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The effect of dietary quercetin on the glutathione kinetics in the gut mucosa of weaned piglets

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Table of contents

List of abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
BW	Body weight
CDNB	1-chhloro-2,4-dinitrobenzene
CON	Control
COX-2	Cyclooxygenase-2
DEM	Diethyl maleate
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
FCR	Feed conversion ratio
FD4	Fluorescein isothiocyanate-dextran 4
GCL	Glutamate cysteine ligase
ɣ-GC	ɣ-Glutamylcysteine
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione- S -transferase
HPLC	High-performance liquid chromatography
HQUE	High quercetin
LQUE	Low quercetin
MDA	Malondialdehyde
MPLN	Melphalan
MQUE	Medium quercetin
NADPH	Nicotinamide adenine dinucleotide phosphate
NDA	Naphthalene-2,3-dicarboxialdehyde
NO	Nitric oxide
OCC1	Occluding 1 protein
PCA	Perchloric acid solution
PCNA	Proliferating cell nuclear antigen
PCZ	Procarbazine
PGC-1α	PPARγ coactivator-1α
ROS	Reactive oxygen species
SEM	Standard errors
SI	Small intestine

SOD	Superoxidase dismutase
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substance
TEER	Trans-epithelial electrical resistance

Abstract

In pig production, weaning is the point that piglets face the greatest challenge after birth. During this period, the piglets are suffering from oxidative stress and disturbances in intestinal barrier function which could lead to diarrhea, stunting, loss of appetite and even death. The aim of this Master thesis was to test a dietary supplementation which could help the piglets deal with weaning stress and therefore improve the efficiency in pig production. The supplementation with quercetin was tested; a flavonoid which is widespread in plants and has been reported to have anti-oxidant and anti-inflammatory functions both *in vivo* and *in vitro*. The experimental piglets (n = 224) were weaned at 21 days of age and were divided into 4 equal groups. During the 42 days of experiment, a diet with quercetin concentration of either 0, 100, 300 or 900 mg/kg feed was given for the first 14 days and the same quercetin-free starter feed was given to all groups for the rest of experiment. The parameters that have been tested are performance and health, oxidative status glutathione kinetics in different tissues and intestinal barrier integrity. Results showed that dietary quercetin significantly increased the glutathione peroxidase (GPx) activity in plasma at day 5, and the glutathione S-transferase (GST) activity in liver at day 5 and in plasma at day 12. Also, quercetin significantly increased the GPx activity in distal small intestinal mucosa and increased the glutathione reductase (GR) activity in liver at day 12. Quercetin treatment caused a significantly higher oxidized glutathione / reduced glutathione (GSSG/GSH) ratio in red blood cells and liver and a significantly higher malondialdehyde (MDA) content in liver and distal small intestine mucosa. Thus, the effect of quercetin on glutathione kinetics in weaned piglets is inconsistent and future studies may be required.

Key words: dietary quercetin, animal performance, oxidative stress, gut integrity, weaning piglets

1.Introduction

The objective of this study is to test the effect of quercetin as a dietary supplementation on helping the piglets deal with weaning stress, since weaning is the point that piglets face the greatest challenge after birth. Test has been done to analysis the effect of quercetin on oxidative stress in different organs and the effect on small intestine functionality in piglets. The tested parameters of this study are growth performance, GSH related enzymes, small intestine functionality and redox status in liver, plasma and small intestine plasma. The hypothesis of this study is that the dietary quercetin may help to alleviate the oxidative stress of the weaned piglets and thereby improve the growth performance of the animals.

In the next chapters, a literature review will be given in chapter 2 regarding the weaning stress, the general characteristics of quercetin, and its effects on antioxidation, anti-inflammatory, functionality of small intestine and animal performance. The material and methods are given in chapter 3. Chapter 4 describes the results, and the discussion and conclusion are in chapter 5 and chapter 6.

2.Literature review

2.1 General introduction

2.1.1 Weaning stress

In pig production, weaning is the point that piglets face the greatest challenge after birth. During this period, piglets are undergoing changes in environment, diet and habits, which could all contribute to the increased disease susceptibility and digestion disorders (Moeser et al., 2007). Unfavorable situations such as diarrhea, stunting and loss of appetite could lead to economic loss for producers (Hong et al., 2006). Thus, the health state of weaned piglets has a significant importance in pig production.

One of the factors that contribute to weaning stress is the change in antioxidant system during this period. Numerous studies reported that weaning affects the oxidative and redox status of piglets. Studies at Ghent university have shown that in gut mucosa of weaned piglets the oxidative and redox status change significantly (Degroote et al., 2012), which suggest that oxidative damage is one of the reasons for the fragile health state of weaned piglets. Also, research about weaned piglets conducted in 2012 showed a decrease in mRNA expression of genes related to antioxidant enzymes, such as SOD and PPARγ coactivator-1α (PGC-1α). These results suggested that during the weaning transition the antioxidant system in piglets is affected. This hypothesis is supported by the increase in concentration

of MDA, NO, and H_2O_2 in weaned piglets which indicate increased oxidative stress (Zhu et al., 2012).

Also, disturbances in intestinal barrier function is one of the problems that is associated which lead to weaning stress. The intestinal barrier is mainly composed by a single layer of epithelial cells (Moeser et al., 2007). As the first line of defense in the body, the intestinal barrier prevents harmful chemicals and microbes from entering the body. However, during the weaning period, the integrity of intestinal barrier is under challenge. In a study of weaned piglets conducted in 2013, the trans-epithelial electrical resistance (TEER) during the two weeks after weaning has been tested by using Ussing chambers. The decrease in TEER of weaned piglets reflects that the intestinal mucosal barrier is under challenge during this period (Hong et al., 2006). Also, the expression of genes related to tight junction proteins decreased during weaning, which lead to the similar conclusion that the intestinal barrier function of weaned piglets is disturbed.

2.1.2 Flavonoids and quercetin

2.1.2.1 Flavonoids

Flavonoids are polyphenolic compounds containing diphenylpropans (C6C3C6), they are a large group of secondary metabolites ubiquitously distributed throughout the plant kingdom. Flavonoids all have a similar basic chemical structure with three ring structures. Depending on the saturation and substituents in the central ring different classes of flavonoids are distinguished. They are classified into six major categories: isoflavones, anthocyanidins, flavones, flavonols, flavan-3-ols and flavonones, and more than 4000 different molecules have been identified to date (Ravishankar et al., 2013).

The major sources of flavonoids in the human diet are fruits, vegetables and beverages, such as tea and coffee (Merken & Beecher, 2000), and the majority of flavonoids are present as glycosides in which one or more sugar groups are bound to the phenolic groups through glycosidic linkage, although a small proportion is present as aglycones. In fact, they are ingested in daily quantities of 1-2 g by humans who eat diets typical of those found in Western world (Kuhnau, 1976).

Many of the flavonoids have been reported to interfere in cell activities or body metabolism. For example, they have been found to have functions such as antimicrobial, anticancer, antioxidant, anti-hepatotoxic and anti-inflammatory (Rathee et al., 2009). Flavonoids have been reported to have antioxidant functions which have a protective effect on biological systems. Flavonoids are able to prevent oxidative injury by directly reducing the reactive oxygen species (ROS) (Prochazkova et al., 2011). Also, flavonoids are able to act as an antioxidant by inhibiting the enzymes that are responsible for the production of ROS (Heim et al., 2002). Another mechanism by which flavonoids protect the cells and tissues from oxidative damage is to activate the antioxidant enzymes. Other mechanisms through which the flavonoids are able to perform the antioxidant function include chelating trace metals or inhibition to nitric oxide (NO) production that causes mitigation of oxidative stress

(Ravishankar et al., 2013).

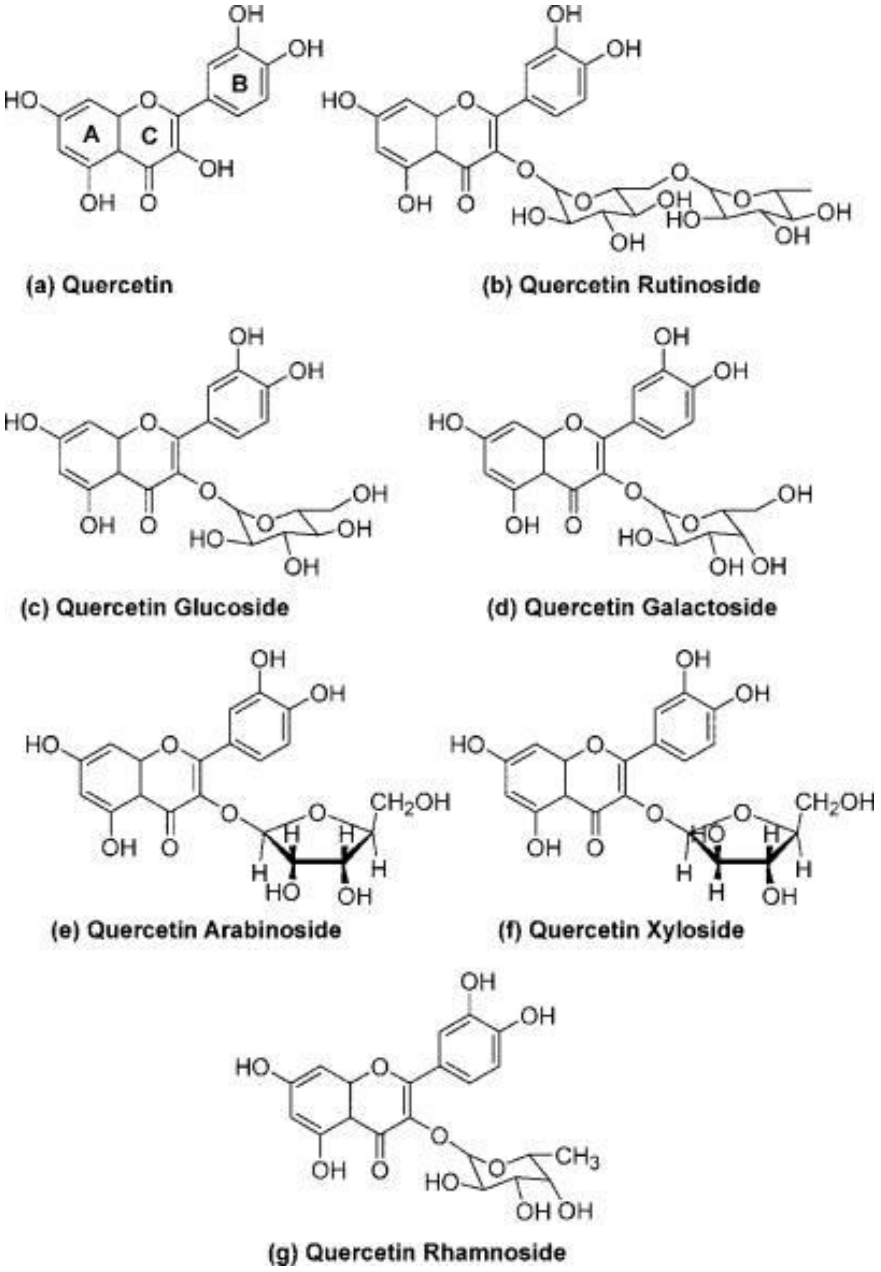

Figure 2.1. Structures of (a) quercetin aglycone, (b) quercetin rutinoside(rutin), (c) quercetin glucoside, (d) quercetin galactoside, (e) quercetin arabinoside, (f) quercetin xylosides, and (g) quercetin rhamnoside (Meyers et al., 2008)

Furthermore, flavonoids have also been found to have anti-inflammatory activity in both proliferative and exudative phases of inflammation. Hesperidin is a flavonoid present in citrus plants. Studies have shown that hesperidin deficiency in the diet has a negative effect on animal health and leads to problems such as abnormal capillary leakiness (Garg et al., 2001). Also, research has shown that hesperidin combined with diosmin has a protective effect against inflammatory disorders. The mechanism behind this anti-inflammatory effect might be related to the antioxidant effect of flavonoids (Jean & Bodinier, 1994).

