria by using a 16s rRNA gene sequencing technique. However, they did find that ZnO supressed gram-positive commensal microbes such as *Lactobacillus amylovorous, Lactobacillus reiteri, and Streptococcus alactolyticus*. In an *in vitro* study conducted by Li et al. (2001) using culture based techniques, it was indicated that 2500-3000 ppm mg/kg of ZnO did not effect the *E. coli* population in the

ileal digesta. The data from these studies suggests that decrease in PWD and increase in growth performance when high levels of ZnO are supplemented is not associated with elimination of ETEC in the GIT. However, Li et al. (2001) did find that inclusion of 3000 ppm mg/kg ZnO increased mucus thickness, villous height, villous width, villous height: crypt depth ratio and decreased crypt depth in the small intestines of 33 day old pigs weaned at 21 days. The data from an *in vivo* study by Zhang and Guo (2009), using piglets weaned at 24 days, showed that inclusion of 2000 ppm mg/kg ZnO or tetrabasic zinc chloride reduced the expression of PWD and decreased paracellular permeability measured as urinary recovery of lactulose and mannitol. Through analyses of mRNA expression of tight junction proteins occluding and ZO-1 in the ileal mucosa, Zhang and Guo (2009) also found that both ZnO and tetrabasic zinc chloride significantly increased the expression of tight junction proteins in the ileal epithelium. Results from these studies suggest that the supplementation of ZnO suppressed PWD and increased growth performance is not related with elimination of ETEC, but rather that improved intestinal barrier and immune function could be accredited.

The bioavailability of Zn compounds is generally less then 50% and there is a increasing concern of use of Zn due to the levels excreted into the environment, with possible antibacterial effects on beneficial soil and water bacteria (Pluske et al. 2002). Due to these reasons some European countries have banned the use of high levels of ZnO in diets for pigs (Kim et al. 2012).

4.3 Organic acids

Organic acids are characterised as weak and short-chain fatty acids and are a widely distributed in nature as components in herbal and animal tissue (Costa et al. 2013). They have for a long time been used as preservatives to increase food shelf-life, where their mode of action is related to food pH and the degree of non-dissociated acid available to inhibit spoilage by microorganisms (Piva and Grilli 2007). It also appears that organic acids used as feed preservatives exert their antimicrobial action after ingestion, resulting in varying degrees of gastrointestinal microbial control in relation to their bioavailability (Piva and Grilli 2007). The bactericidal effects of organic acids are based on the ratio between dissociated and non-dissociated acid. The form in which the acid

appears depends on the pK_a value of the organic acid and the pH of the environment (Halas 2009). This ratio can then be calculated using the Henderson-Hasselbach equation: $pH = pK_a + \log [A^-]/[HA]$, where $[A^-]$ and [HA] are the concentrations of dissociated and non-dissociated acid respectively (Halas 2009). It is not only the concentration of organic acids that impairs the bacterial metabolism, but more the level of non-dissociated organic acids.

The antimicrobial mode of action is two-fold. Adding organic acids to the diet lowers the gastric pH, which results in increased gastric retention time and improved activity of proteolytic enzymes (Costa et al. 2013). They may also lower the buffering capacity of the diet, inhibit proliferation and colonisation of undesirable microorganisms in raw materials, feed and GIT of animals, act on physiology if the gastrointestinal mucosa, and ease the availability of nutrients, improving the digestion, absorption and retention (Costa et al. 2013). Organic acids have been reported to help with problems such as PWD and growth performance in the immediate post-weaning period (Busser et al. 2011). Also, the organic acids in their non-dissociated form can penetrate through the bacterial cell wall and destroy some vital cell functions (Riemensperger et al. 2012). This ability is primarily a characteristic of organic acids with a low carbon number, such as formic acid, acetic acid, propionic acid and lactic acid (Partanen et al. 2001). Organic acids can be categorised into two groups based on their effects, where the first one comprises lactic, fumaric and citric acids which are characterised by an indirect effect reducing bacterial populations by decreasing pH in the stomach (Castro 2005). The second group comprises formic, acetic, propionic and sorbic acids, and are characterised by a direct effect of lowering the pH in the digestive tract on the cell wall of gram-negative bacteria and thus preventing replication of deoxyribonucleic acid (Castro 2005).

It has been reported that inclusion of organic acids in the diet of swine can enhance growth performance and modulate swine intestinal fermentation and microbial proteolysis (Gong et al. 2008). Out of all the current available alternatives to in-feed antibiotics and ZnO (including probiotics, prebiotics, fermented liquid feed, minerals, and organic acids), organic

acids are probably the most promising, however to obtain an efficacy comparable to antibiotics in relation to health and growth promotion, a combination of organic acids and levels needs to be investigated (Knarreborg et al. 2002)

It has been found supplementing weaner diets with organic acids improves the growth performance and feed conversion ratio, however the responses have varied greatly for a number of reasons including differences in type and amount of organic acids added to the diet, composition of the diet itself, age of animals and existing levels of performance (Busser et al. 2011), and in particular the diet's buffering capacity (de Lange et al. 2010). The positive effects of feeding organic acids on gut health and development, and indirectly on pig health and productivity, may be attributed to multiple factors, including; antimicrobial activity of non-dissociated organic acids, lowering digesta pH particular in the stomach aiding protein digestion, decreasing stomach emptying rate, stimulating enzyme production and activity in small intestine, and providing nutrients preferred by intestinal tissue and by this enhancing mucosal integrity and function (de Lange et al. 2010).

Partanen et al. (2007)found that formic acid has a tendency to decrease bacterial nitrogen in different parts of the small intestine and improved the apparent ileal digestibility of protein sources, certain essential amino acids, lipids, calcium and phosphorus. Compared to the effect of supplementation of antibiotics, it showed a similar or higher effect. The growth efficiency was significantly increased by the addition of formic acid on market pigs, and when combined with potassium sorbate the effect was further enhanced.

Hansen et al. (2007) found that the adding formic, lactic and acetic acids to the diet increased the concentrations of organic acids, which in a combination with a lower pH resulted in reduced colonisation of enterobacteria in the lower parts of the GIT. Tsiloyiannis et al. (2001) tested six organic acids (propionic, lactic, formic, malic, citric and fumaric acid) and their effect on controlling PWD and found that all six organic acids had a positive effect on controlling PWD and in general improved the performance of the piglets. All six treatments reduced the incidence and severity of

diarrhoea in the weaned piglets, although none of them proved to be capable of lowering the mortality rates significantly. In relation to growth performance all six groups showed a significantly ($P < 0.05$) better performance than the negative control group. The diet containing lactic acid showed the best improvement in all tested parameters compared to the five other organic acid groups. When studying benzoic, lactic and sorbic acids *in vitro*, Piva and Grilli (2007) found that lactic acid failed to inhibit microbial activity in the caecum and benzoic only showed a general reduction of antimicrobial activity. The data from a study by Namkung et al. (2004) supplementing with organic acid blends of formic, acetic, phosphoric and acetic acids, indicated that they can be used as an alternative to in-feed antibiotics, and when lactic acid was added to the mix as well the results were similar to those fed a diet supplemented with antibiotics.

Activity	Organic Acid	Comparison to control diet[1]	Reference
	Formic	positive	Manzanilla et al. (2004)
	Fumaric and citric	positive	Falkowski and Aherne (1984)
	Formic, propionic, potassium sorbate and benzoate	positive	Partanen et al. (2007)
	Formic and lactic	positive	Jongbloed et al. (2000)
	Formic, benzoic, sorbic and ca-butyrate	equal	Overland et al. (2007)
	citric and sorbic	positive	Grilli et al. (2010)
	Formic acid, ammonium formate coated with potassium sorbate	positive	Partanen, Karhapää, and Siljander-Rasi (2006)
	Formic and lactic	positive	Partanen, Karhapää, and Siljander-Rasi (2006)
	propionic, lactic, formic, malic, citric and fumaric acid	positive	Tsiloyiannis et al. (2001)
	Formic, acetic, lactic, phosphoric and citric acid	positive	Namkung et al. (2004)
Intestinal microbiota modulation	Formic	equal	Manzanilla et al. (2004)
	Formic, propionic, potassium sorbate and sodium benzoate	equal	Partanen et al. (2007)
	citric and sorbic	positive	Grilli et al. (2010)
	Formic, benzoic, sorbic and ca-butyrate	positive	Overland et al. (2007)
	Formic acid, ammonium formate coated with potassium sorbate	equal	Partanen, Karhapää, and Siljander-Rasi (2006)
	Formic and lactic	equal	Partanen, Karhapää, and Siljander-Rasi (2006)
	Formic, acetic, lactic, phosphoric and citric acid	positive	Namkung et al. (2004)
Feed digestibility	Formic	equal	Manzanilla et al. (2004)
	Fumaric and citric	equal	Falkowski and Aherne (1984)
	Formic and lactic	positive	Jongbloed et al. (2000)
Stomach pH	Formic	equal	Manzanilla et al. (2004)
	Formic, acetic, lactic, phosphoric and citric acid	positive	Namkung et al. (2004)
	Formic and lactic acids	positive	Hansen et al. (2007)
Histological modification	Formic, acetic, lactic, phosphoric and citric acid	positive	Namkung et al. (2004)
	Formic	equal	Manzanilla et al. (2004)
Volatile fatty acids	Formic	equal	Manzanilla et al. (2004)
	citric and sorbic	positive	(Grilli et al. 2010)
	Sorbic	positive	(Overland et al. 2007)
	Formic, benzoic and ca-butyrate	equal	(Overland et al. 2007)
Intensity and severity of PWD	propionic, lactic, formic, malic, citric and fumaric acid	positive	Tsiloyiannis et al. (2001)
	Formic and lactic acids	positive	Hansen et al. (2007)
	Formic, acetic, lactic, phosphoric and citric acid	positive	Namkung et al. (2004)

Table 1. Main actions of growth promotion from use of organic acids as feed additives for pig.
[1]Positive and negative results represents significant difference between the control and treated groups ($p<0.05$).

From the results from these studies and others, summarised in table 1, formic acid seems to be an attractive option as it enhances growth performance in lower dosage than those of other organic acids. However, formic acid is difficult to handle as it is volatile and corrosive and has a strong pungent odour (Partanen et al. 2007). Partanen et al. (2007) suggested that this could be solved by absorbing it in a carrier, however when investigated they found that growth performance wasn't improved when the formic acid was in a carrier compared to no carrier.

4.4 Phytochemicals

Phytochemicals are non-nutritive plant chemicals, which have disease preventative properties (Riemensperger et al. 2012). Essential oils, or volatile or ethereal oils, are phytochemicals and are aromatic oily liquids which can be acquired from plant material (flowers, buds, seed, leaves, twigs, bark, herbs, woods, fruits and roots) (Yan et al. 2010). These compounds are produced in the plant as a defence mechanism against external factors such as physiological stress, environmental factors and protection from predators and pathogens (Costa et al. 2013). These substances are usually not present in a pure state within the herbal cells, but in form of complexes, which in some cases have a better antimicrobial activity in the animal body (Costa et al. 2013). Cinnamaldehyde is a metabolic and aromatic compound found in the bark and leaf of *Cinnamonum* spp., and has traditionally been used in traditional human and veterinary medicine practiced by ethnic groups for treatment of various maladies, such as gastritis (Ferme et al. 2008). The possible mechanisms of action affecting the growth promotion in animals include changes in the intestinal microbiota, increased digestibility and nutrient absorption, enhanced nitrogen absorption, improved immune response, morphological and histological changes if the GIT and antioxidant activity (Costa et al. 2013). It has also been discovered to have bactericidal effects, inhibiting growth of assorted pathogenic and food spoilage bacteria and is at the same time considered non toxic and safe for use (Ferme et al. 2008). Essential oils act by altering the permeability of the cytoplasmic membrane to hydrogen ions (H^+) and potassium (K^+), and the change leads to disruption of essential cellular processes such as electron transport, protein translocation oxidative phosphorlylation as well as other enzyme-dependent reactions resulting in loss of chemiosmotic control (Costa et al. 2013). The antimicrobial affect is credited to it

targeting the FtsZ protein, which plays an important role in cell division of pathogenic bacteria, first binding to the FtsZ protein inhibiting its assembly and then disturbing the formation of the Z ring thus inhibiting the of cell division (Domadia et al. 2007).

Michiels et al. (2007) found that *trans*-cinnamaldehyde was very effective at low doses against coliform bacteria, whilst it hardly inhibited lactobacilli, however they had no clear understanding of the high selectivity and exceptionally high activity of the *trans*-cinnamaldehyde. This was also found in another study undertaken by Michiels et al. (2009). Both of these studies were undertaken *in vitro*. Yan and Kim (2012) conducted an *in vivo* study where they considered the effect of eugenol and cinnamaldehyde on growth performance, nutrient digestibility, blood characteristics, faecal microbial shedding and faecal noxious gas content in growing pigs. They concluded that both eugenol and cinnamaldehyde significantly decreased the faecal *E. coli* concentration compared to the control diet without having any affect on faecal lactobacilli concentrations. They also found that neither had any effect on growth performance. Another trial was undertaken by Riemensperger et al. (2012), where it was found that cinnamaldehyde, *in vitro*, can synergistically improve the effect of organic acids on the inhibition of bacteria.

As the mode of action of organic acids and phytochemicals are different to AGP´s, it is unlikely that one of the alone is going to directly substitute. However, by using a combination of an organic acid blend and essential oils a broader spectrum of activity is achieved, as well as a wider range of effect in the GIT due to their effects in different parts of the GIT(Riemensperger et al. 2012). Organic acids exert their activity in feed and upper part of the GIT, whilst essential oils exert their activity more in the distal parts of the GIT (Langhout 2000).

4.5 Permeabilising substance

Gram-negative bacteria, such as ETEC, possess in addition to their cell membrane a cell wall, a barrier, which prevents toxic compounds from entering the bacteria and destroying vital functions (Riemensperger et al. 2012). The outer cell wall allows only limited diffusion of hydrophobic substances trough its lipopolysacharie (LPS) covered

surface (Vaara 1992). They are the principle component of the outer envelope and carry a net negative charge, and in large part, these are responsible for the impermeability of these microorganism to compounds (Denyer and Maillard 2002). The gram-negative bacteria contains porins, hydrophilic channels, which regulates permeability and these allow diffusion of small hydrophilic molecules within a limited size range (Denyer and Maillard 2002). This outer membrane can be disturbed by permeabilising substances, which make the bacteria more susceptible to toxic compounds (Riemensperger et al. 2012). Ethylenediamine tetraacetic acid (EDTA) and some organic acids, (e.g. citric acid) are ion chelators, and are examples of permeabilising agent which may lead to an increase in biocidal activity in gram-negative bacteria (Denyer and Maillard 2002). EDTA in particular binds cations (e.g Mg^{2+}), which are essential for stabilising the strong negative charges of the core oligosaccharide chain of the LPS, and consequently releases LPS from the outer leaflet and phospholipids from the interior of the outer membrane is left exposed (Denyer and Maillard 2002).

The effect of adding a permeabilising substance was found to support the theory in a *in vitro* trial by Riemensperger et al. (2012), indicating that the effect of organic acids can be synergistically increase inhibition of bacteria when a permeabilising substance is added. Riemensperger et al. (2012) then did an *in vivo* study using pigs weaned at 28±2 days of age, feeding diets supplemented with a mix of organic acids (formic, propionic and acetic acid), the phytochemical cinnamaldehyde and the permeabilising substance. The data showed a significant increase in growth performance. However, it could not be concluded from the results if inclusion of cinnamaldehyde and a permeabilising substance can further enhance the effect of organic acids in vivo as the control was a negative control group with no additives added. Riemensperger et al. (2012) assumed that the improved growth rate was due to reduction in bacterial load in the GIT, however it can only be speculated as no measurements where taken to prove this.

5. Conclusions

PWD, associated with the proliferation of some serotypes of *E. coli*, is a frequent and large cost to producers. New and sustainable alternatives to in-feed AGPs and ZnO are needed due to bans and (or) environmental and consumer concerns. Through research,

organic acids and essential oils have shown promise due to their antimicrobial effects. Research ha shown that use of a single organic acid or essential oil cannot substitute AGPs, as their mode of action is different. However a mix of organic acids and essential oils, combined with a permeabilising substance to facilitate the entry of the organic acids and essential oils, could be able to achieve results similar to AGP´s

From the literature review in this paper I hypothesise that supplementing diets with a blend of organic acids (formic, propionic and acetic acid), an essential oil (cinnamaldehyde) and a permeabilising substance for weaner diets infected with ETEC will decrease the prevalence of PWD and increase the growth performance of the pigs.

References:

Bhandari, S. K., B. Xu, C. M. Nyachoti, D. M. Giesting, and D. O. Krause. 2008. "Evaluation of alternatives to antibiotics using a *Escherichia coli* K88 model of piglet diarrhoea: Effects on gut microbial ecology." *Journal of Animal Science* 86:836-847. doi: 10.2527/jas.2006-822.

Broeck, W. Van den, E. Cox, B. Oudega, and B.M. Goddeeris. 2000. "The F4 fimbria antigen of *Escherichia coli* and its receptors." *Veterinary Microbiology* 71:223-244.

Busser, E. V. De, J. Dewulf, L. De Zutter, F. Haesebrouck, J.Callens, T. Meyns, W. Maes, and D. Maes. 2011. "Effect of administration of organic acids in drinking water on faecal shedding of E.*coli*, performance parameters and health in nursery pigs." *The Veterinary Journal* 188:184-188. doi: 10.1016/j.tvjl.2010.04.006.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phllips. 2003. "The European ban on growth-promoting antibiotics and emerging consequenses for human and animal health." *Journal of Antimicrobial Chemotherapy* 52:159-161.

Castro, M. 2005. "Use of additives on the feeding of monogastric animals." *Cuban Journal of Agricultural Science* 39:439-445.

Costa, L. B., F. B. Luciano, V. S. Miyada, and F. D. Gois. 2013. "Herbal extracts and organic acids as natural feed additives in the pig diet." *South African Joural of Animal Science* 43 (2):181-193.

Cromwell, G. L. 2002. "Why and how antibiotics are used in swine production." *Animal biotechnology* 13 (1):7-27. doi: 10.1081/ABIO-120005767.

Cutler, R., and I. Gardner. 1988. *A Blue Print for Pig Health Research*. Canberra, Australia: Pig Research Council.

de Lange, C. F. M., John R. Pluske, J. Gong, and C. M. Nyachoti. 2010. "Strategic use of feed ingredients and feed addities to stimulate gut health and development in young pigs." *Livestock Science* 134:124-134. doi: 10.1016/j.livsci.2010.06.117.

Denyer, S. P., and J. -Y. Maillard. 2002. "Cellular impermeability and uptake of biocides and antibiotics in gram-negative bacteria." *Journal of applied Microbiology Symposium supplement* 92:35S-45S.

Domadia, P., S. Swarup, A. Bhunia, J. Sivaraman, and D. Dasgupta. 2007. "Inhibition of bacterial cell division protein FtsZ by cinnamaldehyde." *Biochemical Pharmacology* 74:831-840.

Fairbrother, J. M., E. Nadeau, and C. L. Gyles. 2005. "*Escherichia coli* in postweaning diarrhoea in pigs: an update on bacterial types, pathogenesis, and prevention strategies." *Animal health research Reviews* 6 (1):17-39. doi: 10.1079/AHR2005105.

Falkowski, J. F., and F. X. Aherne. 1984. "Fumaric and citric acid as feed additives in starter pig nutrition." *Journal of Animal Science* 58:935-938.

Ferme, D., M. Malnersic, L. Lipoglavsek, C. Kamel, and G. Avgustin. 2008. "Effect of Sodium Monensin and Cinnamaldehyde on the growth and Phenotypic characteristics of *Prevotella bryantii* and *Orevotella ruminicola*." *Folia Microbiologia* 53 (3):204-208.

Fleckenstein, J. M., P. R. Hardwidge, G. P. Munson, D. A. Rasko, H. Sommerfelt, and H. Steinsland. 2010. "Molecular mechanisms of enterotxigenic *Escherichia coli* infection." *Microbes and infection* 12:89-98. doi: 10.1016/j.micinf.2009.10.002.

Gong, J., H. Yu, T. Liu, M. Li, W. Si, C. F. M. de Lange, and C. Dewey. 2008. "Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract." *Livestock Science* 116:318-322.

Grilli, E., M. R. Messina, M. Tedeschi, and A. Piva. 2010. "Feeding a microincapsulated blend of organic acids and nature identical compounds to weaning pigs improved growth performance and intestinal metabolism." *Livestock Science* 133:173-175. doi: 10.1016/j.livsci.2010.06.056.