2.1.2.2 Quercetin

Quercetin is not the most predominant flavonoid in the diet, but it is widespread and has been widely studied in a variety of models (Percival, 2005). Quercetin is characterized as a flavonol on the basis of the presence of an oxygen-containing ring between 2 benzene rings (Garg et al., 2001). In plants, however, quercetin occurs most frequently in its glycoside form, such as rutin (quercetin 3-rhamno-glucoside) and quercetrin (quercetin 3-rhamnoside) (Garg et al., 2001). In this form, quercetin is bound to mono- or oligosaccharides via a glycosidic bond with the 3' hydroxyl group on the oxygen-containing ring. The presence of the saccharides increases the aqueous solubility of quercetin (Garg et al., 2001). The structures are shown in Figure 2.1.

Quercetin is present in a number of plant-derived foods. Apples with their skin (4.42 mg/g), raw celery (3.50 mg/g), raw chives (4.77 mg/g), unsweetened cocoa (20.13 mg/g), cooked onion (19.36 mg/g), yellow chili peppers (50.63 mg/g), dill (55.15 mg/g), and especially canned capers (180.77 mg/g) are food items that contains a higher-than-average content of quercetin (Garg et al., 2001).

Several functions have been demonstrated both *in vivo* and *in vitro* for quercetin, including antioxidant function (Moskaug et al., 2005), anti-inflammatory (Camuesco et al., 2004), protection towards intestinal integrity (Ulluwishewa et al., 2011) and improvement of animal performance (Goliomytis et al., 2014a).

2.2.Quercetin biological activities

2.2.1 Anti-oxidative function of quercetin

Oxidative stress is defined as a disturbed balance between antioxidants and pro-oxidants in the cell environment, with a shift toward the pro-oxidant side (Meyers et al., 2008). The increased production of reactive oxygen species which exceed the antioxidant defense ability will cause oxidative damage to lipids, proteins and DNA. Further consequences of oxidative stress would be damage in cells, tissues and organs which would cause subsequent disease development and aging (Moskaug et al., 2005).

Quercetin has been reported to possess an anti-oxidative function. The structure of quercetin allows it to donate hydrogens atoms to scavenge free radicals through the reaction R• + ROO• → ROOR. Figure 2.2 shows the result of a systematic screening conducted in 2002 using ferric ion-reducing activity assays which showed that quercetin has the highest anti-oxidative activity in the 4 compounds tested (Benzie & Strain, 1996; Halvorsen et al., 2002).

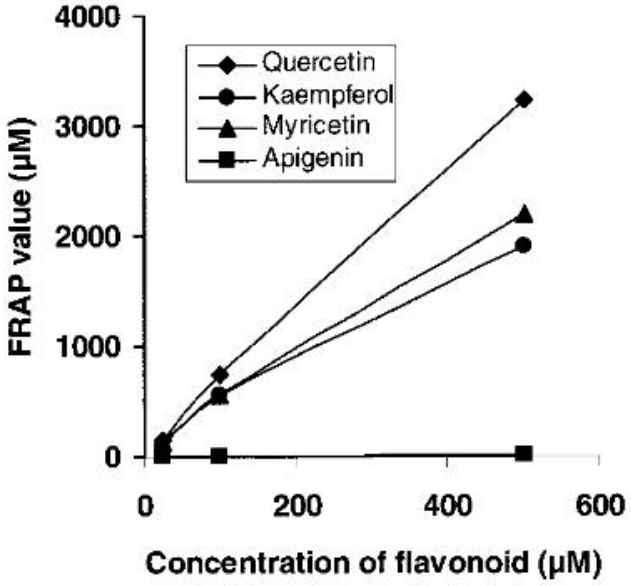

Figure 2.2 Total antioxidant activity of pure flavonoids as measured with ferric ion-reducing activity assays.(Halvorsen et al., 2002)

2.2.1.1 Effect of quercetin on the glutathione redox status

Glutathione (γ-glutamylcysteinylglycine; GSH) is a tripeptide which is involved in many important activities in the animals' body, including antioxidant functions and nutrient metabolism. Also, GSH participates in activities inside cells, including gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production and immune response, and protein glutathionylation. The deficiency of GSH will contribute to oxidative stress, which plays an important role in aging and the development of many diseases including Parkinson's disease, liver disease, cancer, and heart attack (Wu et al., 2004). Quercetin can interact with the GSH in different ways like directly affecting the GSH/GSSG ration or affecting the enzymes involved.

In antioxidant defense, GSH plays an important role in the protection of animal tissues against reactive oxygen species (Nygren et al., 2005). Numerous studies have been done in order to have a better understanding of the role of GSH under oxidative stress. Studies focused on GSH-deficiency models also showed more oxidative damage than control (Magalhaes et al., 2004).

While GSH plays important roles in animal tissues, the ratio between GSH and its oxidized form glutathione disulfide (GSSG) is an important indicator of the cellular redox state. The intracellular ratio of GSH/GSSG is the major redox couple in animal cells because the ratio of GSH/GSSG will increase under oxidative stress, which provides a bio-marker to test the effect of different supplements on anti-oxidative function.

Studies show that quercetin increases the GSH/GSSG ratio through up-regulation of GSH as well as the down-regulation of GSSG , and in result reduces oxidative stress (Table 2.1). Studies by Molina et

al. (2003) tested the effect of quercetin on mice through gastric intubations. The results showed that quercetin has a protective effect against oxidative stress in hepatic tissues by increasing the ratio of GSH/GSSG. In another study, Galvez et al. (1994) used orally administrated quercitrin on rats. The result showed that compared with the control group, the treatment group has higher GSH/GSSG ratio in hepatic tissue, ileal mucosa and colonic mucosa. Meyers et al. (2008) demonstrated that both dietary quercetin and dietary dried onion have an effect on upregulation of GSH/GSSG ratio in liver, but the effect was not found in cardiac tissues or plasma.

2.2.1.2 Effect of quercetin on glutathione related enzymes

The GSH related enzymes contribute to the anti-oxidative system in animals. Figure 2.3 shows the function of GSH related enzymes during GSH synthesis and utilization.

Researches have shown that enzymes related to antioxidant system including glutathione peroxidase (GPx), glutathione reductase (GR), glutathione- S -transferase (GST), catalase and superoxide dismutase have high levels of expression when animals are under oxidative stress. This hypothesis has been tested by using antioxidants in epithelial colon cancer cells, which showed a significant change in the activity of related enzymes (Burrow et al., 2011). Quercetin has been used as an antioxidant to test the effect on enzymes related to antioxidant system. The result shows that quercetin could upregulate the level of glutamate cysteine ligase (GCL), GST, GPx, GR, which are all enzymes related to the GSH redox cycle (Arredondo et al., 2010; Granado-Serrano et al., 2012; Odbayar et al., 2009; Sanders et al., 2001).

2.2.1.2.1 Glutathione peroxidase (GPx)

GPx is the enzyme that catalyzes the conversion between glutathione and glutathione disulfide (GSSG) (Lee et al., 2006). Studies have shown that when GPx activity is inhibited, there are higher level of oxidative damage in cells and tissues (Chatziargyriou & Dailianis, 2010). Quercetin has been reported to have a protective function towards tissues and cells under oxidative stress by regulating the activity and mRNA of GPx.

In vivo studies conducted by Molina et al. (2003) demonstrated that mice treated with quercetin by gastric intubation shows higher GPx activity in liver compared with the control group. The study conducted by Coskun et al. (2005) on diabetic rats also found that quercetin has a protective effect on streptozotocin (STZ)-induced oxidative stress. The study shows that the activity of GPx in pancreatic homogenates increased significantly. Another study using STZ-induced diabetic rats conducted by Sanders et al. (2001) also shows that a dose of 10mg/kg/day quercetin lead to an increase of renal and cardiac GPx activity.

Table 2.1. Literature review related to the effect of quercetin on glutathione redox ratio (GSH/GSSG) in animals

Reference	Animal Species	Compounds	Method	Trial Set-up	Sampling day & Tissue	GSH/GSSG
(Molina et al., 2003)	Mice (male Swiss mice aged 7—12 weeks)	Quercetin	Gastric intubation	•Control group: day 0 to day 30: ethanol treatment •Treatment group : day -15 to day -1: quercetin treatment (25, 50 and 75mg/kg body weight); day 0 to day 30: ethanol treatment	•day 31 •Liver •Blood	•Hepatic tissue ↑(all concentrations)
(Galvez et al., 1994)	Rats (Female Wistar rats approx. 170 g)	Quercitrin	Oral administration	•20 animals/group •Control group: 1 ml isotonic saline solution/day •Treatment group: quercetin dissolved in 1ml isotonic saline solution/day (25mg quercetin/kg solution)	•day 3 & day 7 •Liver •Jejunal mucosa •Ileal mucosa •Colonic mucosa	•Hepatic tissue ↑ •Jejunal mucosa = •Ileal mucosa ↑ •Colonic mucosa ↑
(Meyers et al., 2008)	Mice (male swiss Webster mice 25-30g)	Quercetin	Dietary quercetin	•12 animals/group •Control group: purified control diet •Treatment group: purified diet + quercetin (200ug quercetin/g diet)	•day 7 •Liver •Blood •Heart	•Hepatic tissue ↑ •Cardiac tissue = •Plasma =
(Meyers et al., 2008)	Mice (male swiss Webster mice 25-30g)	Dried Onion	Dietary quercetin	•12 animals/group •Control group: purified control diet •Treatment group: purified diet + dried onion (200ug quercetin/g diet)	•day 7 •Liver •Blood •Heart	•Hepatic tissue ↑ •Hepatic mitochondria ↑ •Cardiac tissue = •Plasma =

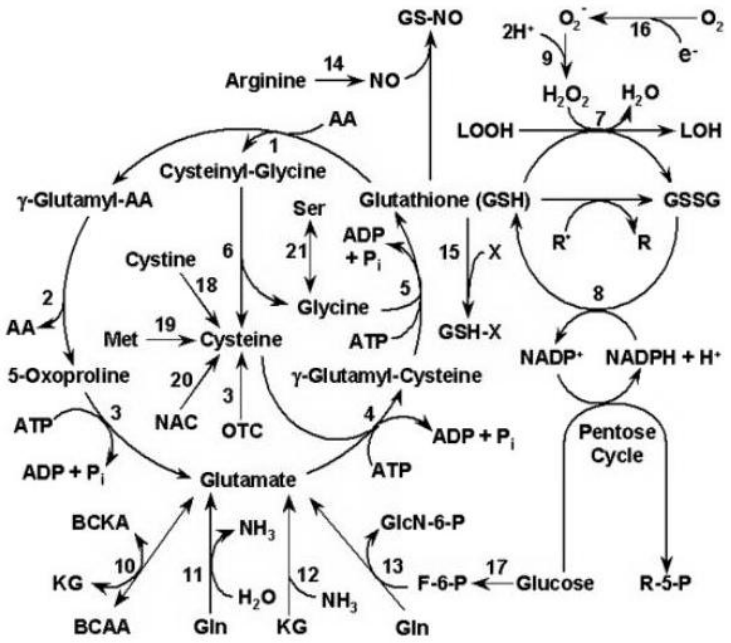

Figure 2.3 Glutathione synthesis and utilization in animals. Enzymes that catalyze the indicated reactions are: 1) γ-glutamyl transpeptidase, 2) γ-glutamyl cyclotransferase, 3) 5-oxoprolinase, 4) γ-glutamylcysteine synthetase, 5) glutathione synthetase, 6) dipeptidase, 7) glutathione peroxidase, 8) glutathione reductase, 9) superoxide dismutase, 10) BCAA transaminase (cytosolic and mitochondrial), 11) glutaminase, 12) glutamate dehydrogenase, 13) glutamine:fructose-6-phosphate transaminase (cytosolic), 14) nitric oxide synthase, 15) glutathione S-transferase, 16) NAD(P)H oxidase and mitochondrial respiratory complexes, 17) glycolysis, 18) glutathione-dependent thioldisulfide or thioltransferase or nonenzymatic reaction, 19) transsulfuration pathway, 20) deacylase, and 21) serine hydroxymethyltransferase. Abbreviations: AA, amino acids; BCKA, branched-chain α-ketoacids; GlcN-6-P, glucosamine-6-phosphate; GS-NO, glutathione–nitric oxide adduct; KG, α-ketoglutarate; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; NAC, N-acetylcysteine; OTC, L-2-oxothiazolidine-4-carboxylate; R•, radicals; R, nonradicals; R-5-P, ribulose-5-phosphate; X, electrophilic xenobiotics (Wu et al., 2004)

Same result has been reported. that quercetin decreases oxidative stress and increases the activity and mRNA expression of GPx in rat liver (Dias et al., 2005).