Halas, D. 2009. "Management and nutritional approaches to reduce post-weaning diarrhoea and improve production in weaner pigs, with an emphasis on weaning age, diet composition, and selected food additives." Ph. D., Facutly of Health Sciences, Murdoch University.

Hansen, C. F., A. L. Riis, S. Bresson, O. Høyjbjerg, and B. B. Jensen. 2007. "Feeding organic acids enhances the barrier function against pathogenic bacteria of the piglet stomach." *Livestock Science* 108:206-209. doi: 10.1016/j.livsci.2007.01059.

Heo, J. M., F. O. Opapeju, J. R. Pluske, J. C. Kim, D. J. Hampson, and C. M. Nyachoti. 2012. "Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds." *Journal of Animal Physiology and Animal Nutrition* 97:201-237. doi: 10.1111/j.1439-0396.2012.01284.x.

Højberg, O., N. Canibe, H. Damgaad, M. S. Hedemann, and B. B. Jensen. 2005. "Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets." *Applied and environmental microbiology* 71 (5):2267-2277. doi: 10.1128/AEM.71.5.2267-2277.2005.

Hopwood, D. E., and D. J. Hampson. 2003. "Interactions between the intestinal microflora, diet and diarrhoea, and their influences on piglet health in the immidiate post-weaning period." In *Weaning the pig: Concepts and consequences*, edited by J. R. Pluske, J. Le Dividich and M. W. A. Verstegen, 199-218. United Kingdom: Nottingham University Press.

Jongbloed, A. W., Z. Mroz, R. Van der Weij-Jongbloed, and P. A. Kemme. 2000. "The effects of microbialphytase, organic acids and their interaction in diets for growing pigs." *Livestock Production Science* 67:113-122.

Kim, J. C., C. F. Hansen, B. P. Mullan, and J. R. Pluske. 2012. "Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract." *Animal Feed Science and Technology* 173:3-16. doi: 10.1016/j.anifeedsci.2011.12.022.

King, D. E. 2003. "Gastrointestinal disorders in neonatal pigs." In *Gastrointestinal Physiology and Nutrition*, edited by R. J. Xu and P. D. Cranwell, 275-308. United Kingdom: Nottingham University Press.

Knarreborg, A., N. Miquel, T. Granli, and B. B. Jensen. 2002. "Establishment and application of an in vitro methodology to study the effects of organic acids on coliform and lactic acid bacteria in the proximal part of the gastrointestinal tract of piglets." *Animal Feed Science and Technology* 99:131-140.

Langhout, P. 2000. "New feed additives for broiler chickens." *World poultry* 16 (3):22-27.

Li, B. T., A. G. Van Kessel, W. R. Caine, S. X. Huang, and R. N. Kirkwood. 2001. "Small intestinal morphology and bacterial populations in ileal digesta and feces

of newly weaned pigs receiving a high dietary level of zinc oxide." *Canadian Journal of Animal Science* 81:511-516.
Lusk, J. L., F. B. Norwood, and J. R. Pruitt. 2006. "Consumer demand for a Band on antibiotic drug use in pork production." *American Journal of Agricultural Economics* 88 (4):1015-1033.
Manzanilla, E. G., J. F. Perez, J. F. Martin, C. Kamel, F. Baucells, and J. Gasa. 2004. "Effect of herbal extracts and formic acid on intestinal equilibrium of early-weaned pigs." *Journal of Animal Science* 82:3210-3218.
Michiels, J., J. Missotten, D. Fremaut, S. De Smet, and N. A. Dierick. 2007. "*In Vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora." *Livestock Science* 109:157-160. doi: 10.1016/j.livsci.2007.01.132.
Michiels, J., J. Missotten, D. Fremaut, S. De Smet, and N. A. Dierick. 2009. "*In Vitro* characterisation of the antimicrobial activity of selected essential oil components and binary combinations against the pigs gut flora." *Animal Feed Science and Technology* 151:111-127. doi: 10.1016/j.anifeedsci.2009.01.004.
Nagy, B., and P. Z. Fekete. 1999. "Enterotoxigenic *Escherichia coli* (ETEC) in farm animals." *Veterinary Research* 30:259-284.
Namkung, H., M. Li, J. Gong, H. Yu, M. Gottrill, and C. F. M. De lange. 2004. "Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs." *Canadian Journal of Animal Science* 84 (4):697-704.
Overland, M., N. P. Kjos, M. Borg, and H. Sørum. 2007. "Organic acids in diets for entire male pigs." *Livestock Production Science* 109:170-173.
Partanen, K., T. Jalava, J. Valaja, S. Perttilä, H. Siljander-Rasi, and H. Lindberg. 2001. "Effect of dietary carbadox or formic acid and fibre level on ileal and faecel nutrient digestibility and microbial metabolite concentrations in ileal digesta of the pig." *Animal Feed Science and Technology* 93:137-155.
Partanen, K., M. Karhapää, and H. Siljander-Rasi. 2006. "Performance, carcass quality, and gastric alterations in fattening pigs fed additives containing formic acid either coated with sorbate or mixed with lactic acid." *Agriculture and food science* 15:324-339.
Partanen, K., H. Siljander-Rasi, J. Pentikainen, S. Pelkonen, and M. Fossi. 2007. "Effects of weaning age and formic acid-based feed additives on pigs from weaning to slaughter." *Archieves of Animal Nutrition* 61 (5):336-356. doi: 10.1080/17450390701556866.
Piva, A., and E. Grilli. 2007. "Role of Benzoic, Lactic and Sorbic acids in *In Vitro* Swine cecal Fermentation." *Veterinary Research Communication* 31:401-404. doi: 10.1007/s11259-007-0066-8.
Pluske, J. R. 2013. "Feed- and feed additives-related aspects of gut health and development in weanling pig." *Journal of Animal Science and Biotechnology* 4:1-7.
Pluske, J. R., D. W. Pethick, D. E. Hopwood, and D. J. Hampson. 2002. "Nutritional influences on some major enteric acterial diseases of pigs." *Nutrition Research reviews* 15:333-372. doi: 10.1079/NRR200242.
Riemensperger, A. V., D. Bachinger, S. Schaumberger, R. Urbaityte, and S. Pasteiner. 2012. "The effect of an organic acid blend, cinnamaldehyde and a permeabilising substance on the inhibition of bacterial growth in vitro and

growth performance of weaning pigs." *Veterinary Medicine Zootechnika* 60 (80):59-66.
Smith, M. G., D. Jordan, T. A. Chapman, J. J. -C. Chin, M. D. Barton, T. N. Do, and V. A. Fahy. 2010. "Antimicrobial resistance and virulance gene profiles in multi-drug resistant enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhoe." *Veterinary Microbiology* 145:299-307. doi: 10.1016/j.vetmic.2010.04.004.
Tokach, M. D., S. S. Dritz, R. D. Goodband, and J. L. Nelssen. 2003. "Nutritional requirements of the weaned pig." In *Weaning of the pig: Concepts and Consequences*, edited by J. R. Pluske, J. Le Dividich and M. W. A. Verstegen, 259-299. United Kingdom: Notthingham University Press.
Tsiloyiannis, V. K, S. C. Kyriakis, J. Vlemmas, and K. Sarris. 2001. "The effect of organic acids on the control of post-weaning oedema disease of piglets." *Research in Veterinary Science* 70:281-285.
Vaara, M. 1992. "Agents tha increase the permeability of the outer membrane." *Microbiological reviews* 56 (3):395-411.
Vondruskova, H., R. Slamova, M. Trckova, Z. Zraly, and I. Pavlik. 2010. "Alternative to antibiotic growth promoters in prevention of diarrrhoea in weaned piglets: a review." *Veterinarni Medicina* 55:199-224.
Wegener, H. C. 2003. "Antibiotics in animal feed and their role in resistance development." *Current opinion in Microbiology* 6:438-445. doi: 10.1016/j.mib.2003.09.009.
Wellock, I. J., P. D. Fortomaris, J. G. M. Houdijk, and I. Kyriazakis. 2007. "Effect of weaning age, protein nutrtion and enterotoxigenic *Escherichia coli* challenge on the health of newely weaned piglets." *Livestock Science* 108:102-105. doi: 10.1016/j.livsci.2007.01.004.
Williams, I. H. 2003. "Growth of the weaned pig." In *Weaning the pig: Concepts and concequenses*, edited by J. R. Pluske, J. Le Dividich and WM. W. A. Verstegen, 17-36. Netherland: Wageningen Academic Publishers.
Yan, L., and I. H. Kim. 2012. "Effect of eugenol and cinnamaldehyde on the growth performance, nutrient digestibility, blood characteristics, faecal microbial shedding and fecal noxious gas content in growing pigs." *Asian-Australian Journal of Animal Science* 25 (8):1178-1183.
Yan, L., J. P. Wang, H. J. Kim, Q. W. Meng, X. Ao, S. M. Hong, and I. H. Kim. 2010. "Influence of essential oil supplementation and diets with different nutrient densities on growth performance, nutrient digestibility, blood characteristics, meat quality and fecal noxious gas content in grower-finisher pigs." *Livestock Science* 128:115-122. doi: 10.1016/j.livsci.2009.11.008.
Zhang, B., and Y. Guo. 2009. "Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning pigs." *British Journal of Nutrition* 102:687-693. doi: 10.1017/S0007114509289033.
Zhang, Y. Q., and R. J. Xu. 2003. "Anatomy and histology of the gastrointestinal tract." In *The Neonatal Pig: Gastrointestinal Physiology and Nutrition*, edited by R. J. Xu and P. Cranwell, 1-30. United Kingdom: Notthingham University Press.
Zimmerman, J. J., L. A Karriker, A. Ramirez, K. J. Scwartz, and G. W. Stevenson. 2012. *Diseases of Swine*. 10th ed. Iowa: John Wiley & Sons, inc. .

Assessment of diets supplemented with a mixture of an organic acid blend, cinnamaldehyde and permeabilising substance or ZnO in relation to their effects on post-weaning diarrhoea and performance in weaned piglets experimentally infected with enterotoxigenic *E. coli*.

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Abstract: The effects of feeding a diet supplemented with organic acids, cinnamaldehyde and a permeabilising substance (OACP), or zinc oxide (ZnO), on post-weaning diarrhoea (PWD) and performance in pigs infected with enterotoxigenic *E. coli* (ETEC) were examined. Pigs (n=72) weaned at 22 ± 1 days of age (7.2 ± 1.02 kg; mean ± SD) were stratified into three diets (n=24) in pens of four. Treatments comprised: (i) base diet (control, no antimicrobials), (ii) base diet + 3 g/kg ZnO, and (iii) base diet + 1.5 g/kg OACP, fed on *ad libitum* for 3 weeks. All pigs were infected with ETEC (9 mL, 1.03×10^{10} CFU/mL, serotype O149:K98:K88) on days 4, 5 and 6. The prevalence of PWD was lower in pigs fed ZnO (P=0.026). Pigs fed ZnO had higher daily gain (P=0.013) and feed intake (P=0.004) than control- or OACP-fed pigs in the 21-day study, with no difference (P>0.05) between control- or OACP-fed pigs. Feed conversion ratio was similar (P>0.05) for all diets. Faecal F4 *E. coli* numbers increased from day 4 to day 11 (P=0.003), with no difference between treatments (P=0.630) or other bacterial populations (P=0.005). Plasma urea was higher on day 4 than d 11 (P=0.001) with no differences between treatments (P=0.220). Plasma albumin and haptoglobin were unaffected by time or treatment (P>0.05). C-reactive protein levels were higher on day 11 than day 4 (P=0.010), and ZnO-fed pigs had lower levels compared to other pigs (P=0.039). No differences between days or treatments were found for blood cell counts (P>0.05). Pigs fed diet OACP had more short-chain fatty acids in their faeces (P=0.029). These data failed to show a decrease in PWD and no increase in performance for pigs supplemented with OACP compared to pigs not supplied with antimicrobials, whilst pigs fed ZnO performed best with less PWD.

Introduction

Weaning under current commercial conditions is stressful for the piglet due to multifactorial nutritional, environmental and physiological factors (Pluske 2013), which may decrease the voluntary feed intake, rendering the piglets more susceptible to gastrointestinal diseases and dysfunction (Heo et al. 2012). Post weaning diarrhoea (PWD) is associated with the proliferation of enterotoxigenic β- haemolytic strains of *Escherichia coli* (*E. coli*) (Wellock et al. 2007), and can be one of the most frequent causes of heavy economic loss in the pig herd (Vondruskova et al. 2010). Traditionally, dietary antibiotics and (or) mineral compounds such as zinc oxide (ZnO) and (or) copper sulphate ($CuSO_4$) have been used to control PWD (Gong et al. 2008). Due to concerns of antibiotic resistance strains of the bacteria, the European Union placed a ban on use of in-feed antibiotic growth promoters (AGP) in 2006 (Riemensperger et al. 2012), and there is pressure in other parts of the world to reduce or eliminate in-feed AGP (Lusk, Norwood, and Pruitt 2006), as well as environmental concerns for accumulation of minerals such as zinc (Pluske 2013). Due to this and the increasing concern amongst consumers in relation to use of antibiotics in pig production (Lusk, Norwood, and Pruitt 2006), research has focused on developing alternative strategies whilst maintaining animal health and performance.

The inclusion of organic acids in weaner diets has been reported to help with the control of PWD and growth performance (Busser et al. 2011). Acetic, formic and propionic acids have a direct effect on the wall of Gram-negative bacteria by decreasing pH in the digestive tract, thus inhibiting replication of deoxyribonucleic acid (Castro 2005). Formic acid has been found to decrease colonisation of enterobacteria in the lower part of the gastrointestinal tract (GIT) (Hansen et al. 2007), and significantly increased growth efficiency when supplemented to market pigs (Partanen et al. 2007) and having a positive effect on controlling PWD (Tsiloyiannis et al. 2001). Propionic acid has also displayed positive effects on controlling PWD and a general improvement in performance (Tsiloyiannis et al. 2001). Furthermore acetic acid, when fed in a combination with formic, phosphoric and citric acid, was found to have positive effects on performance (Namkung et al. 2004).

Essential oils (EO) are phytochemicals acquired from plant material (Yan et al. 2010) that have disease preventative properties (Riemensperger et al. 2012), and are the

plants' natural defence mechanism against predators and pathogens (Costa et al. 2013). Cinnamaldehyde is an essential oil, which has been found to be effective against coliform bacteria whilst hardly affecting lactobacilli *in vitro*. In an *in vivo* trial by Yan and Kim (2012), a decrease in faecal *E. coli* concentrations was observed with no effect on the concentration of faecal lactobacilli.

Organic acids and EO have different modes of action to AGP, and it is therefore unlikely that one alone is going to able to substitute AGP to control PWD. A combination of an organic acid blend and EO would give a broader spectrum of activity as organic acids exert their activity in feed and upper part of the GIT whilst EO exert their activity more in the distal part of the GIT (Langhout 2000).

Gram-negative bacteria, such as *E. coli*, possess a cell wall in addition to their cell membrane, which prevents toxic compounds from entering the bacteria and destroying vital functions (Riemensperger et al. 2012). A permeabilising substance can disturb this cell wall, making the bacteria more susceptible to toxic compounds. This was confirmed by Riemensperger et al. (2012) in an *in vitro* study, and when repeated *in vivo* showed a significant increase in growth performance.

The hypothesis tested in this experiment was that feeding a diet to pigs after weaning supplemented with a blend of organic acids (propionic, formic and acetic), cinnamaldehyde and a permeabilising substance will decrease the prevalence of PWD and increase growth performance following infection with enterotoxigenic *E. coli*. This product was examined against a diet containing ZnO, which is still commonly used in the post-weaning period to mitigate disease and improve performance.

Materials and methods

This study was reviewed and approved by the Animal Ethics Committee of Murdoch University (R2631/14). Animals were handled according to the Australian code of Practice for the care and use of animals for scientific purpose (NHMRC 2013).

Animals, Experimental design, Diets and Housing

A total of 72 entire male pigs (Large White × Landrace) weighing 7.2 ± 1.02 kg (mean ± SD) and weaned at 22 ± 1 days of age were used. The pigs were obtained from a commercial farm (Waroona, Western Australia) on the day of weaning and transported

to an experimental animal facility at Murdoch University. Due to supply issues, piglets in arrived in two batches 3 days apart. On arrival, the pigs were weighed and faecal rectal swabs were taken and cultured for baseline presence of β-haemolytic *E. coli*. Pigs were randomly allocated to their experimental diet treatment in individual pens of four according to a completely randomised block distribution and live weight (6 pens per treatment).

Treatments were three different diets: (i) a base diet without any antimicrobial compounds (Control), (ii) the base diet added with 3 g/kg ZnO (to represent current industry practice), and (iii) the base diet added with 1.5 g/kg mixture of organic acids, cinnamaldehyde, and permeabilising substances (OACP; Biotronic Top 3®, Biomin Australia Pty Ltd, Carlingford, Australia). The base diet comprised mainly of wheat, soybean meal, barley and whey and was formulated to meet the animals' requirements according to National Research Council (2012) (10.4 MJ NE/kg, 0.9 g standardised ileal digestible lysine/MJ DE). Diet compositions and analysed nutrient contents are presented in Table 1. The diets, along with water, were offered on an *ad libitum* basis for three weeks after weaning.

Pigs were kept in pens of metal wire-meshed construction with plastic flooring and with a space allowance of at least 0.6 m^2 per pig. Each pen was equipped with a nipple water drinker and a plastic feeding trough. Pens were in three different rooms, with 6 pens and 2 pens per treatment in each room. The ambient temperature was maintained at 26.3 ± 1.0 °C. Pigs were monitored twice daily and weighed weekly. Feed residue from each pen was weighed weekly and feed wastage recorded daily to calculate feed intake, gain and feed conversion ratio. Feed intake was measured as feed disappearance.

Induction of post weaning diarrhoea with enterotoxigenic Escherichia coli and Measurements of post weaning diarrhoe

Pigs were infected with an enterotoxigenic *E. coli* (ETEC, serotype O149:K98:K88; toxins LT, ST, and STb) on days 4, 5 and 6 after weaning. Inoculation cultures of ETEC were prepared as described by (Heo et al. 2009). All pigs were orally dosed, using mild constraint, with the inoculum via a drench gun to provide 9 mL aliquots of 1.03×10^{10} colony forming units (CFU)/mL of ETEC per pig on d 4, 5 and 6.

Table 1. Composition of experimental diets (g/kg, as-fed basis).

	Control	Zinc oxide	OACP[A]
Ingredient			
Barley	100.0	100.0	100.0
Wheat	492.2	489.2	490.7
Soybean meal	150.0	150.0	150.0
Blood meal	20.0	20.0	20.0
Fishmeal	84.1	84.1	84.1
Whey powder	100.0	100.0	100.0
Canola Oil	34.2	34.2	34.2
L-lysine	2.71	2.71	2.71
DL-methionine	2.30	2.30	2.30
L-threonine	1.30	1.30	1.30
L-tryptophan	0.13	0.13	0.13
Vitamin/Mineral premix[B]	1.0	1.0	1.0
Limestone	5.2	5.2	5.2
Dicalcium phosphate	4.4	4.4	4.4
Salt (NaCl)	2.0	2.0	2.0
Zinc Oxide	0.0	3.0	0.0
Choline Chloride 60%	0.4	0.4	0.4
Biotronic[C]	0.0	0.0	1.5
Calculated composition			
NE, MJ/kg	10.39	10.35	10.37
Protein	213	213	213
Fat	54	54	54
ND Fibre[E]	95	95	95
AD Fibre[F]	28	28	28
Calcium	9.0	9.0	9.0
Digestible phosphorus	4.5	4.5	4.5
Total lysine	14.1	14.1	14.1
SID[D] Lysine	13.5	13.5	13.5
SID Meth + Cysteine	8.1	8.1	8.1
SID Threonine	8.5	8.5	8.5
SID Tryptophan	2.4	2.4	2.4
SID Isoleucine	7.7	7.7	7.7
SID Leucine	14.8	14.7	14.8

[A]OACP: a mixture of organic acid blend, cinnamaldehyde and permeabilising substance.

[B]Provided the following nutrients (per kg of air-dried diet): vitamins: A, 7000 IU; D3, 1400 IU; E, 20 mg; K, 1 mg; thiamine, 1 mg; riboflavin, 3 mg; pyridoxine, 1.5 mg; cyanocobalamin, 15 μg; calcium pantothenate, 10.7 mg; folic acid, 0.2 mg; niacin, 12

mg; biotin, 30 μg. Minerals: Co, 0.2 mg (as cobalt sulfate); Cu, 10 mg (as copper sulfate); iodine, 0.5 mg (as potassium iodine); iron, 60 mg (as ferrous sulfate); Mn, 40 mg (as manganous oxide); Se, 0.3 mg (as sodium selenite); Zn, 100 mg (as zinc oxide); BJ Grower 1, BioJohn Pty Ltd., WA, Australia.