In vitro study also shows the upregulation of GPx in animal cells. Granado-Serrano et al. (2012) reported that 50μM quercetin increases the expression and activity of GPx in human hepatoma HepG2 cells after 4h incubation. Nagata et al. (1999) also demonstrated same effect in rat hepatocytes (BL-9). In the study, an oxidative cell damage was induced by H2O2, and the treatment of quercetin shows a protective function by mediating the activity of GPx. Alia et al. (2006) also reported the positive effect of quercetin on antioxidant defense system of human hepatoma cell line. Doses of quercetin from 0.1 to 1 μM increased the expression of GPx.

2.2.1.2.2 Glutathione- S -transferase (GST)

Glutathione S-transferase can catalyze the conjugation of GSH with a wide range of harmful compounds such as the breakdown products of lipid peroxidation or DNA hydroperoxides (Hayes & Pulford, 1995; Nordberg & Arner, 2001) (Figure 2.3). The resulting products are further transformed

into water-soluble metabolites and are able to be excreted (Lee, 1991). Together with GSH, GST plays an important role in anti-oxidative system which protects mammalian cells against electrophilic compounds. And it has been reported that when the concentration of GSH is low, class pi GST might serve as a scavenging protein (van Zanden et al., 2003). Quercetin has been reported to have a protective function towards tissues and cells under oxidative stress by regulate the activity and expression of GST. *In vivo* study conducted on male Wistar rats in 2015 demonstrated that quercetin treatment (20 mg/kg BW) increases hepatic GST activity and protected against procarbazine induced oxidative damage (Olayinka et al., 2015). Another *in vivo* study conducted in 2014 also proved a similar effect of quercetin treatment (20 mg/kg BW) against melphalan induced oxidative damage. The result showed that the GST activity in rat livers has been increased significantly compared with non-quercetin groups (Olayinka et al., 2014). Also, Odbayar et al (2009) have reported the upregulating effect of dietary quercetin on GST mRNA expression. The study was conducted on male Sprague-Dawley rats, and the results showed that dietary quercetin (5, 10, 20 g quercetin/kg diet) increases GST expression in the liver .

2.2.1.2.3 Glutathione reductase (GR)

Glutathione reductase (GR) is responsible for catalyzing GSSG into GSH, which maintains the oxidative and redox status and helps to minimize oxidative damage in cells. Studies showed that the decrease in GR caused by direct inhibition of the enzyme led to increased oxidative damage (Romero-Ramos et al., 2003).

In vitro study in 2012 showed that after 4h or 18h exposure ito 50µM quercetin, the expression and activity of GR in human hepatoma HeoG2 cells has significantly increased (Granado-Serrano et al., 2012). The *in vivo* study conducted on male Swiss mice revealed the protective effect of quercetin by gastric intubation in mouse liver. The results showed that the activity of GR has been increased compared with the non-quercetin group (Molina et al., 2003). Another study proved that an oral treatment of quercetin restrained the oxidative stress in rats heart and significantly increased the activity of GR (Liu et al., 2014).

2.2.1.2.4 Glutamate cysteine ligase (GCL)

Glutamate Cysteine Ligase is the enzyme that catalyzes L-cysteine and L-glutamate into gamma-glutamyl cysteine which will further be used for the synthesis of GSH. Since cysteine is the limiting amino acid in this synthesis, the activity of GCL is quite important. Research proved that increased activity of GCL induced by GCL overexpression has a protective function against oxidative damage to cells. Also, another study conducted by Krejsa et al. proved that after the increase of oxidative stress there is a rapid increase of the activity of GCL (Cortes-Wanstreet et al., 2009; Krejsa et al., 2010).

In vitro study demonstrate the protective effect of quercetin against oxidative stress. The cells (COS-1 cells and HepG2 cells) were treated with quercetin dissolved in dimethylsulfoxide (DMSO) for 17h

before the mRNA was analyzed. The results showed an increased expression of GCL in the cells (Moskaug et al., 2005). Another study proved the protective effect of quercetin against H_2O_2 in 24h-pretreated neuronal cultures, which described the increase of GCL gene expression (Arredondo et al., 2010). The study of Myhrstad et al. (2002) also demonstrated the upregulation effect of quercetin on the GCL gene expression in cultured COS-1 cells. Also, the research conducted in 2012 showed that after 4h exposure in 50μM quercetin, the expression of GCL in human hepatoma HeoG2 cells has significantly increased (Granado-Serrano et al., 2012).

2.2.1.3 Liver oxidative and redox status

As a metabolic active tissue, liver is of major importance in maintaining oxidative balance in animals. Liver contains relatively high amounts of GSH and GSH-related enzyme activity and liver is the major producer and exporter of GSH (Toroser et al., 2006; Wu et al., 2004). When animals are under oxidative stress, parameters describing the oxidative and redox status in liver are often affected. Research showed increased lipid peroxidation, decreased GSH content, decreased GST and SOD activity, decreased AA level, decreased catalase activity in liver under oxidative stress (Olayinka et al., 2014; Sanders et al., 2001). Quercetin has been used as an anti-oxidant in hepatic oxidative status studies.

The study of Molina et al. (2003) (Table 2.2) shows that quercetin treatment through gastric intubation improved the GSH/GSSG ratio in liver. Also, the level of hepatic SOD, GPx, GR has also been improved, and the level of malondialdehyde (MDA) which is a marker for lipid peroxidation in liver has been decreased by the quercetin treatment. Another study conducted on rats also shows the improved oxidative status in liver by dietary quercetin treatment (Odbayar et al., 2009). Study conducted by Van Le Thanh, B., et al. (2016) demonstrated the protective effect of quercetin on pig liver. The GSH/GSSG ratio, vitamin A and vitamin E in liver has been increased and the hepatic MDA level has been decreased. Other studies have also showed the effect of quercetin treatment on liver by the increased GSH/GSSG ratio, regulation of related enzymes, and reduced lipid oxidation (Galvez et al., 1994; Meyers et al., 2008; Olayinka et al., 2014; Sanders et al., 2001).

2.2.1.4 Small intestine oxidative and redox status

The intestines are one of the major consumers of the liver-derived GSH (Wu et al., 2004). Increased production of free radicals in intestines could lead to the increased oxidative stress and result in detrimental situations such as inflammatory bowel disease and delay in growth (Galvez et al., 1994). Studies have been conducted to find dietary supplements to reduce the oxidative stress in intestines. Quercetin has been reported to have protective effect towards intestinal oxidative status in mammalian animals.

Galvez et al. (1994) (Table 2.2) has conducted the study using rats to test the effect of orally administrated quercetin on the intestinal anti-oxidative system. Here quercitrin has been used, which is one of the most frequently occurred glycoside form of quercetin. The results showed that the

GSH/GSSG ratio has been increased significantly in ileum and colonic mucosa. Also, the MDA level, which reflects the lipid oxidation level, has been decreased in jejunum and colon. Another study also demonstrated the protective effect of dietary quercetin on piglets intestine where pure quercetin has been used. The results showed the increased GPx and SOD level and decreased MDA level, which indicated improvement in intestinal oxidative status (Van Le Thanh et al., 2016).

2.2.1.5 Blood oxidative and redox status

The whole-blood fractional synthesis rate of GSH is relatively high, in human blood, all the GSH could be completely replaced in 1.5d. Thus, whole blood may contribute up to 10% of whole body GSH synthesis in humans (Wu et al., 2004). Animals under oxidative stress shows increased level of MDA concentration in plasma (Olayinka et al., 2014; Sanders et al., 2001; Van Le Thanh et al., 2016). As an antioxidant, quercetin has been used to protect blood oxidative status in researches.

The study conducted by Van Le Thanh et al. (Table 2.2) on piglets proved that dietary quercetin has improved the plasma oxidative status by increasing vitamin A level and decreasing MDA level. Also, the study using rats with induced oxidative stress showed that quercetin could improve the plasma ɤ-glutamyl transpeptidase (ɤ-GT) and protect the stability of blood oxidative status (Olayinka et al., 2014).

Table 2.2 Literature review related to the positive effects of quercetin on mammalian tissues

Reference	Animal Species	Compounds	Method	Trial Set-up	Sampling day & Tissue	GSH/GSSG	Effect on antioxidant status
(Molina et al., 2003)	Mice (male Swiss mice aged 7—12 weeks)	Quercetin	Gastric intubation	•Control group: day 0 to day 30: ethanol treatment •Treatment group : day -15 to day -1: quercetin treatment (25, 50 and 75mg/kg body weight); day 0 to day 30: ethanol treatment	•day 31 •Liver	•Hepatic tissue ↑	•SOD/CAT/GPx/GR/ ↑ (all concentrations) •MDA in liver ↓ (all concentrations)
(Odbayar et al., 2009)	Rats (Male Sprague-Dawley rats)	Quercetin	Dietary quercetin	Feed an experimental diet containing either 0,2.5,5,10 or 20 g/kg quercetin for 15 days	•day 15 •Liver	not tested	•GST mRNA ↑ (5 and 20 g/kg)
(Meyers et al., 2008)	Mice (male swiss Webster mice 25-30g)	Quercetin	Dietary quercetin	•12 animals/group •Control group: purified control diet •Treatment group: purified diet + quercetin (200ug quercetin/g diet)	•day7 •Liver •Blood •Heart	•Hepatic tissue ↑ •Cardiac tissue = •Plasma =	not tested
(Meyers et al., 2008)	Mice (male swiss Webster mice 25-30g)	Dried Onion	Dietary quercetin	•12 animals/group •Control group: purified control diet •Treatment group: purified diet + dried onion (200ug quercetin/g diet)	•day 7 •Liver •Blood •Heart	•Hepatic tissue ↑ •Hepatic mitochondria ↑ •Cardiac tissue = •Plasma =	not tested

Table 2.2 Continued

(Galvez et al., 1994)	Rats (Female Wistar rats approx. 170 g)	Quercitrin	Oral administration	•20 animals/group •Control group: 1 ml isotonic saline solution/day •Treatment group: quercetin dissolved in 1ml isotonic saline solution/day (25mg quercetin/kg solution)	•day 3 & day 7 •Liver •Jejunal mucosa •Ileal mucosa •Colonic mucosa	•Hepatic tissue ↑ •Jejunal mucosa = •Ileal mucosa ↑ •Colonic mucosa ↑	•GST/GPx/GR = •Hepatic MDA ↓ •Jejunal MDA ↓ •Ileal MDA = •Colonic MDA ↓
(Sanders et al., 2001)	Rats (Male Sprague-Dawley rats)	Quercetin	Daily intraperitoneal injection	•8 animals/group •Control group: dimethyl sulfoxide vehicle for 14 days •Treatment group: quercetin (10 mg/kg/day) for 14 days	•day 15 •Liver •Heart •Kidney •Brain	•Hepatic tissue ↑	•Hepatic GPx ↑ •Brain GPx ↑ •Hepatic TBARS ↑ •Renal GPx ↑ •Cardiac GPx ↑
(Olayinka et al., 2014)	Rats (Wistar rats 160-170g)	Quercetin	Oral intubation	•5 animals/group •Control group: normal diet •Treatment group 1: MPLN (0.2 mg/kg BW) for 7 days •Treatment group 2: pre-treat with quercetin (20 mg/kg BW) for 7 days + MPLN (0.2 mg/kg BW) for 7 days •Treatment group 3: co-treat with quercetin (20 mg/kg BW) and MPLN (0.2 mg/kg BW) for 7 days •Treatment group 4: only quercetin (20 mg/kg BW) for 7 days	•day 15 •Liver •Blood	•Hepatic tissue ↑	•Hepatic GST, SOD,CAT ↑ •Hepatic MDA ↓ •Plasma γ-GT↑
(Gonzalez-Segovia et al., 2008)	Guinea pigs(250-350g)	Quercetin	Orally treated	•5 animals/group •Control group: untreated •Treatment group 1: H.polori infected animal •Treatment group 2: infected animal+quercetin(100 mg/kg) for 15 days •Treatment group 3: only quercetin(100 mg/kg) for 15 days	•day 16 •Stomach	not tested	•Lipid peroxidation ↓

Table 2.2 Continued

(Van Le Thanh et al., 2016)	Pig (castrated male weaned pigs, 5.5 - 6.5 kg)	Quercetin	Dietary quercetin	•12 animals/group •Control group: diet with 0.8mg/kg DON for 17days •Treatment group 1: diet with 3.1 mg/kg DON for 17days •Treatment group 2: diet with 3.1 mg/kg DON+ vitamins for 17days •Treatment group 3: diet with 3.1 mg/kg DON+ organic selenium /GSH for 17days •Treatment group 4: diet with 3.1 mg/kg DON+quercetin for 17days •Treatment group 5: diet with 3.1 mg/kg DON+ combination of treatment 2,3,4 for 17days	•day 18 •Liver •Blood •Intestine	•Hepatic tissue ↑	•Plasma: MDA↓,Vit-A↑ •Hepatic: MDA↓,SOD↓,Vit-A↑,Vit-E↑ •Ileum: MDA↓ •Jejunal:GPx↑, SOD↑

2.2.2 Anti-inflammatory function of quercetin

Inflammation is a complicated biological process which links to many aspects such as immune system, blood vessels and molecular mediators. Inflammatory cytokines such as TNF-a, IL-1, IL-6, IL-8, IL-12 and IL-18 are responsible for the development of inflammatory processes. Increased gene expression and protein production of these cytokines has been observed in various inflammation situations (Gonzalez-Segovia et al., 2008; Guo et al., 2007; Pie et al., 2004). Also, cyclooxygenase and lipoxygenase play an important role as inflammatory mediators (Nijveldt et al., 2001).