[C]Biotronic Top 3®, Biomin Australia Pty Ltd, Carlingford, Australia.

[D]SID; standardised ileal digestible

[E]ND fibre; neutral detergent fibre

[F]AD fibre; acid detergent fibre

Faecal swabs were taken on days 0, 3, 5, 7, 9 and 11 to assess faecal shedding of ETEC, by inserting a cotton swab into the anus. Swabs were inoculated on sheep blood (50 mL/L) agar plates (Path West Laboratories, WA). Plates were incubated overnight at 37 °C and assessed based on morphology and haemolysis. Scores were given on a six-point scale (from 0 to 5) according to number of streaked sections containing viable haemolytic *E. coli*, where 0 was no growth and 5 was growth out in the fifth section of the plate (Heo et al. 2009).

Faecal consistency and prevalence of diarrhoea of individual pigs were visually assessed daily, by the same person, for 21 days after weaning. A score between 1 and 4 was given, where (1) firm well formed faeces, (2) soft formed faeces, (3) soft and loosing shape or (4) watery liquid consistency where the latter was considered diarrhoea. To allow for statistical analysis the scores were converted into percentiles (1 = 0%, 2 = 33.3%, 3 = 66.7% and 4 = 100%). The diarrhoea index (DI) was calculated as the mean proportion of days pigs had diarrhoea with respect to 14 days after weaning (Heo et al. 2009). Faecal samples from eight "focus pigs" (medium weight pigs within the treatment group) were collected per treatment, on days 4 and 11 after weaning, for volatile fatty acid (VFA) analysis and DNA extraction for real time PCR. Samples were stored at -20°C until analysed.

Blood sampling

Blood samples were collected on d 4 and 11 from 2 pigs per pen (12 samples per treatment). Samples were collected via jugular vein puncture into either a lithium heparin or K_3EDTA coated tube. Samples were immediately placed on ice. The heparin tube was centrifuged at 3,000 × g for 10 min at room temperature. Plasma was then collected and stored at -20°C until analysed for plasma urea nitrogen (PUN), haptoglobin, albumin and C-reactive protein contents. Whole blood samples collected in K_3EDTA tube were subjected to blood cell count assessment on the same day.

Analytical methods

Diet samples were analysed for dry matter, gross energy, crude protein, crude fibre, neutral detergent fibre, acid detergent fibre, and zinc. Dry matter content was determined using AOAC official method 930.15 (AOAC 1997)). The N content was

determined using combustion method 990.03 (AOAC 1997) and crude protein content was calculated as N content × 6.25. Crude fat content was determined using AOAC official method 2003.06 (AOAC 1997). The NDF and ADF contents were determined using the AOAC official methods 925.10 (AOAC 1997). Gross energy content was determined using a Ballistic Bomb Calorimeter (SANYO Gallenkamp, Loughborough, UK). Zinc content was determined using inductively coupled atomic emission spectroscopy. The pH levels in the feeds were assessed by preparing a 1:9 feed in water solution, which were then measured using a pH metre (ROSS Ultra® pH/ATC Triode®, Thermo Fisher Scientific inc.).

Faecal samples were analysed for volatile fatty acid (VFA) content using gas chromatography. Samples were prepared as described by (Kim et al. 2009), except that the thawed digesta samples were diluted 1:2 (w/v). Plasma urea nitrogen (PUN), haptoglobin and albumin contents were determined using a Beckman Coulter/Olympus Reagent Kit (OSR6134), an in-house method NTM-62 based on (Eckersall et al. 1999) and Randox Ranbut Reagent kit (RB1007), respectively. All kits and methods were performed on an Olympus AU400 Clinical Chemistry Analyser at the Department of Agriculture and Food, Animal Health Laboratories (South Perth, WA). C-reactive protein levels were determined by the use of porcine ELISA kits (R&D Systems, catalogue # DY2648). Whole blood cell count was done using an automatic haematology analyser (ADIVA 2120, Bayer Healthcare, Siemens, Germany).

DNA extraction and quantitative real time polymerase chain reaction

Faecal samples were extracted for DNA and processed using quantitative real time polymerase chain reaction (qPCR) to screen for relative total counts of microbiota, *E. coli, E. coli* with F4 fimbria (the specific *E. coli* used for oral infection in this experiment), *Enterobacteriaceae spp.* and *Lactobacillus spp.* The QIAamp® DNA Stool Mini Kit (Qiagen) was used to extract DNA frozen stool sample then stored at -20 °C until qPCR was performed. Primers and probes used are presented in Table 2.

Total counts of *E. coli, Enterobacteriaceae spp.* and *Lactobacillus spp.* qPCR were prepared using RT-PCR buffer and enzyme mix (Applied Biosystems, Foster City USA) and were first set for 95 °C for 10 minutes, then cycled 40 times at 95 °C for 15 seconds and 65 °C for 30 seconds. The *E. coli with* F4 fimbria qPCR were prepared

using SYBR green Low ROX (Applied Biosystems, Foster City, USA) were first set for 95 °C for 10 minutes, then cycled 40 times at 95 °C for 15 seconds, 65 °C for 30 seconds and 72 °C for 60 seconds. Samples were run and quantified on ViiA7 PCR machine (Applied Biosystems, Foster City, USA), and number of bacteria was expressed as bacteria per gram of faeces.

Statistical analysis

Statistical analyses of production data were performed using a one-way ANOVA in SPSS (Version 21, IBM Corporation) with dietary treatment as the independent variable, and batch as a random factor (to account for a difference in start weight between batches of pigs). Plasma measures, faecal VFA and faecal bacterial count measured on days 4 and 11 were analysed by repeated-measures ANOVA. Pen was used as the experimental unit for performance, faecal score, faecal ETEC excretion, and diarrhoea index. Pig was used as the experimental unit for plasma measurements, blood cell counts, faecal VFA concentration, and qPCR. Statistical significance was accepted at $P<0.05$ and $0.05<P<0.10$ was considered a trend.

Table 2. Primers and probes used for quantitative real time PCR.

Target	Primer/probe	Reference	PCR product length (bp)
Total *E. coli*	Forward	Bartosch et al. (2004)	195
	Reverse		
	Probe	Designed by Y. Chen, EMAI (unpublished)	
Enterobacteriaceae spp.	Forward	Bartosch et al. (2004)	195
	Reverse		
	Probe	Designed by Y. Chen, EMAI (unpublished)	
Lactobacillicus spp.	Forward	Walter et al. (2001)	190
	Reverse	Modified from Heilig et al. (2002)	
	Probe	Modified from Delroisse et al. (2008)	
E. coli F4 fimbria	Forward	Franklin et al. (1996)	764

Results

One animal was removed from the trial prior to the ETEC challenge due to ill thrift.

Diets

Analysed diet composition did not greatly vary from calculated values. As expected, the Zn concentration was lower in Control and OACP diets (Table 3). The pH levels were as expected, with the ZnO diet having the highest pH, and the OACP diet having the lowest pH.

Table 3. Analysed composition of diets (g/kg as-fed basis).

Analysed composition	Control	Zinc oxide	OACP[A]
Dry matter	926.5	925.7	926.0
Gross energy, MJ/kg	17.56	17.26	17.64
Protein	221.10	217.80	224.10
Crude fibre	19.50	19.70	18.10
NDF	104.30	89.20	88.20
ADF	33.60	32.7	30.90
Zinc	0.22	2.55	0.22
pH	6.26	6.63	6.20

[A]OACP: a mixture of organic acid blend, cinnamaldehyde and permeabilising substance.

Incidence and severity of post weaning diarrhoea and shedding of enterotoxigenic Escherichia coli

Approximately 4% of pigs fed ZnO had PWD during the 3 weeks after weaning, and it was higher for pigs fed OACP or control diets (45% and 33%, respectively). This correlated with the DI, it being lower in the pigs fed ZnO compared to pigs fed either the OACP or the control diet (P=0.026). A trend for ZnO having a higher *E. coli* score than the other 2 treatments was found on day 7 (P=0.067). For all other days there was no difference between treatments (P>0.1;Table 4).

Performance data

Pigs fed ZnO diet were heavier than pigs fed the control diet on days 14 (P=0.024) and 21 (P=0.038), with pigs fed the diet with OACP being the same as the other two groups (P>0.05). Daily gain was higher in pigs fed ZnO compared to the control group for the first (P=0.008) and the overall 3-week period after weaning (P=0.013). Daily gain was higher for pigs fed ZnO or OACP compared to control group in the second week.

Table 4. The effects of dietary treatment on post weaning diarrhoea (PWD), the diarrhoea index and *E.coli* score in pigs challenged with *E. coli* on d 4, 5 and 6 after weaning

Treatment[A]	Control	ZnO	OACP	SEM	*P-value*
No. pigs with PWD[B]	8/24	1/23	11/24		
% pigs with PWD	33.0	4.0	45.0		
Diarrhoea index (%)[C]	5.06[a]	0.62[b]	6.25[a]	1.526	0.026
E.coli score[D]					
d 0	0.042	0.304	0.250	0.114	0.225
d 3	0.125	0.087	0.042	0.059	0.594
d 5	1.500	1.740	1.710	0.332	0.854
d 7	1.460	2.090	1.170	0.283	0.067
d 9	0.380	0.220	0.380	0.177	0.766
d 11	0.420	0.170	0.380	0.140	0.424
Overall	0.653	0.768	0.653	0.109	0.686

[ab] Means within a row with different superscript are significantly different ($P<0.05$).

[A] Treatment: Control, ZnO: Control + 3000 ppm added zinc oxide, OACP: Control + 1.5 g/kg mixture of organic acid blend, cinnamaldehyde and a permeabilising substance (Biotronic Top 3®, Biomin Australia Pty, Ltd, Carlingford, Australia).

[B] Post weaning diarrhoea was defined as pigs having a faecal consistency score 4.

[C] The mean proportion of days with diarrhoea with respect to 14 days after weaning.

[D] Agar plates were scored from 0 – 5 according to number of streaked sections containing viable haemolytic *E. coli*, where 0 was no growth and 5 was growth out in the fifth section of the plate

Abbreviations used: SEM standard error of the mean, PWD Post weaning diarrhoea

Table 5. The effects of different starting weaner diets on performance in pigs experimentally infected with enterotoxigenic *E. coli.*

Treatment[A]	Control	ZnO	OACP	SEM	*P - Value*
LW, kg					
d 0	7.29	7.17	7.24	0.106	0.761
d 7	9.06	9.85	9.43	0.253	0.122
d 14	12.07[a]	13.58[b]	13.00[ab]	0.343	0.024
d 21	16.34[a]	18.20[b]	17.38[ab]	0.456	0.038
ADG, g/d					
d 0-7	253[a]	381[b]	312[ab]	24.3	0.008
d 8-14	430[a]	533[b]	510[b]	21.3	0.011
d 15-21	611	661	626	26.7	0.421
d 0-21	431[a]	525[b]	483[ab]	19.1	0.013
ADFI, g/d					
d 0-7	336[a]	454[b]	390[ab]	21.8	0.007
d 8-14	543[a]	725[b]	668[b]	33.6	0.006
d 15-21	835	942	871	28.9	0.057
d 0-21	571[a]	707[b]	643[ab]	23.6	0.004
FCR, kg/kg					
d 0-7	1.34	1.18	1.26	0.059	0.218
d 8-14	1.25	1.36	1.30	0.045	0.282
d 15-21	1.37	1.43	1.39	0.039	0.515
d 0-21	1.32	1.34	1.33	0.029	0.847

[ab]Means within a row with different superscript are significantly different (P<0.05).

[A]Treatment: Control, ZnO: Control + 3000 added ppm zinc oxide, OACP: Control + 1.5 g/kg mixture of organic acid blend, cinnamaldehyde and a permeabilising substance (Biotronic Top 3®, Biomin Australia Pty, Ltd, Carlingford, Australia).

Abbreviations used: LW=live weight, SEM=standard error of the mean, ADG=Average daily gain, ADFI=Average daily feed intake, FCR=Feed conversion ratio

(P=0.011). No differences were found for the third week after weaning (P=0.421) (Table 5).

Pigs fed the ZnO diet consumed more food compared to the control group for week one (P=0.007), and the overall 3-week period after weaning (P=0.004). Pigs fed ZnO or OACP were both consuming more food compared to the control group for week 2 (P=0.006). For week three after weaning a trend was found for pigs fed ZnO to consume more food than the control group (P=0.057). There was found no difference in feed efficiency (P>0.05) between treatment groups (Table 5).

Quantitative real time PCR

No interaction was detected between day and treatment for *E. coli* count. The amount of *E. coli* F4 fimbria increased from d 4 to d 11 (P=0.003) and there was no difference between treatments (P=0.630). For all other bacterial populations there was no difference between treatments or days (P>0.005) (Table 6).

Blood metabolites, immune indices and C-reactive protein

The levels of urea decreased from d 4 to d 11 (P=0.001), however there was no difference between treatments (P=0.220). There was no difference over time or between treatments in levels of haptoglobin or albumin (P>0.05). The levels of C-reactive protein increased from d 4 to d 11 (P=0.010), with pigs fed ZnO having lower levels compared to pigs fed control or OACP diet (P=0.039) (Table 7). There was no difference between days or between treatments for levels of haemoglobin, lymphocytes, neutrophils or the lymphocyte to neutrophil ratio (P>0.05) (Table 8).

Changes in VFA composition

Pigs fed the OACP diet had the higher levels of total VFA compared to pigs fed ZnO or the control diet (P=0.013), however there was no difference between days (P=0.475). The percentage of SCFA (sum of acetic, propionic and butyric acids) was higher on d 11 compared to d 4 (P=0.016), and pigs fed the ZnO diet had higher levels compared to pigs fed OACP or control diet (P=0.029). The BCFA (sum of valeric, caproic, isobutyric and isovaleric acids) percentage decreased from d 4 to d 11 (P<0.001), with pigs fed ZnO having a lower percentage compared to OACP and the control diet.

Table 6. Changes in selected bacterial populations in pigs experimentally infected with enterotoxigenic *E. coli* and fed different diets after weaning.

	Treatment[A]				*P - Value*		
	Control	Zinc Oxide	OACP	SEM	Day	Treatment	Day × Treatment
Total E. coli, log_{10} g/faeces							
d 4	7.69	7.37	7.99	0.136	0.794	0.344	0.837
d 11	7.54	7.48	7.87				
E. coli F4 fimbria[B], log_{10} g/faeces							
d 4	5.55	5.20	5.70	0.198	0.002	0.630	0.130
d 11	5.93	7.06	6.66				
Enterobacteriaceae spp., log_{10} g/faeces							
d 4	8.61	8.36	8.57	0.128	0.715	0.952	0.683
d 11	8.46	8.52	8.34				
Lactobacillus spp., log_{10} g/faeces							
d 4	7.12	7.36	7.49	0.061	0.115	0.364	0.066
d 11	7.42	6.78	6.99				

[A]Treatment: Control, ZnO: Control + 3000 ppm added zinc oxide, OACP: Control + 1.5 g/kg mixture of organic acid blend, cinnamaldehyde and a permeabilising substance (Biotronic Top 3®, Biomin Australia Pty, Ltd, Carlingford, Australia).

[B] Specific *E. coli* used for oral infection in the experiment.

Abbreviations used: SEM=standard error of the mean

Table 7. Changes in selected blood metabolites in pigs experimentally infected with enterotoxigenic *E. coli* and fed different diets after weaning.

			Treatment[A]				P - Value		
	d 4	d 11	Control	Zinc Oxide	OACP	SEM	Day	Treatment	Day × Treatment
Haptoglobin, mg/mL									
d 4	-	-	1.22	0.98	1.33	0.086	0.325	0.210	0.285
d 11	-	-	1.36	0.84	0.92				
PUN, mmol/L									
d 4	-	-	3.28	3.10	2.70	0.109	0.001	0.220	0.435
d 11	-	-	2.80	2.38	2.42				
Albumin, mmol/L									
d 4	-	-	0.028	0.023	0.027	0.001	1.000	0.457	0.267
d 11	-	-	0.026	0.026	0.025				
CRP, μg/mL	9.4	15.3	15.1[a]	6.5[b]	14.5	1.412	0.010	0.039	0.030

[ab] Means within a row not having the same superscript are significantly different (P<0.05).

[A] Treatment: Control, ZnO: Control + 3000 ppm added zinc oxide, OACP: Control + 1.5 g/kg mixture of organic acid blend, cinnamaldehyde and a permeabilising substance (Biotronic Top 3®, Biomin Australia Pty, Ltd, Carlingford, Australia).

Abbreviations used: SEM=standard error of the mean, CRP=C-reactive protein, PUN= Plasma urea nitrogen

Table 8. Changes in haemoglobin and selected white blood cell counts for indices of immune activation in pigs experimentally infected with enterotoxigenic *E. coli* and fed different diets after weaning.

	Treatment				*P-value*		
	Control	Zinc Oxide	OACP	SEM	Day	Treatment	Day × Treatment
Haemoglobin, %							
d 4	114.6	109.1	106.9	1.320	0.853	0.561	0.096
d 11	108.4	111.9	109.3				
Lymphocyte, %							
d 4	45.85	43.05	45.07	1.271	0.701	0.378	0.104
d 11	37.66	46.60	46.99				
Neutrophil, %							
d 4	45.81	46.91	46.18	1.483	0.708	0.938	0.135
d 11	54.29	43.46	44.06				
Lymphocyte:Neutrophil ratio, %/%							
d 4	1.132	1.292	1.158	0.074	0.794	0.651	0.372
d 11	1.523	0.958	1.009				

[A] Treatment: Control, ZnO: Control + 3000 ppm added zinc oxide, OACP: Control + 1.5 g/kg mixture of organic acid blend, cinnamaldehyde and a permeabilising substance (Biotronic Top 3®, Biomin Australia Pty, Ltd, Carlingford, Australia).

Abbreviations used: SEM standard error of the mean

Table 9. Changes in volatile fatty acid composition in pigs experimentally infected with enterotoxigenic *E. coli* and fed different diets after weaning.

			Treatment[A]				*P - Value*		
	Day 4	Day 11	Control	Zinc Oxide	OACP	SEM	Day	Treatment	Day × Treatment
Total VFA, mmol/kg	50.4	55.1	49.7[a]	49.1[a]	61.1[b]	1.565	0.475	0.013	0.832
SCFA, %[B]	88.0	91.2	88.2[a]	91.4[b]	88.3[a]	0.532	0.016	0.029	0.861
BCFA, %[C]	12.0	4.9	9.6[a]	6.9[b]	9.6[a]	0.422	0.000	0.021	0.709
Acetic acid (%)	51.9	54.5	54.1	50.7	54.9	0.841	0.028	0.139	0.279
Propionic acid (%)	22.3	21.8	21.4	22.0	23.3	0.822	0.586	0.673	0.756
Butyric acid (%)	14.7	15.9	15.9	15.6	13.4	0.734	0.660	0.416	0.117
Isobutyric acid (%)	2.5	1.9	2.0	2.3	2.4	0.181	0.061	0.650	0.946
Valeric acid (%)	4.4	3.6	2.9[a]	4.9[b]	4.9[a]	0.240	0.064	0.003	0.578
Isovaleric acid (%)	3.9	2.6	3.0	3.5	3.6	0.328	0.024	0.683	0.929
Caproic acid (%)	1.2	0.6	0.7	1.1	1.0	0.099	0.017	0.344	0.943

[ab]Means within a row not having the same superscript are significantly different (P<0.05).

[A]Treatment: Control, ZnO: Control + 3000 ppm added zinc oxide, OACP: Control + 1.5 g/kg mixture of organic acid blend, cinnamaldehyde and a permeabilising substance (Biotronic Top 3®, Biomin Australia Pty, Ltd, Carlingford, Australia).

[B]Sum of acetic, propionic and butyric acids.

[C]Sum of valeric, caproic, isobutyric and isovaleric acids.

Abbreviations used: VFA volatile fatty acids, SCFA Short chain fatty acids, BCFA Branched chain fatty acids, SEM standard error of the

(P=0.021). Acetic acid increased from d 4 to d 11 (P=0.028) with no difference between treatments (P=0.139). There were higher levels of valeric acid for pigs fed ZnO or OACP (P=0.003), and a trend for a decrease from d 4 to d 11 (P=0.064). Isovaleric and caproic acid both decreased from d 4 to d 11 (P=0.024 and P=0.017 respectively), and there was no difference between treatments (P=0.683 and P=0.344). There was a trend for a decrease of isobutyric acid from d 4 to d 11 (P=0.061), and there was no difference in either day or treatment for propionic and butyric acid (P>0.05) (Table 9).

Discussion

The hypothesis tested in the present study was that feeding a diet supplemented with a blend of organic acids (propionic, formic and acetic), cinnamaldehyde and a permeabilising substance would decrease prevalence of PWD and increase growth performance of weanling pigs experimentally infected with ETEC.