Inflammation has been known to be associated with many negative effects in mammalian animals. In weaning piglets, the change in diet and environment often lead to inflammation in intestines, and the effect could impair digestion and absorption of nutrients, which could lead to unfavorable situations such as diarrhea, loss of appetite, decrease in body weight and even death in some cases (McCracken et al., 1999; Pie et al., 2004). Other studies has also shown that inflammation is one of the critical factors during tumor progression. The study of Coussens et al. (2002) has demonstrated that many cancers arise from infection, chronic irritation and inflammation. These results revealed the significance of anti-inflammatory studies.

Quercetin has been reported to have protective effects on animals cells and tissues against inflammation. The study conducted by Gonzalez-Segovia et al. demonstrated the anti-inflammatory effect of quercetin using guinea pigs. The results showed that the quercetin-treated group has lower level of mononuclear cell infiltration in gastric mucosa. Also, the expression and production of inflammatory mediators such as TNF-a, IL-1, IL-6 and IL-8 was significantly decreased (Gonzalez-Segovia et al., 2008). In another study, the effect of quercetin has been investigated by using the mRNA levels of cyclooxygenase-2 (COX-2), which is significantly elevated *in vivo* during inflammation. The result (Figure 2.4) shows that quercetin inhibited basal COX-2 expression in a dose-dependent manner in unstimulated Caco-2 cells (O'Leary et al., 2004).

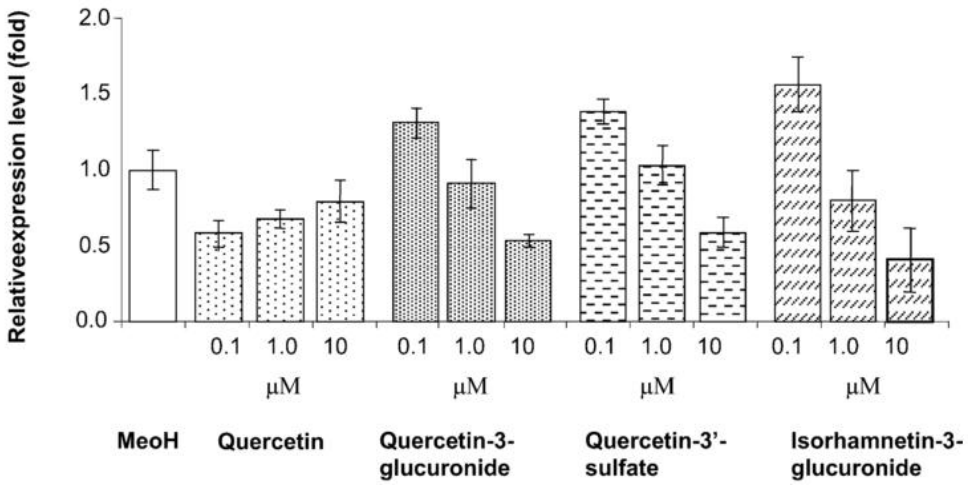

Figure 2.4 Effect of quercetin plasma metabolites (0.1, 1.0, 10µM) on the gene expression of COX-2 in unstimulated Caco-2 cells following 16-h treatment.(O'Leary et al., 2004)

2.2.3 Effect of quercetin on functionality of small intestine

One of the main interests within animal nutrition is the relation between nutrition and gut health, especially that of the small intestine. Nutritionists and feed manufacturers are becoming increasingly aware of the fact that the major gut functions, digestion, absorption and intestinal barrier (as the first line of defense against invasive pathogens) in monogastric animals should be optimized to achieve a high production performance. The intestine allows the digestion and absorption of nutrients while also functioning as a barrier. The gastrointestinal epithelium serves as a physical barrier which protect the animal against pathogens, toxins and antigens. The intestinal barrier also regulate the passive diffusion of solutes and macromolecules (Suzuki & Hara, 2011; Ulluwishewa et al., 2011; Vergauwen et al., 2015). Gut health is challenged during animal growth at different occasions such as weaning of piglets.

2.2.3.1 Effect of quercetin on permeability

The intestinal tract has a single continuous layer of cells which separates the animal circulatory system from the lumen (Suzuki et al., 2011). The barrier function is of great importance because there are various compounds or pathogens in the lumen of the intestine that could lead to mucosal inflammation if they cross the epithelial barrier. When the intestinal permeability increases, the animal is under risk of being attacked by unfavorable compounds and pathogens. Increased intestinal permeability is observed in association with several autoimmune diseases. Immunoglobulins, mucous, intracellular tight junctions and some antimicrobial products contribute to the intestinal barrier function. Among these components, the tight junction is the major determinant of the intestinal physical barrier (Arrieta et al., 2006; Suzuki et al., 2011).

2.2.3.1.1 Tight junctions

Intercellular tight junctions are the major determinant of intestinal barrier function. The tight junctions connect the enterocytes to each other. They prevent macromolecular paracellular passage in a healthy intestine after weaning (Stokes et al., 1997). The tight junction can be found close to the apical side of the para-cellular space (Figure 2.5). The impairment of tight junctions in intestine is linked to various unfavorable situations such as inflammatory bowel disease, necrotizing enterocolitis and Crohn's disease (Suzuki & Hara, 2011). Tight junction is an important structural component of the epithelial barrier, and different aspects have been shown to correlate with the tight junction functionality, like oxidative stress, viability and electrical resistance.

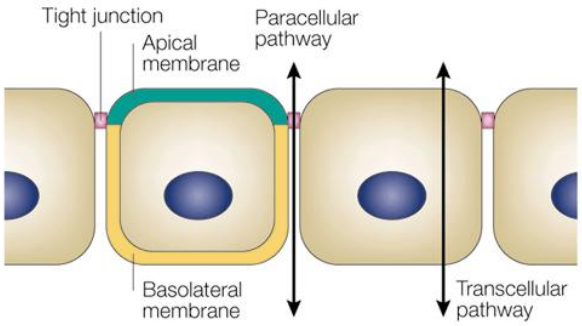

Figure 2.5 Two distinct pathways across cellular sheets (Tsukita et al., 2001)

Oxidative stress has negative effects on intestinal functionality. Research results showed increased intestinal permeability in H_2O_2 treated membranes, and the effect could be ameliorated by using anti-oxidants such as ascorbic acid or Trolox. The study shows that when the oxidative stress is increased in the porcine small intestinal epithelial cell line IPEC-J2 by diethyl maleate (DEM) treatment, the cells show a significant loss of tight junction proteins from the cell-cell contact complexes. The result also show a redistribution of tight junction proteins in the cell body under the effect of increased oxidative stress (Vergauwen et al., 2015).

Viability is the ability of organs, cells or tissues to maintain or recover viability (Pegg, 1989). The study of Vermeer et al. (2009) found out that a reduction of tight junction proteins could lead to a decrease of cell viability. The research showed that the decreased immunostaining of tight junction proteins suggesting disruption of barrier function led to the access of virus to the epithelial basolateral surface, which increased infections. As a result, the loss of epithelial integrity correlated with increased epithelial cell death. Similar results have been reported by using IPEC-J2 cells. The results showed that the decreased para-cellular tight junction level is accompanied by decrease IPEC-J2 cell viability (Vergauwen et al., 2015).

Transepithelial electrical resistance (TEER) reflects the functional integrity of a monolayer of cells and also the stability of tight junctions. A decreased electrical resistance suggests impairment of the intestinal barrier function. The study of Vergauwen et al. (2015) showed that when the tight junction is

impaired by DEM treatment, the trans-epithelial electrical resistance has been significantly reduced. Another study conducted by Zhuo et al. (2005) also demonstrated that in human trabecular meshwork cells, the increased expression of tight junction–related protein is associated with an upregulation of TEER.

2.2.3.1.2 Studies related to the effect of quercetin on intestinal permeability

Quercetin has been reported to have protective effects on intestinal permeability (Table 2.3). The study conducted by Suzuki et al. (2009) has used human intestinal Caco-2 cells to test the effect of quercetin. Their results showed that the treatment with quercetin reduced the Lucifer yellow flux, increased the TEER and the total protein expression of claudin-4. Similar results has been reported by Amasheh et al. (2008). Their results showed that quercetin has increased the activity of claudin-4 promotor, increased the cytoskeletal association of ZO-2, occludin, claudin-1 and increased TEER. Also, the study of Mercado et al. (2013) reviewed the effect of quercetin that the treatment increased the TEER, claudin-5, claudin-7, and the expression of claudin-4 while reduced the transepithelial mannitol permeability and claudin-2.

2.2.3.2 Effect of quercetin on morphology

The gastrointestinal tract has different possibilities to adapt or to react morphologically to changing conditions such as for instance birth, weaning, altered diet or altered composition of the intestinal microflora (Sangild, 1997; van Beers-Schreurs et al., 1998). The intestine can change its surface by growing in length and increasing or decreasing the height of villi. When the presence of villus increases, the surface of small intestine will be increased which would lead to the improvement of absorption ability and healthy status of animals. Epithelial cells, the enterocytes, cover the villus. The enterocytes on the upper third of a villus are fully equipped for their digestive and absorptive function. They have microvilli on their apical part bordering the gut lumen (Buddington, 1997). Diminishment of number or quality of villus or microvilli can be caused by food components and many microorganisms.

Quercetin has been reported to has protective effects on intestinal morphology (Table 2.3). The research of Bala et al. (2015) used sea buckthorn leaf extract which contains quercetin to test the function on mice small intestine. The treatment has a positive effect by increasing the jejunum crypts, villi number, villus height, villus cellularity, cryptal Paneth cells and cryptal stem cells proliferation. Similar result has been reported by the research conducted on mice. The results showed improvement in both intestinal length and villus height (Liu et al., 2013). Also, Nishijima et al. (2009) reported the effect of dietary quercetin on the intestinal morphology in rats. The rats feeding quercetin has shown increased cryptal depth in both jejunum and ileum, and the ileum villus thickness has also been increased.

2.2.3.3 Effect of quercetin on regeneration capacity

Damage and leakage of intestine may permit paracellular passage of unfavorable substances. It is of

great importance that the enterocytes layer could regenerate and repair after injures. The epithelial cells on the villus are continuously replaces by proliferation in the crypts, migration of cells from the crypts up to the villus. Unfavorable situations such as increased oxidative stress could lead to impaired wound healing capacities. The regenerative capacity could be rescued by using anti-oxidant such as Trolox (Vergauwen et al., 2015).

Quercetin has also been reported to have protective effects on the regenerative capacity of intestinal tissues (Table 2.3). *In vivo* study has used mice to test the effect of quercetin on regeneration capacity of villus. The sea buckthorn leaf has been reported to contain quercetin. In the study, the mice has been feed extract of sea buckthorn leaf (SBL-1) 30 minutes before irradiation. The results showed that the animals that were fed SBL-1 has significantly higher cryptal stem cells proliferation compared with the control group (Bala et al., 2015).

Tabel 2.3 Literature review related to the effect of quercetin on small intestine

Reference	Animal Species/ Cell type	Compounds	Method	Trial Set-up	Effect on small intestine
(Suzuki & Hara, 2009)	Human intestinal Caco-2 cells	Quercetin	Transwell culture system	•48 h •Incubated with or without 10-100 μM quercetin	•Lucifer yellow flux ↓ •TEER ↑ •Total protein expression of claudin-4 ↑
(Amasheh et al., 2008)	Intestinal Caco-2 cells	Quercetin	Cell culture	•72 h •Incubated with 0, 50, 100, 150, 200 μM quercetin	•Claudin-4 promotor activity↑ •Cytoskeletal association of ZO-2, occludin and claudin-1 ↑ •TEER ↑
(Mercado et al., 2013)	LLC-PK1 renal epithelial cell l	Quercetin	Cell culture	•48 h •Incubated with 0, 100, 200, 400 μM quercetin	•TEER↑ •Transepithelial mannitol permeability↓ •Claudin-4 expression ↑ •Claudin-5↑ •Claudin-7↑ •Claudin-2↓
(Bala et al., 2015)	Mice (Male Swiss mice)	Standardized sea buckthorn leaf extract (SBL-1)	Oral administration	•Treated 30 min prior to irradiation •Control group: 3 animals, administered sterile water •Treatment 1: 18 animals, irradiated, no SBL-1 •Treatment 2: 18 animals, SBL-1 (30mg/kg BW) only. •Treatment 3: 18 animals,SBL-1 administered 30 min prior to irradiation	•Jejunum crypts ↑ •Villi number ↑ •Villus height ↑ •Villus cellularity ↑ •Cryptal Paneth cells ↑ •Cryptal stem cells proliferation ↑
(Liu et al., 2013)	Mice (male BALB/c mice)	*Chimonanthus nitens* var. salicifolius (CS) aqueous extract	Intragastric administration	•11 days •Treatments: 0 (control group) 5, 10, 20 g/kg BW	•Intestinal length ↑ (20g/kg BW) •Villus height ↑ (20g/kg BW)
(Nishijima et al., 2009)	Rats (Male Wistar rats)	Quercetin	Dietary administration	•6 weeks •Treatment diets: control diet + quercetin; control diet + rutin; pectin diet + quercetin; pectin diet + rutin	•Jejunum crypts ↑ •Ileum crypts ↑ •Ileum villus thickness ↑

2.2.4 Animal performance

Studies have been conducted to explore the effect of quercetin on animal performance, these studies were summarized in Table 2.4. Researches has proven that quercetin has positive effects on growth performance, meat quality and production efficiency in different animal species. However, studies on pig are limited and findings are not always coherent with each other. For example, the study of Van Le Thanh et al. (2016) reported that quercetin did not mitigate the oxidative stress caused by DON-ZEN in weaned piglets.

2.2.4.1 Effect of quercetin on growth performance

Quercetin has been reported to have positive effect on the growth performance of animals (Table 2.4). A study on Tilapia fish showed that quercetin supplementation from a dose of 200 to 1600 mg/kg diet all resulted in significantly higher growth rate (Zhai & Liu, 2013). Also, a research on broiler chickens proved that the animals with dietary quercetin at dose of 200 and 300 mg/kg feed showed better growth performance (Sohaib et al., 2015).

2.2.4.2 Effect of quercetin on meat quality

More researches about dietary quercetin has been conducted to test the effect on meat quality (Table 2.4). Goliomytis et al. (2014b) have demonstrated the effect of dietary quercetin on the meat quality of broilers. The results showed that the oxidative stability of meat has been increased as well as the relative heart weight. Study of Zhai et al. (2013) also showed lower lipid levels in serum and whole body. Two papers of Andres et al. in 2013 and 2014 has shown similar results of quercetin on lambs. The quercetin supplementation with a dose of 2g/kg feed has increased the meat oxidative stability and decreased the meat saturated fatty acid proportion, meat discolouration and meat microbial population growth. Study of Sohaib et al. (2015) also shown increased breast meat lipid stability and decreased saturated fatty acid proportion by dietary quercetin. The research of Liu et al. (2014) has proved that dietary quercetin on laying hens could improve the laying rate and best effect were shown with doses around 367 to 369 mg/kg feed.

Tabel 2.4 Literature review related to the effect of dietary quercetin on animal performance

Reference	Animal Species	Compounds	Method	Trial Set-up	Animal performance
(Goliomytis et al., 2014b)	Chicken (broiler)	Quercetin	Dietary supplementation	•50 broilers per treatment •Control: normal diet for 42 d •Treatment 1: diet with quercetin (0.5 g/kg feed) •Treatment 2: diet with quercetin (1 g/kg feed)	•Meat oxidative stability ↑(1 g/kg of feed) •Relative heart weight ↑(1 g/kg of feed) •Feed intake = •Feed conversion ratio ↓(1 g/kg of feed)
(Zhai & Liu, 2013)	Fish (Tilapia)	Quercetin	Dietary supplementation	•100 fishes per treatment •7 weeks •Dietary quercetin levels of the 5 treatment groups: 0 (control group), 200, 400, 800 and 1600 mg/kg	•Growth rate ↑ (all concentrations) •Final body weight ↑ (all concentrations) •Lipid levels in serum and whole body ↓ •Feed conversion rate =
(Andres et al., 2014; Andres et al., 2013)	Lamb (fattening lambs)	Quercetin	Dietary supplementation	•8 animals per treatment •5 weeks •Control: diet with palm oil •Treatment 1: diet with palm oil and quercetin (2 g/kg feed) •Treatment 2: diet with linseed (85 g/kg feed) •Treatment 3: diet with linseed (85 g/kg feed) and quercetin (2 g/kg feed)	•Meat: saturated FA proportion ↓ •Meat oxidative stability ↑ •Meat discolouration ↓ •Meat microbial population growth ↓
(Liu et al., 2014)	Chicken (Hessian laying hens)	Quercetin	Dietary supplementation	•60 animals per treatment •8 weeks •Dietary quercetin levels of the 4 treatment groups: 0 (control group), 200, 400, 600 mg/kg	•Laying rate ↑ (best effect: 367 to 369 mg/kg feed) •Egg quality =
(Sohaib et al., 2015)	Chicken (broiler)	Quercetin	Dietary supplementation	•30 animals per treatment •6 weeks •Control: 0 quercetin and 0 α-tocopherol •Feed composition of 9 treatment groups: 3 levels of quercetin (100, 200, 300 mg/kg feed) in combination with 3 levels of α-tocopherol (150, 225, 300 mg/kg feed)	•Growth performance ↑ (all concentrations except T1) •Breast meat lipid stability↑ •Breast meat saturated FA proportion ↓

3.Materials and methods

3.1 Introduction

There is increasing evidence that oxidative stress is an important mechanism in the weaning induced villus atrophy and integrity loss of the small intestine of weaned piglets. Therefore, the possible alleviating effect of quercetin, a well-known anti-oxidative and anti-inflammatory polyphenol, on animal performance, blood, liver and small intestinal oxidative and redox status, the small intestinal permeability, morphology and regeneration capacity was tested in this study with newly weaned piglets.

3.2 Animals and experimental design

Two hundred twenty four suckling piglets (males and females, Topigs hybrid sow x Piétrain boar) have been weaned at 3 weeks of age and assigned to the 4 treatments (Table 3.1):

1) Control (CON): 56 piglets, 8 pens. Basal weaner and starter diets, no quercetin included in the feed (Diet FE1-T1).
2) Low quercetin (LQUE): 56 piglets, 8 pens. Basal weaner and starter diets, quercetin is provided into the weaner diet at 100 mg/kg feed (Diet FE1-T2)
3) Medium quercetin (MQUE): 56 piglets, 8 pens. Basal weaner and starter diets, quercetin is provided into the weaner diet at 300 mg/kg feed (Diet FE1-T3)
4) High quercetin (HQUE): 56 piglets, 8 pens. Basal weaner and starter diets, quercetin is provided into the weaner diet at 900 mg/kg feed (Diet FE1-T4)

All treatments have been replicated in 8 pens of 7 piglets (32 pens, 224 piglets in total) (Figure 3.1). Eight animals per treatment (one from each pen) have been sacrificed and sampled on day 5/day 6 and another 8 animals per treatment (one from each pen) on day 12/day 13 post-weaning. The experimental unit for feed intake and water intake is pen (n=8 per treatment). Live weight and average daily gain have been determined individually. For variables determined in blood, liver and the digestive tract, one animal per pen with a body weight close to the average pen body weight has been sampled, and the piglet is then considered as experimental unit (n=8 per treatment).

The animals were housed in a stable with controlled temperature (25 to 30℃) and controlled light:dark cycle (day 0 to day 5: 23L/1D; day 6 to day 42: 16L/8D). Each pen was equipped with feeder, water nipple and slatted floor (Figure 3.2). The water meter, water nipple, feeder and faecal score (Table 3.2) have been checked daily.

Table 3.1 Allocation of the treatments over the stable

Pen	Animals per pen	Block	Treatment	Pen	Animals per pen	Block	Treatment
1	7	1	CON	17	7	5	MQUE
2	7	1	LQUE	18	7	5	LQUE
3	7	1	MQUE	19	7	5	HQUE
4	7	1	HQUE	20	7	5	CON
5	7	2	CON	21	7	6	HQUE
6	7	2	HQUE	22	7	6	MQUE
7	7	2	LQUE	23	7	6	LQUE
8	7	2	MQUE	24	7	6	CON
9	7	3	MQUE	25	7	7	HQUE
10	7	3	LQUE	26	7	7	CON
11	7	3	HQUE	27	7	7	LQUE
12	7	3	CON	28	7	7	MQUE
13	7	4	MQUE	29	7	8	LQUE
14	7	4	CON	30	7	8	HQUE
15	7	4	LQUE	31	7	8	CON
16	7	4	HQUE	32	7	8	MQUE

3.3 Feed

In this trial, water has been provided *ad libitum* throughout the whole trial and a specialized drinking water system has been used to enable to record daily pen water intake. The diets have been fed as mash and ad libitum. Two different basal diets have been included in this experiment: basal weaner diet FE1 and basal starter diet FE2.

3.3.1 Basal weaner diet FE1

The basal weaner diet FE1 has been fed from d0 to d13 post-weaning. The experimental diets (FE1 T1-T4) have been produced from one batch of the basal weaner diet (FE1).

The basal weaner feed is composed of barley, wheat (58% starch), corn, soybean meal (soya 49 ARG Hypro), toasted soybean (Danex), sweet whey powder, lactose, sugar beet pulp, wheat gluten meal (amytex 80% RE), linseed oil (3%), lactic acid, monocalcium phosphate, potato protein (Protastar Avebe) and feed grade lime stone. Synthetic amino acid L-lysine HCl, DL-methionine, L-threonine, L-tryptophan and L-valine have been added to meet the requirements of piglets. Diets have been

formulated with special attention to vitamin E/dl-alpha tocopherol acetate (50 mg/kg), L-ascorbic acid (0 mg/kg), selenium/sodium selenite (0.35 mg/kg), copper/copper sulphate pentahydrate (15 mg/kg), iron/iron sulphate heptahydrate (100 mg/kg), cysteine (0.25% - digestible), methionine (0.40% - digestible), 2382 kcal/kg net energy content, 17.7% crude protein, 5% ether extract, 3.89% crude fibre.

Quercetin has been supplemented to the diet by adding the pure compound (>95%) to the diet, in a hydrated formula (monohydrate-dehydrate). No glycosides, aglycones and rutinosides has been supplemented. Quercetin has been supplied by Sigma-aldrich, product code Q4951.

Figure 3.1 Experimental stable with 32 pens

Figure 3.2 Examples of pens with feeder, water nipples and slatted floor

3.3.2 Basal starter diet FE2

The basal starter diet FE2 has been fed from d14 to d42. The experimental diet (FE2) has been offered to all treatments (T1-T4) during the respective feeding period.