Supplementing weaner diets with OACP did not decrease the prevalence of PWD, which does not support the hypothesis. The prevalence of PWD decreased only for the pigs supplemented with ZnO, as both the number of pigs with PWD and DI index were decreased. Although there was a decrease in the incidence of PWD for the ZnO-treated group the shedding of ETEC was similar for all treatment groups. An increase in ETEC shedding was seen on d 5, as expected, and returned to pre-infection levels d 9. These data support other studies (Li et al. 2001, Zhang and Guo 2009) suggesting that supplementation of ZnO supressed PWD and increased growth performance but was not related to the elimination of ETEC, but to an improved intestinal barrier and immune function. In a study by Roselli et al. (2003) it was concluded that ZnO protects cells from ETEC by the inhibition of bacterial adhesion and internalisation, preventing disruption of barrier integrity. This inhibition of adhesion reduces levels of endotoxins produced and therefore decreases the severity of PWD. This explains the decrease in DI but not in ETEC shedding for the ZnO treatment group. The equal levels of shedding between groups indicate that the level of infection was likely equal between groups. Pigs can also be resistant to PWD, as they don't express the receptors necessary for the *E. coli* to attach (Fairbrother, Nadeau, and Gyles 2005, Hopwood and Hampson 2003). Other pigs may have receptors that are only weakly adhesive, and the presence of receptors also vary between individuals within a litter (Hopwood and Hampson 2003). These factors will have a strong influence on the occurrence of PWD (Hopwood and Hampson 2003). As no genotype screening for these receptors was done at the start of the

trial, there is no indication if the pigs were distributed equally into treatment groups in relation to receptors.

Li et al. (2006) found that when supplementing 3 g/kg ZnO an increase in villus height in the upper small intestine was found. This improvement was accossiated with increase in growth rates and feed efficiency. It has also been suggested that reduced fermentation of digestible nutrients in the GIT, due to the supplementation of ZnO, might leave more energy available for the animal to utilise for growth, hence the increase in growth rates (Højberg et al. 2005). Findings in the present study of increased growth and feed intake for the ZnO treatment group is in agreement with other studies (Hill et al. 2001, Slade et al. 2011, Højberg et al. 2005, Li et al. 2006).

In the present study, supplementation with OACP gave statistically similar production performance as ZnO supplemented diets, however it was numerically lower. An abundance of research has been conducted on organic acids and their effects on performance in weanling pigs, and has been summarized by Partanen and Mroz (1999) and Mroz (2005), among others. Meta-analysis of the data show that organic acids improve growth performance, however the large variations exist due to factors such as form and type of organic acid, inclusion level, production of intraluminal SCFA, differences in amount of fermentable carbohydrate substrates in diet for bacterial growth, colonisation and activity leading to SCFA production, weaning age, presence or absence of bacterial receptors, and hygiene and welfare (Mroz 2005).

The decrease in PUN levels from d 4 to d 11 in the present study could be due to a decrease in microbial fermentation of nitrogenous compounds in the large intestine due to a decrease in proliferation of microbes. Catabolism of amino acids by microbes produces NH_3, which is converted to urea in the liver. The urea synthesised in the liver is either excreted as urine or diffused back into the caecum and combined into microbial nitrogen (Kim et al. 2008). A decrease in urea could also be linked to more efficient utilisation of dietary protein or decrease in protein breakdown (Shen et al. 2012). As the piglets get older (weigh more and overcome the post-weaning growth check) their protein requirements increase from 13.1 g/d (5-7 kg LW) to 23.1 g/df(7-11 kg LW) (National Research Council 2012). In the present study, only one diet phase was fed for the entire trial period; thus as piglets entered the higher weight bracket there would be an increase in utilisation (ie less excess N) as the requirements increased.

Acute phase proteins (APP) are proteins that are a part of the acute phase response, the early defence or innate immune system triggered by different stimuli such as trauma,

infection, stress and inflammation (Cray, Zaias, and Altman 2009). Acute phase proteins are divided into two categories, negative and positive APP, which increases or decreases with stimuli respectively. Haptoglobin, a positive APP, increases in concentration according to deteriorated health status, infection, inflammation or trauma (Eckersall et al. 1999). However no increase in haptoglobin levels was found in the present study. C-reactive protein is another positive APP and would therefore be expected to increase in levels between days, which was observed in this study. The difference between treatments observed in the present study would indicate that ZnO had a positive effect on the pigs compared to the control and OACP treatment, being less affected by the *E. coli* infection.

It has been established that APP have different induction sensitivities, and hence some react to a lesser extent than others to the same infection/inflammation (Heegaard et al. 2011). When measuring APP levels in pigs infected with ETEC, Houdijk et al. (2007) found that CRP concentrations increased more than 10-fold, whilst haptoglobin concentrations only increased by three-fold.

In this regard, the lack of any differences found in haemoglobin and white blood cell counts in the present study indicate that the overall level of infection was insufficient to cause a major health issue for the pigs. The lymphocyte to neutrophil ratio has been used previously as an indicator of the pigs' responses to stress (Sugiharto, Hedemann, and Lauridsen 2014), and the lack of any difference in the current study supports this notion.

The gut microbiota is important as it strongly effects physiological, developmental, nutritional and immunological processes of the pig, influencing both health and performance (Richards, Gong, and de Lange 2005, Metzler, Bauer, and Mosenthin 2005). It also helps protect the pig from colonisation of pathogens, as well as protection from overgrowth of non-pathogenic and pathogenic species (Richards, Gong, and de Lange 2005). None of the dietary treatments used in present trial had any major effect on the bacterial populations tested. For the group of pigs supplemented with OACP a decrease in *E. coli* and an increase in *Lactobacillus spp.* were expected as inclusion of organic acids in the diet decrease the intestinal pH, increase the proteolytic enzyme activity producing a more favourable environment for *Lactobacillus spp.* and supressing the proliferation of *E. coli* (Li, Jiang, and Ma 2003). However, this was not supported by our data. Højberg et al. (2005) found a decrease in *Lactobacilli* activity when piglets were supplemented with 2.5 g/kg dietary ZnO, and suggested that the influence of ZnO on the gut microbiota worked in similar ways as some AGP, reducing Gram-positive commensals rather than potentially pathogenic Gram-negative bacteria.

The increase in SCFA detected was as expected as the OACP diet was supplemented with organic acids. SCFA are carbohydrates with less than 6 carbon atoms, and are the major end products of bacterial fermentation reactions in the colon (Rossi et al. 2010). The addition of organic acids aids the acidification of the stomach, thus increasing proteolytic enzyme activity, which then may increase the digestibility of protein and amino acids (Metzler, Bauer, and Mosenthin 2005). The results from the pH test, showing a lower pH for the diet that was supplemented with OACP, should therefor lead to acidification of the digesta, which was observed. It has also been found that an increase in SCFA´s stimulates gut epithelial cell proliferation and villus size, hence increasing surface area for absorption (Richards, Gong, and de Lange 2005), and some studies have shown that increased production of SCFA can reduce bacterial numbers (Knarreborg et al. 2002, Naughton and Jensen 2001). However, the lack of any improvement in growth and feed conversion efficiency or any decrease in *E. coli* and *Enterobacteriaceae* numbers in the present study indicates that the increase in total SCFA levels in pigs fed OACP was unrelated to these measurements.

While this study explored many aspects and measurements in relation to production, immune status, gut health and microbiota populations of weaner pigs infected with *E. coli* fed varying diets some key limitations were evident. Data indicated that the infection model was severe enough as to ellicit a strong immune response (as measured by APP) thus the positive effect of OACP product would have been muted as the pathogen load was less that may be observed in commercial conditions thus differences between dietary treatments were less (ie not statistically different). Additionally, the present trial was limited in the number of piglets and pen replicates by the supply, facilities and time constraints. Further research should increase replicates so as to be able to detect smaller differences (increase the statistical power of the experiment).

Conclusion

In conclusion, this experiment demonstrated that feeding a diet supplemented with a blend of organic acids (propionic, formic and acetic), cinnamaldehyde and a permeabilising substance did not decrease the prevalence of PWD and did not increase growth performance of weanling pigs experimentally infected with enterotoxigenic *E. coli*.

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References

AOAC. 1997. *Official Methods of Analysis*. 16th ed. Washington DC: Association of Official Analytic Chemis.

Bartosch, S., A. Fite, G. T. Macfarlane, and M. E. T. McMurdo. 2004. "Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota." *Applied and Environmental Microbiology* 70 (6):3575-3581.

Busser, E. V. De, J. Dewulf, L. De Zutter, F. Haesebrouck, J.Callens, T. Meyns, W. Maes, and D. Maes. 2011. "Effect of administration of organic acids in drinking water on faecal shedding of E.*coli*, performance parameters and health in nursery pigs." *The Veterinary Journal* 188:184-188. doi: 10.1016/j.tvjl.2010.04.006.

Castro, M. 2005. "Use of additives on the feeding of monogastric animals." *Cuban Journal of Agricultural Science* 39:439-445.

Costa, L. B., F. B. Luciano, V. S. Miyada, and F. D. Gois. 2013. "Herbal extracts and organic acids as natural feed additives in the pig diet." *South African Joural of Animal Science* 43 (2):181-193.

Cray, C., J. Zaias, and N. H. Altman. 2009. "Acute phase response in animals: a review." *Comparative Medicine* 59 (6):517-526.

Delroisse, J. M., A. L. Bouulvin, I. Parmentier, R. D. Dauphin, M. Vandenbol, and D. Portelle. 2008. "Quantification of *Bifidobacterium spp.* and *Lactobacillus spp.* in rat fecal samples by real-time PCR." *Microbiological Research* 163 (6):663-670.

Eckersall, P. D., S. Duthie, S. Safi, D. Moffatt, N. U. Horadagoda, S. Doyle, R. Parton, D. Bennet, and J. L. Fitzpatrick. 1999. "An automated biochemical assay for haptoglobin: prevention of interference from albumin." *Comparative Haematology International* 9:117-124.

Fairbrother, J. M., E. Nadeau, and C. L. Gyles. 2005. "*Escherichia coli* in postweaning diarrhoea in pigs: an update on bacterial types, pathogenesis, and prevention strategies." *Animal health research Reviews* 6 (1):17-39. doi: 10.1079/AHR2005105.