The basal starter feed is composed of barley, wheat (58% starch), soybean meal (soya 49 ARG Hypro), corn, toasted soybean (Danex), sweet whey powder, linseed oil (3%), sugar been pulp, wheat gluten

meal (Amytex 80% RE), monocalcium phosphate and feed grade lime stone. Synthetic amino acids L-lysine HCl, DL-methionine, L-threonine, L-tryptophan and L-valine have been added to meet the requirements of piglets. Diets have been formulated with special attention to vitamin E/dl-alpha tocopherol (50 mg/kg), L-ascorbic acid (0 mg/kg), selenium/sodium selenite (0.35 mg/kg), copper/copper sulphate pentahydrate (15 mg/kg), iron/iron sulphate heptahydrate (100 mg/kg), cysteine (0.25% - digestible), methionine (0.40% - digestible), 2397 kcal/kg net energy content, 18% crude protein, 5% ether extract, 3.89% crude fibre.

3.4 Sampling procedure

3.4.1 Animal performance

The piglets have been weighed on d0, d5, d12, d14 and d42 individually. The average daily gain (ADG, kg/d) was calculated for day 0 to day 14 (d0-d14), day 14 to day 42 (d14-d42), and day 0 to day 42 (d0-d42). Average daily feed intake (ADFI, g/d) (only measured at pen level) and feed conversion ratio (FCR, kg feed / kg growth) were determined for the periods: d0-d14, d14-d42 and d0-d42.

3.4.2 Blood samples

The pigs were sampled at two time points post-weaning, being day 5 and day 12. From each pen, the piglet with the closest body weight to average body weight of the pen has been selected. Because the sampling of the animals was labor-intensive, the samplings for each time point were spread over a two day period. Therefore, piglets were sampled at day 5 and 6 post-weaning, but still considered sampled at time point d5 post-weaning. A similar approach was applied to time point day 12 post-weaning, where animals were actually sacrificed at day 12 or day 13 post-weaning. Blood sample has been collected from the jugular vein and divided into 10 mL heparinized tubes containing 200 µl 1mM bathophenanthrolinedisulfonic acid disodium salt hydrate (BPDS) . Red blood cells were obtained by centrifuging uncoagulated blood (3000g, 10 min) and removing the supernatant. To this residue, 100 µL of a70% meta-phosphoric acid solution was added to break up the red blood cells followed by intense vortex and centrifugation (3000 g, 10 min). A portion of the resulting acid extract (0.5 mL) is added to a tube containing 50 µL internal standard solution (ɤ-Glu-Glu). Samples were then stored at -80 ºC pending further analysis.

Blood plasma was harvested by collecting blood in a 15mL tube containing 300 µL ethylenediaminetetraacetic acid solution (EDTA). The blood was then centrifuged (15 min at 3000 g), and the supernatant has been transferred into vials. The plasma was stored at -20 ºC until further analysis.

3.4.3 Small intestinal samples

After exsanguination, the whole small intestine has been taken out and the total length has been measured. Two segments with length of 20 cm and 10 cm has been cut out at 5% of the total length of the small intestine. Three segments with length of 10 cm, 20 cm and 10 cm has been cut out at 75% of the total length of the small intestine. In the discussion of this thesis, the 5% small intestine has been defined as duodenum and the 75% small intestine has been defined as jejunum.The first 10 cm segment at 75% was collected for Ussing Chamber measurements. The 10 cm segments at both 5% and 75% were collected for histomorphology measurement. For the measurement of anti-oxidant parameters, the 20 cm segments at both 5% and 75% were collected.

For the segment of 20 cm, the sample was carefully excised and placed on ice cold surface. Segments were taken and then rinsed with cold saline (0.9% NaCl) to remove particulate matter. The segments were opened longitudinally to expose the epithelial surface and the mucosal layer was harvested by gentle scraping of the epithelium using a glass slide kept on ice.

About 1 g of the obtained mucosal samples were homogenized with 10 ml perchloric acid solution (PCA , 10% v/v in BPDS 1 mmol/L). Therefore, 5 mL PCA 10% was added to the sample in a glass tube and then homogenized at 900 rpm with the Braun homogenizer. This content was transferred to a centrifuge tube. Residues on the stamper and in the glass tube are then flushed with another 5 mL PCA 10% into the centrifuge tube. This homogenate is promptly centrifuged at 15000 g at 4 ºC for 15 min. A portion of the resulting acid extract (0.5 ml) is added to a tube containing 50 µL internal standard solution (ɤ-Glu-Glu). Samples were stored at -80 ºC pending further analysis. A sample of homogenate was taken for the determination of the protein content before centrifugation.

About 1 g of the obtained mucosal samples were homogenized with 10 mL phosphate buffer solution. 5 mL phosphate buffer solution was added to the sample in a glass tube and then homogenized at with the homogenizer. This content was transferred to a centrifuge tube. Residues on the stamper and in the glass tube are then flushed with another 5 mL phosphate buffer into the centrifuge tube. This homogenate is promptly centrifuged at 15000 g at 4 ºC for 15 min. The resulting phosphate buffer extract was transferred to four 2 mL vails and stored at -80 ºC pending further analysis. A sample of homogenate was taken for the determination of the protein content before centrifugation.

3.4.4 Liver samples

After exsanguination, the whole liver has been taken out and weighed. About 1 g of liver samples were cut out and homogenized with 10 ml PCA 10% following the same procedure for small intestine samples. About 2 g of liver sample were homogenized with 10 ml phosphate buffer following the same procedure for small intestine samples. Samples were stored at -80 ºC pending further analysis. A

sample of homogenate was taken for the determination of the protein content before centrifugation.

A sample of bile from the gallbladder was collected using syringe. The bile samples were stored at -20 °C pending further analysis.

3.5 Analysis

3.5.1 Glutathion redox-status (GSSG/GSH)

To determine the glutathione redox status, the concentrations of GSH and GSSG need to be determined in these specific tissues. During this procedure, the tissue samples that were homogenized in 10% PCA solution were used. The method includes a sample preparation and deravatiosation step. The method was adapted for the Yoshida (1996) and Reed et al., (1980) Before derivatization, 50 µL internal standard solution is added to 0.5 mL of standard solutions for determination of response factors. To the 0.5 mL of acid extracts or standard solutions containing the internal standard, 50 µL of 100 mmol/L iodoacetic acid solution is added. The acid solution (pink in color) is brought to pH 8-9 (purple in color) by the addition of 0.48 mL of KOH (2 mol/L)-KHCO3 (2.4 mol/L) and allowed to incubate in the dark at room temperature for 10 min.Next, 0.8 mL of 2,4-dinitrofluorobenzene is added, and the reaction mixture is capped, mixed with a vortex mixer, and stored at 4°C overnight. The final solution is stored in the dark at 4°C until HPLC analysis and is stable for at least 2 weeks. Samples are centrifuged and the supernatans is used for HPLC analysis. Accurate and reproducible measurements of derivatized samples stored for up to 6 weeks, however can be obtained using the internal standard method during HPLC analysis.

Derivatized thiols were separated on a EC250/4.6 Nucleosil 120-7 NH2 (aminopropyl column; Machery-Nagel, Duren, Germany) protected by the same NH2 guard column (CC8/4).

The injection volume was 100 µL. Chromatographic runs were performed at a flow-rate of 1.5 mL/min, starting at 80% A / 20% B for 5 min, followed by a 10 min linear gradient to 1% A / 99% B and a 10 min isocratic period at 1% A / 99% B. The column was then re-equilibrated to the initial conditions for 15 min. The column temperature was maintained at 40°C.. The UV detector was set at 365 nm for absorption measurements.

GSH and GSSG were identified by retention times of authentic standards. GSH and GSSG were determined by using the internal and external standards.

3.5.2 Glutathion related enzymes

3.5.2.1 Glutathione peroxidase activity(GPx)

The activity of GPx was determined by the reduction rate of the NADPH absorbance at 340nm using spectrophotometer (Hernandez et al., 2004). The measurement is based on following reactions: GPx reduces and reduces H2O2, that is added to the sample, backo water. Together with this reaction, GSH is turned into GSSG by the enzyme GR, which uses NADPH as reductant. To determine the activity of GPx the amount of NADPH used to reverse GSSG to GSH is measure by spectrophotometer. The concentration of NADPH is directly proportional to the activity of GPx. One unit of GPx will cause the formation of 1 umol NADP+ from NADPH per min at pH 8.0 at 25°C.

3.5.2.2 Glutathione S-transferase (GST)

Activity of GST was determined according to the method of (Habig & Jakoby, 1981). This method is to measure the GST activity by measuring the conjugation of 1-chhloro-2,4-dinitrobenzene (CDNB) with GSH. The conjugation is accompanied by an increase in absorbance at 340nm. The rate of increase is directly proportional to the GST activity in the samples One unit of GST will conjugate 1.0 umol of CDNB with GSH per minute at 25°C.

3.5.2.3 Glutathione reductase (GR)

Activity of GR was determined according to the method of Carlberg and Mannervik (1985), based on the reduction of GSSG by NADPH in the presence of GR. The reaction was measured by the decrease in the absorbance at 340nm using an extinction coefficient (ε^{mM}) of 6.22 for NADPH. One unit of GR will cause the oxidation of 1.0 µmole of NADPH at 25°C(pH = 7.5).

3.5.2.4 Glutamate cysteine ligase (GCL)

Activity of GCL was determined according to the method of White et al, based on the reaction of naphthalene-2,3-dicarboxialdehyde (NDA) with the ɣ-glutamylcysteine(ɣ-GC) produced in a medium containing ATP, glutamic acid, cysteine and sample (White et al., 2003). Fluorescence intensity of product formed was measured at 472 nm excitation and 528 nm emission using a fluorescence plate reader (Thermo Scientific, Marietta, OH, USA). One unit of GCL us defined as nmol of NDA-ɣ-GC formed per minute at 25°C (pH=7.5).

3.5.3 Ex vivo measurement for intestinal permeability of FD4

Intestinal mucosal permeability was assessed *ex vivo* by measuring the trans-location of macro molecular markers using the Ussing chamber technique described by the method of Michiels et al (2013). Fresh segments of proximal or distal SI were first rinsed with saline, then collected in duplicate, tripped from the seromuscular layer, pinned onto 1.07 cm^2 sliders and mounted into modified Ussing chambers (Dipl.-Ing.Muβler Scientific Instruments, Aachen, Germany). All tissues

were mounted within 15 minutes following euthanasia. Tissue were surrounded by 6.5 mL Ringer solution (115 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L $NaHCO_3$, 2.4 mmol/L Na2HPO4, 0.4 mmol/L NaH_2PO_4, 1.25 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$) with 6.24 mmol/L of mannitol or glucose in the luminal and serosal side, respectively. The system was water-jacketed to 37℃ and oxygenated with 95% O_2 and 5% CO_2 gas flow. After an equilibration period of 20 min, 4kDa fluorescein isothiocyanate-dextran (FD4, Sigma-Aldrich, Bornem, Belgium) was added to the mucosal side to a final concentration of 0.8 mg/mL of FD4. Samples from serosal side were taken at 20, 40, 60 and 100 min after adding markers. Meanwhile, the same volume of Ringer solution was also taken from the mucosal side to keep the volume balance across sides. Fluorescence intensity of FD4 in the medium was measured at excitation wavelength of 485 nm and emission wavelength of 538 nm using a fluorescence plate reader (Thermo Scientific, Marietta, OH, USA).

The apparent permeation coefficient (Papp) was calculated as: Papp (cm/s): (dc/dt) * V/c0/A, whereby dc/dt is the change of serosal concentration in the 20- to 100-min period; V is the volume of the chamber, c0 is the initial marker concentration in the mucosal reservoirs and A is the area of the exposed intestine in the chambers (cm^2).

3.5.4 ELISA for Caspase, OCC1 and PCNA

The concentration of specific tight junction proteins and markers for apoptosis and mitosis of the proximal and distal intestinal tissue samples was investigated using commercially available enzyme-linked immunosorbent assays (ELISA) of occludin (SEC228Hu), proliferating cell nuclear antigen (PCNA) (SEA591Hu) and caspase-3 (SEA626Hu) (Cloud-Clone Corporation®, Houston, TX, USA). All tissue samples were crushed, dissolved in phosphate-buffered saline solution (PBS, pH 7.4, 0.01 M), sonicated 6 times for 5 sec at 4°C, and kept on ice for 30 min. Subsequently the samples were centrifuged for 2 min at 13,400 rpm at 4°C, after which the supernatant was isolated. Then, the samples were diluted to a total protein concentration of 10 ng/µL. Samples were processed on a sandwich ELISA plate and the experiment was performed according to the manufacturer's instructions and absorbance was measured at 450 nm at 25°C. Values of protein expression were determined per mL of sample.

3.5.5 Malondialdehyde

MDA content was determined according to the method of Grotto et al.(Grotto et al., 2007) This method is to measure the MDA content by measuring thiobarbituric acid reactive substance (TBARS) concentrations. TBARS were formed from the reaction of MDA with 2-thiobarbituric acid in an acid environment. After extraction in 1-butanol, the absorbance of the colored complex was measured colorimetrically at 532 nm. A standard curve with 1,1,3,3-tetramethoxypropane was used.

3.5.6 Vitamin E content in liver

The vitamin E content in liver was determined according the method of Claeys, E., et al.(Claeys et al., 2016). Homogenized liver sample were weighed in an amber centrifuge tube of 75 ml. Forty milliliters of absolute ethanol (containing 5gl^{-1} ascorbic acid, freshly prepared) were added to extract the α-tocopherol and the contents were homogenized using an Ultra Turrax device. The tubes were held in a water bath at 78°C for 30 min, mixed manually about every 10 min and cooled to room temperature under running tap water. Subsequently, 7mL distilled water were added to the tubes and the tubes were cooled in an ice bath for at least 15 min. The tubes were centrifuged at 1100 × g for 10min and the supernatant (ethanol layer)was transferred to another centrifuge tube with screwcap. Twenty milliliters of KCl (57.5gl－1) and 5mL hexane (containing 0.02gL－1 BHT) were added and the mixture was shaken vigorously, and then centrifuged for 5 min at 720 × g. The supernatant (hexane layer) was transferred to an amber test tube using a Pasteur pipette. The extraction with 5mL hexane was repeated and the supernatant was added to the same test tube. The total hexane phase was evaporated using nitrogen gas and the residue was re dissolved in 1mL methanol, and then filtered through a 0.2μm cellulose filter(Machery Nagel Chromafil RC-20/25) using a syringe of 5mL. The filtrate was collected in an amber vial of 1.5mL which was closed with a crimp cap.

Analysis was carried out immediately after extraction as well as a series of standard solutions of known concentrations of α-tocopherol. Standard solutions were injected separately and were used as an external standard. 10 μL of liver sample solution were injected and the separation was carried out using two solvents: solvent A was a methanol – water solution (94:6, v/v) and solvent B was pure HPLC-grade methanol. The separation started with 0.5min of solvent A, followed by a linear gradient to 100% solvent B in the next 11.5min and keeping solvent B at 100% for another 19 min. Then, the system returned to 100% solvent A by a linear gradient in the next 13.5min and this was held for 1 min. Total separation time was 44.5min.

3.6 Statistical analysis

After carrying out the above analyzes, the results were processed to a well-organized set of data. The obtained variables are:

- Animal performance:

  Body weight

  Average daily gain

  Average daily feed intake

  Feed conversion rate

- Blood:
    - GPx activity
    - GST activity
    - MDA concentration
    - GSH concentration, GSSG concentration, GSSG/GSH ratio
- Proximal small intestine:
    - GR activity
    - GPx activity
    - GST activity
    - MDA concentration
    - GSH concentration, GSSG concentration, GSSG/GSH ratio
- Distal small intestine:
    - GR activity
    - GPx activity
    - GST activity
    - MDA concentration
    - GSH concentration, GSSG concentration, GSSG/GSH ratio
    - *Ex vivo* FD4 fluxes value
    - Caspase concentration, OCC1 concentration, PCNA concentration
- Liver:
    - GR activity
    - GPx activity
    - GST activity
    - MDA concentration
    - GSH concentration, GSSG concentration, GSSG/GSH ratio
    - Vitamin E concentration

Data were analyzed using SPSS Statistics 23. The univariate procedure was used for each parameter to

study the influence of quercetin with the fixed factor treatment and the block run as a random effect (P-u value in tables). Fisher's least significant difference (LSD) test were performed for all pairwise comparisons between means. Data are expressed as means and their standard errors (SEM). As some effects were found inconsistent across quercetin dose, data were further explored by using an orthogonal contrast between control versus quercetin (3 levels of quercetin taken together) (P-c value in tables). $P < 0.05$ was considered significant.

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4. Results

4.1 Animal performance

Table 4.1 shows the growth performance of post-weaning piglets fed feeds with different concentration of quercetin supplementation. The results show no significant effect of quercetin on body weight of piglets at day 0, day 5 and day 14. At day 29 and day 42, the results show significant difference between treatment QUE300 and QUE900 (P = 0.033 and 0.046, respectively). At day 29 and day 42, the QUE900 body weight are 9.0% and 9.0% higher than QUE300.

The results of average daily gain showed no significant difference between treatments. But there is a trend of increased average daily gain for QUE900 between day 0 to day 42 (P = 0.056), during which period the QUE900 is 5.6%, 7.1% and 13.5% higher than CON (P = 0.177), QUE100 (P = 0.108) and QUE300 (P = 0.007) respectively. Also, a trend has been shown from d14 to day 29 (P = 0.093), during which period QUE900 is 5.7%, 6.7% and 11.0% higher than CON (P = 0.154), QUE100 (P = 0.098) and QUE300 (P = 0.015).

The results of average daily feed intake also show no significant effect of treatment. But a statistical trend has been observed for day 14-42 (P = 0.069) and day 0-42 (P = 0.059). During day 14-42, the QUE900 is 7.5%, 7.4% and 8.9% higher than CON (P = 0.038), QUE100 (P = 0.043) and QUE300 (P = 0.018). During day 0-42, the QUE900 is 8.3%, 8.9% and 10.3% higher than CON (P = 0.038), QUE100 (P = 0.032) and QUE300 (P = 0.015).

The results of feed conversion showed no significant treatment difference. No significant effect has been observed by using contrasts test.

4.2 Enzyme activities related to glutathione redox cycle

4.2.1Results from day 5 post-weaning

The results of the treatment effect on enzyme activities from day 5 are presented in Table 4.2. The enzymes has been tested in plasma, liver and small intestine mucosa.

The activity of GPx is not significantly affected by different concentration of quercetin supplementation in all tissues. But a statistical trend (P = 0.062) of decreased activity has been shown in quercetin groups in plasma which showed that QUE100 has significantly lower activity than CON. Significant effect has been shown when using contrast test (P = 0.047) which showed lower GPx activity in quercetin groups than quercetin-free groups.

Tabel 4.1 Effect of treatment on animal performance during the first 42 days post-weaning

	TREATMENT				SEM	P-value	
	CON	QUE 100	QUE 300	QUE 900		P-u	P-c
Body weight							
d0 post-weaning (kg)	5.88	5.89	5.88	5.9	0.045	0.987	0.950
d5 post-weaning (kg)	6.01	6..00	5.86	6.00	0.058	0.262	0.502
d14 post-weaning (kg)	6.85	6.73	6.54	7.06	0.161	0.18	0.717
d29 post-weaning (kg)	11.59^{ab}	11.42^{ab}	11.06^{a}	12.06^{b}	0.222	**0.033**	0.792
d42 post-weaning (kg)	17.92^{ab}	17.78^{ab}	17.11^{a}	18.65^{b}	0.355	**0.046**	0.873
Average Daily Gain							
d0-d5 (kg/d)	0.03	0.02	0.00	0.02	0.01	0.245	0.346
d5-d14 (kg/d)	0.09	0.08	0.08	0.12	0.015	0.225	0.886
d14-d29 (kg/d)	0.32^{ab}	0.31^{ab}	0.30^{a}	0.33^{b}	0.009	0.093	0.987
d29-d42 (kg/d)	0.49	0.49	0.47	0.51	0.016	0.391	0.993
d0-d14 (kg/d)	0.07	0.06	0.05	0.08	0.012	0.249	0.695
d14-d42 (kg/d)	0.40	0.39	0.38	0.41	0.011	0.16	0.989
d0-d42 (kg/d)	0.29^{ab}	0.28^{ab}	0.27^{a}	0.30^{b}	0.009	0.056	0.866
Daily Feed Intake							
d0-d5 (kg/d)	0.07	0.07	0.06	0.07	0.007	0.614	0.406
d5-d14 (kg/d)	0.18	0.17	0.15	0.2	0.013	0.157	0.705
d14-d29 (kg/d)	0.44	0.43	0.42	0.47	0.014	0.11	0.855
d29-d42 (kg/d)	0.73	0.74	0.73	0.78	0.018	0.148	0.383
d0-d14 (kg/d)	0.14	0.13	0.12	0.14	0.009	0.209	0.568
d14-d42 (kg/d)	0.57^{a}	0.57^{a}	0.56^{a}	0.61^{b}	0.014	0.069	0.444
d0-d42 (kg/d)	0.40^{a}	0.40^{a}	0.39^{a}	0.43^{b}	0.011	0.059	0.516
Feed Conversion Rate							
d0-d5	0.32	0.26	0.08	0.28	0.154	0.748	0.514
d5-d14	0.50	0.47	0.53	0.60	0.141	0.343	0.655
d14-d29	0.73	0.72	0.71	0.71	0.019	0.92	0.594
d29-d42	0.66	0.66	0.63	0.65	0.013	0.433	0.336
d0-d14	0.49	0.45	0.48	0.57	0.206	0.487	0.426
d14-d42	0.69	0.69	0.67	0.68	0.013	0.425	0.270
d0-d42	0.72	0.72	0.69	0.71	0.015	0.432	0.895

CON = Control diet; QUE100 = quercetin is provided into the diet at 100 mg/kg feed; QUE300 = quercetin is provided into the weaner diet at 300 mg/kg feed; QUE900 = quercetin is provided into the weaner diet at 900 mg/kg feed; Feed conversion ratio was calculated by dividing the weight gained by feed consumed using the following expression: FCR = weight gain / feed consumed; P-u = P value obtained by using the univariate test to test the treatment effect; P-c = P value obtained by using contrasts test; Values are presented as least square means with SEM (n=16 per treatment); Values with different superscripts within a row are significantly different (P<0.05; LSD post-hoc test).; P value smaller than 0.05 are in bold.

The activity of GST has been affected significantly by treatment in liver (P = 0.009). The QUE100,

QUE300 and QUE900 are 46.4%(P = 0.004), 50.9% (P = 0.002) and 42.4% (P = 0.008) higher than CON. A statistical trend of treatment effect has been observed in distal small intestine mucosa (P = 0.086). The QUE900 shows 18.3% lower GST activity than CON (P = 0.192). But the contrast test suggest no difference between quercetin and quercetin-free treatment. Another statistical trend of increased activity has been shown in plasma by the contrast test (P = 0.068).

The activity of GR are not significantly affected by treatment although statistical trend of increased activity been observed by contrast test in distal small intestine mucosa (P = 0.053). The QUE900 and QUE100 show 16.7% (P = 0.082) and 21.5% (P = 0.028) higher GR activity than CON.

Tabel 4.2 Effect of treatment on enzyme activities that related to GSH redox cycle (day 5)

	TREATMENT				SEM	P-value	
	CON	QUE	QUE	QUE		P-u	P-c
Glutathione peroxidase (GPx)							
Plasma (U/ml)	0.33[a]	0.23[b]	0.29[ab]	0.29 [ab]	0.024	0.062	**0.047**
Liver (U/g)	23.4	21.19	22.57	19.01	1.593	0.213	0.152
Proximal SI mucosa (U/g)	1.67	1.41	1.56	1.50	0.148	0.642	0.320
Distal SI mucosa (U/g)	1.27	1.28	1.43	1.35	0.19	0.925	0.707
Glutathione- S -transferase (GST)							
Plasma (U/ml)	0.003	0.004	0.003	0.003	0.003	0.230	0.068
Liver (U/g)	10.69[a]	15.65 [b]	16.12[b]	15.22 [b]	1.045	**0.009**	**0.009**
Proximal SI mucosa (U/g)	3.32	3.56	3.03	3.94	0.567	0.713	0.771
Distal SI mucosa (U/g)	3.50[a]	2.94 [ab]	2.21 [b]	2.86 [ab]	0.319	0.086	0.268
Glutathione reductase (GR)							
Liver (U/g)	2.18	1.93	2.12	1.87	0.14	0.390	0.322
Proximal SI mucosa (U/g)	6.01	5.46	5.45	5.56	0.407	0.738	0.320
Distal SI mucosa (U/g)	5.63	6.84	6.00	6.57	0.364	0.110	0.053

SI = Small intestine; CON = Control diet; QUE100 = quercetin is provided into the diet at 100 mg/kg feed; QUE300 = quercetin is provided into the weaner diet at 300 mg/kg feed; QUE900 = quercetin is provided into the weaner diet at 900 mg/kg feed; P-u = P value obtained by using the univariate test to test the treatment effect; P-c = P value obtained by using contrasts test; Values are presented as least square means with SEM (n=8 per treatment); Values with different superscripts within a row are significantly different (P<0.05; LSD post-hoc test).; P value smaller than 0.05 are in bold.