Franklin, M. A., D. H. Francis, D. Baker, and A. G. Mathew. 1996. "A PCR-based method of detection and differentiation of K88+ adhesive *Escherichia coli*." *Journal of veterinary Diagnostic Investigation* 8 (4):460-463.

Gong, J., H. Yu, T. Liu, M. Li, W. Si, C. F. M. de Lange, and C. Dewey. 2008. "Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract." *Livestock Science* 116:318-322.

Hansen, C. F., A. L. Riis, S. Bresson, O. Høyjbjerg, and B. B. Jensen. 2007. "Feeding organic acids enhances the barrier function against pathogenic bacteria of the piglet stomach." *Livestock Science* 108:206-209. doi: 10.1016/j.livsci.2007.01059.

Heegaard, P. M. H., A. Stockmarr, M. Piñeiro, R. Carpintero, F. Lampreave, F. M. Campbell, P. D. Eckersall, M. J. M. Toussaint, E. Gruys, and N. S. Sorensen. 2011. "Optimal combination of actue phase proteins for detecting infectious diseases in pigs." *Veterinary Research* 42:50-62.

Heilig, H. G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. De Vos. 2002. "Molecular diversity of *Lactobacillus spp.* and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA." *Applied and environmental microbiology* 68 (1):114-123.

Heo, J. M., J. C. Kim, B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2009. "Feeding a diet with decreased protein content reduces indices of protein fermentation and the indices of postweaning diarrhoea in weaned pigs challenged with an enterotoxigenic strain of *Escherichia coli*." *Journal of Animal Science* 87:2833-2843. doi: 10.2527/jas.2008-1274.

Heo, J. M., F. O. Opapeju, J. R. Pluske, J. C. Kim, D. J. Hampson, and C. M. Nyachoti. 2012. "Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds." *Journal of Animal Physiology and Animal Nutrition* 97:201-237. doi: 10.1111/j.1439-0396.2012.01284.x.

Hill, G. M., D. C. Mahan, S. D. Carter, G. L. Cromwell, R. C. Ewan, R. L. Harrold, A. J. Lewis, P. S. Miller, G. C. Shurson, and T. L. Veum. 2001. "Effect of pharmacological concentrations of zinc Oxide with or without the inclusion of an antibacerial agen on nursery pig performance." *Journal of Animal Science* 79:934-941.

Højberg, O., N. Canibe, H. Damgaad, M. S. Hedemann, and B. B. Jensen. 2005. "Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets." *Applied and environmental microbiology* 71 (5):2267-2277. doi: 10.1128/AEM.71.5.2267-2277.2005.

Hopwood, D. E., and D. J. Hampson. 2003. "Interactions between the intestinal microflora, diet and diarrhoea, and their influences on piglet health in the immidiate post-weaning period." In *Weaning the pig: Concepts and consequences*, edited by J. R. Pluske, J. Le Dividich and M. W. A. Verstegen, 199-218. United Kingdom: Nottingham University Press.

Houdijk, J. G. M., F. M. Campbell, P. D Fortomaris, P. D. Eckersall, and I. Kyriazakis. 2007. "Effects of sub-clinical post-weaning colibacillosis and dietary protein on acute phase proteins in weaner pigs." *Livestock Science* 108:182-185.

Kim, J. C., B. P. Mullan, D. J. Hampson, and J. R. Pluske. 2008. "Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhoea and reduces indices of protein fermentation in the gastrointestinal tract." *British Journal of Nutrition* 99:1217-1225.

Kim, J. C., B. P. Mullan, J. M. Heo, C. F. Hansen, and J. R. Pluske. 2009. "Decreasing dietary particle size of lupins increases apparent ileal amino acid digestibility and alters fermentation characteristics in the gastrointestinal tract of pigs." *British Journal of Nutrition* 102:350-360.

Knarreborg, A., N. Miquel, T. Granli, and B. B. Jensen. 2002. "Establishment and application of an in vitro methodology to study the effects of organic acids on coliform and lactic acid bacteria in the proximal part of the gastrointestinal tract of piglets." *Animal Feed Science and Technology* 99:131-140.

Langhout, P. 2000. "New feed additives for broiler chickens." *World poultry* 16 (3):22-27.

Li, B. T., A. G. Van Kessel, W. R. Caine, S. X. Huang, and R. N. Kirkwood. 2001. "Small intestinal morphology and bacterial populations in ileal digesta and feces of newly weaned pigs receiving a high dietary level of zinc oxide." *Canadian Journal of Animal Science* 81:511-516.

Li, D. F., J. Y. Jiang, and Y. X. Ma. 2003. "Early weaning diets and feed additives." In *The Neonatal Pig: Gastrointestinal Physiology and Nutrition*, edited by R. J. Xu and P. Cranwell, 247-274. United Kingdom: Nothingham University Press.

Li, X., J. Yin, D. Li, X. Chen, J. Zang, and X. Zhou. 2006. "Dietary supplementation with zinx oxide increases IGF-I and IGF-I receptor gene expression in the small intestine of weanling piglets." *Journal of Nutrition* 135:1786-1791.

Lusk, J. L., F. B. Norwood, and J. R. Pruitt. 2006. "Consumer demand for a Band on antibiotic drug use in pork production." *American Journal of Agricultural Economics* 88 (4):1015-1033.

Metzler, B., E. Bauer, and R. Mosenthin. 2005. "Microflora management in the gastrointestinal tract of piglets." *Asian-Australian Journal of Animal Science* 18 (9):1353-1362.

Mroz, Z. 2005. "Organic acids as potential alternatives to antibiotic growth promoters for pigs." *Advances in Pork Production* 16:169-182.

Namkung, H., M. Li, J. Gong, H. Yu, M. Gottrill, and C. F. M. De lange. 2004. "Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs." *Canadian Journal of Animal Science* 84 (4):697-704.

National Research Council. 2012. *Nutritent Requirement of Swine*. 11th Rev.Ed. ed. Washington DC, USA: National Academies Press.

Naughton, P. J., and B. B. Jensen. 2001. "A bioreactor system to study survival of *Salmonella typhimurium* in pig gut content." *Berliner and Münchener Tierärztliche Wochenschrift* 114:1-4.

NHMRC. 2013. Australian code of practice for the care and use of animals for scientific purposes. Canberra: National Health and Research Council.

Partanen, K. H., and Z. Mroz. 1999. "Organic acids for performance enhancement in pig diets." *Nurition Research Reviews* 12:117-145.

Partanen, K., H. Siljander-Rasi, J. Pentikainen, S. Pelkonen, and M. Fossi. 2007. "Effects of weaning age and formic acid-based feed additives on pigs from weaning to slaughter." *Archieves of Animal Nutrition* 61 (5):336-356. doi: 10.1080/17450390701556866.

Pluske, J. R. 2013. "Feed- and feed additives-related aspects of gut health and development in weanling pig." *Journal of Animal Science and Biotechnology* 4:1-7.

Richards, J. D., J. Gong, and C. F. M. de Lange. 2005. "The gastrointestinal microbiota and its role in monogastric nutrition and health with an emphasis on pigs: current understanding, possible modulations, and new technologies for ecological studies." *Canadian Journal of Animal Science* 85 (4):421-435.

Riemensperger, A. V., D. Bachinger, S. Schaumberger, R. Urbaityte, and S. Pasteiner. 2012. "The effect of an organic acid blend, cinnamaldehyde and a permeabilising substance on the inhibition of bacterial growth in vitro and growth performance of weaning pigs." *Veterinary Medicine Zootechnika* 60 (80):59-66.

Roselli, M., A. Finamore, I. Garaguso, M. S. Britti, and E. Mengheri. 2003. "Zinc oxide protects cultured enterocytes from the damage induces by *Escherichia coli*." *The Journal of Nutrition* 133:4077-4082.

Rossi, R., G. Pastorelli, S. Cannata, and C. Corino. 2010. "Recent advances in the use of fatty acids as supplements in pig diets: a review." *Animal Feed Science and Technology* 162:1-11. doi: 10.1016/j.anifeedsci.2010.08.013.

Shen, Y. B., G. Voilqué, J. D. Kim, J. Odle, and S. W. Kim. 2012. "Effects of increasing tryptophan intake on growth and physiological changes in nursery pigs." *Journal of Animal Science* 90:2264-2275.

Slade, R. D., I. Kyriazakis, S. M. Carroll, F. H. Reynolds, I. J. Wellock, L. J. Broom, and H. M. Miller. 2011. "Effect of rearing environment and dietary zinc oxide on the response of group-housed weaned pigs to enterotoxigenic *Escherichia coli* O149 challenge." *Animal* 5 (8):1170-1178.

Sugiharto, S., M. S. Hedemann, and C. Lauridsen. 2014. "Plasma metabolomic profiles and immune responses of piglets after weaning and challenge with *E. coli*." *Journal of Animal Science and Biotechnology* 5:17-24.

Tsiloyiannis, V. K, S. C. Kyriakis, J. Vlemmas, and K. Sarris. 2001. "The effect of organic acids on the control of post-weaning oedema disease of piglets." *Research in Veterinary Science* 70:281-285.

Vondruskova, H., R. Slamova, M. Trckova, Z. Zraly, and I. Pavlik. 2010. "Alternative to antibiotic growth promoters in prevention of diarrrhoea in weaned piglets: a review." *Veterinarni Medicina* 55:199-224.

Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes. 2001. "Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel Electrophoresis." *Applied and environmental microbiology* 67 (6):2578-2585.

Wellock, I. J., P. D. Fortomaris, J. G. M. Houdijk, and I. Kyriazakis. 2007. "Effect of weaning age, protein nutrtion and enterotoxigenic *Escherichia coli* challenge on the health of newely weaned piglets." *Livestock Science* 108:102-105. doi: 10.1016/j.livsci.2007.01.004.

Yan, L., and I. H. Kim. 2012. "Effect of eugenol and cinnamaldehyde on the growth performance, nutrient digestibility, blood characteristics, faecal microbial shedding and fecal noxious gas content in growing pigs." *Asian-Australian Journal of Animal Science* 25 (8):1178-1183.

Yan, L., J. P. Wang, H. J. Kim, Q. W. Meng, X. Ao, S. M. Hong, and I. H. Kim. 2010. "Influence of essential oil supplementation and diets with different nutrient densities on growth performance, nutrient digestibility, blood characteristics, meat quality and fecal noxious gas content in grower-finisher pigs." *Livestock Science* 128:115-122. doi: 10.1016/j.livsci.2009.11.008.

Zhang, B., and Y. Guo. 2009. "Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning pigs." *British Journal of Nutrition* 102:687-693. doi: 10.1017/S0007114509289033.