4.2.2 Result from day 12 post-weaning

The results of the treatment effect on enzyme activities from day 12 are presented in Table 4.3. The enzymes has been tested in plasma, liver and small intestine mucosa.

The activity of GPx shows significant treatment effect in distal small intestine mucosa (P = 0.045). The QUE900 shows 42.7% higher activity than CON (P =0.027). Also, the activity in plasma shows numerical increase. The QUE300 and QUE100 are 24.9% (P = 0.052) and 22.0% (P = 0.083) higher

than CON. No significant effect has been observed by using contrast test.

The activity of GST shows significant treatment effect in plasma (P = 0.031). The QUE900 group has higher GST activity than CON (P = 0.059) and QUE100 (P = 0.005). Also, the QUE 300 group has higher activity than QUE100 (P = 0.04). A statistical trend has been observed in liver (P = 0.079). The QUE100 has 17.8% increase when compared with CON (P = 0.016). No significant effect has been observed by using contrast test.

The GR activity in liver has been affected significantly by the treatment (P = 0.038). The group of QUE300 and QUE100 has 36.5% (P = 0.005) and 21.4% (P = 0.084) higher GR activity than CON.

Tabel 4.3 Effect of treatment on enzyme activities that related to GSH redox cycle (day 12)

	TREATMENT				SEM	P-value	
	CON	QUE	QUE	QUE		P-u	P-c
Glutathione peroxidase (GPx)							
Plasma (U/ml)	0.31	0.37	0.38	0.35	0.026	0.198	0.152
Liver (U/g)	29.65	30.42	28.92	29.73	1.444	0.909	0.985
Proximal SI mucosa (U/g)	1.03	1.27	1.27	1.18	0.089	0.205	0.164
Distal SI mucosa (U/g)	1.01^{a}	1.29^{ab}	0.96^{a}	1.45^{b}	0.129	**0.045**	0.153
Glutathione- S -transferase (GST)							
Plasma (U/ml)	0.002^{ab}	0.002^{a}	0.003^{b}	0.003^{b}	0.000	**0.031**	0.620
Liver (U/g)	3.07^{a}	3.62^{b}	3.17^{a}	3.34^{ab}	0.148	0.079	0.100
Proximal SI mucosa (U/g)	2.65	2.5	2.36	2.57	0.261	0.887	0.556
Distal SI mucosa (U/g)	2.46	2.41	3.11	2.89	0.419	0.589	0.497
Glutathione reductase (GR)							
Liver (U/g)	1.68^{a}	2.04^{ab}	2.30^{b}	1.91^{ab}	0.140	**0.038**	**0.038**
Proximal SI mucosa (U/g)	4.54	4.34	4.5	4.54	0.129	0.650	0.622
Distal SI mucosa (U/g)	4.58	5.06	4.61	4.78	0.221	0.413	0.391

SI = Small intestine; CON = Control diet; QUE100 = quercetin is provided into the diet at 100 mg/kg feed; QUE300 = quercetin is provided into the weaner diet at 300 mg/kg feed; QUE900 = quercetin is provided into the weaner diet at 900 mg/kg feed; P-u = P value obtained by using the univariate test to test the treatment effect; P-c = P value obtained by using contrasts test; Values are presented as least square means with SEM (n=8 per treatment); Values with different superscripts within a row are significantly different (P<0.05; LSD post-hoc test).; P value smaller than 0.05 are in bold.

4.3 Glutathione redox cycle and malondialdehyde content in erythrocytes, liver and intestinal mucosa

4.3.1 Results from day 5 post-weaning

The results of treatment effect on GSH redox cycle and MDA content in erythrocytes, liver and intestinal mucosa at day 5 are presented in Table 4.5. None of the parameters has shown significant treatment effect.

Tabel 4.5 Effect of treatment on GSH redox cycle and MDA content in plasma, liver and intestinal mucosa (day 5)

	TREATMENT				SEM	P-value	
	CON	QUE 100	QUE 300	QUE 900		P-u	P-c
Plasma/Erythrocytes							
GSH (nmol/mL)	147.1	139.65	124.8	133.81	11.226	0.562	0.311
GSSG (nmol/mL)	46.68	42.62	38.18	34.75	3.824	0.195	0.155
Ratio GSSG/GSH	0.34	0.32	0.32	0.25	0.040	0.510	0.408
MDA (nmol/mL)	9.01	8.46	8.62	7.94	0.417	0.361	0.180
Liver							
GSH (nmol/mg)	1.16	1.30	1.14	1.26	0.121	0.761	0.745
GSSG (nmol/mg)	0.12^{a}	0.19^{b}	0.13^{a}	0.14^{ab}	0.020	0.080	0.120
Ratio GSSG/GSH	0.11^{a}	0.17^{b}	0.12^{ab}	0.12^{ab}	0.021	0.206	0.082
MDA (nmol/mg)	171.48	159.93	146.85	155.57	11.263	0.533	0.107
Vitamin E (ug/g)	2.70	2.66	2.62	2.55	0.282	0.986	0.795
Proximal SI mucosa							
GSH (nmol/mg)	1.24	1.55	2.35	0.99	0.631	0.509	0.557
GSSG (nmol/mg)	0.17	0.29	0.55	0.13	0.188	0.448	0.232
Ratio GSSG/GSH	0.20	0.19	0.18	0.18	0.046	0.981	0.535
MDA (nmol/mg)	49.98	47.48	46.94	43.81	3.382	0.646	0.478
Distal SI mucosa							
GSH (nmol/mg)	0.70	0.73	1.70	0.60	0.617	0.573	0.425
GSSG (nmol/mg)	0.17	0.18	0.53	0.18	0.214	0.596	0.333
Ratio GSSG/GSH	0.25	0.26	0.25	0.28	0.031	0.947	0.828
MDA (nmol/mg)	47.53	53.28	49.34	52.18	4.758	0.822	0.479

SI = Small intestine; CON = Control diet; QUE100 = quercetin is provided into the diet at 100 mg/kg feed; QUE300 = quercetin is provided into the weaner diet at 300 mg/kg feed; QUE900 = quercetin is provided into the weaner diet at 900 mg/kg feed; MDA = Malondialdehyde; GSH = Glutathione reduced; GSSG = Glutathione oxidized; P-u = P value obtained by using the univariate test to test the treatment effect; P-c = P value obtained by using contrasts test; Values are presented as least square means with SEM (n=8 per treatment); Values with different superscripts within a row are significantly different ($P<0.05$; LSD post-hoc test).; P value smaller than 0.05 are in bold.

In Liver, a statistic trend has been observed on GSSG concentration ($P = 0.08$). The QUE100 group has 58.2% increase when compared with CON ($P = 0.023$). When use the contrast test, statistical trend has been shown in GSSG/GSH ratio between quercetin groups and quercetin-free groups ($P = 0.082$)

which suggested the quercetin treatment might contributed to the higher GSSG/GSH ratio.

4.3.2 Results from day 12 post-weaning

The results of treatment effect on GSH redox cycle and MDA content in erythrocytes, liver and intestinal mucosa at day 12 are presented in Table 4.6.

In erythrocytes, the treatment has a significant effect on GSSG/GSH ratio (P = 0.027). The QUE900 and QUE300 show 18.8% (P = 0.19) and 43.2% (P = 0.006) increase when compare with CON. The contrast test of this parameter showed a statistical trend of increase in quercetin groups.

Tabel 4.6 Effect of treatment on GSH redox cycle and MDA content in erythrocytes, liver and intestinal mucosa (day 12)

	TREATMENT				SEM	P-value	
	CON	QUE 100	QUE 300	QUE 900		P-u	P-c
Plasma/Erythrocytes							
GSH (nmol/mL)	180.39	179.93	147.17	176.23	12.465	0.211	0.397
GSSG (nmol/mL)	31.18	33.23	36.54	36.33	3.296	0.614	0.284
Ratio GSSG/GSH	0.18[a]	0.19[a]	0.25[b]	0.21[ab]	0.017	**0.027**	0.056
MDA (nmol/mL)	7.00	7.55	7.28	7.36	0.262	0.527	0.180
Liver							
GSH (nmol/mg)	4.26	1.57	1.45	1.46	1.349	0.389	0.335
GSSG (nmol/mg)	0.10	0.08	0.07	0.06	0.021	0.554	0.190
Ratio GSSG/GSH	0.04	0.06	0.05	0.04	0.009	0.521	0.473
MDA (nmol/mg)	123.08	131.76	136.41	133.09	4.673	0.249	0.056
Vitamin E (ug/g)	1.92	1.73	2.39	1.91	0.201	0.144	0.720
Proximal SI mucosa							
GSH (nmol/mg)	2.30	1.36	1.29	1.54	0.335	0.154	0.204
GSSG (nmol/mg)	0.19	0.10	0.10	0.10	0.029	0.128	0.165
Ratio GSSG/GSH	0.08	0.07	0.08	0.07	0.008	0.567	0.360
MDA (nmol/mg)	33.83	31.61	34.13	30.34	1.785	0.399	0.342
Distal SI mucosa							
GSH (nmol/mg)	0.75	0.72	0.61	0.95	0.125	0.308	0.960
GSSG (nmol/mg)	0.09	0.07	0.07	0.10	0.021	0.632	0.777
Ratio GSSG/GSH	0.11	0.10	0.11	0.10	0.010	0.619	0.600
MDA (nmol/mg)	38.80[a]	39.93[a]	48.47[b]	42.27[ab]	2.453	**0.049**	0.088

SI = Small intestine; CON = Control diet; QUE100 = quercetin is provided into the diet at 100 mg/kg feed; QUE300 = quercetin is provided into the weaner diet at 300 mg/kg feed; QUE900 = quercetin is provided into the weaner diet at 900 mg/kg feed; MDA = Malondialdehyde; GSH = Glutathione reduced; GSSG = Glutathione oxidized; P-u = P value obtained by using the univariate test to test the treatment effect; P-c = P value obtained by using contrasts test; Values are presented as least square means with SEM (n=8 per treatment); Values with different superscripts within a row are significantly different (P<0.05; LSD post-hoc test).; P value smaller than 0.05 are in bold.

In liver, none of the parameters shows significant treatment effect. When use contrast test, a statistic trend has been observed on MDA concentration (P = 0.056). The QUE900 and QUE300 has 8.1% (P = 0.144) and 10.8% (P = 0.057) increase of MDA when compared with CON.

In proximal small intestine mucosa, none of the parameters shows significant treatment effect. In distal small intestine mucosa, the effect of treatment is significant in MDA concentration (P = 0.049). The QUE300 has higher MDA than QUE900 (14.7%, P = 0.089), QUE100 (21.4%, P = 0.023) and CON (24.9%, P = 0.011). Also, statistical trend has been observed in this parameter (P = 0.088) which suggests higher MDA in quercetin groups.

4.4 Parameters related to small intestine functionality

Table 4.8 shows the result of treatment effect on the small intestinal functionality. No significant treatment effect has been observed in table 4.8.

Tabel 4.8 Effect of treatment on distal small intestine functionality .

	TREATMENT				SEM	P-value	
	CON	QUE	QUE	QUE		P-u	P-c
Day 5 post-weaning							
FD4 fluxes (10^{-7}cm/s）	10.03	8.77	8.94	7.46	1.967	0.835	0.475
Caspase (ng/mL)	3.88	4.17	4.14	3.73	0.435	0.870	0.785
OCC1 (ng/mL)	0.10^{a}	0.22^{b}	0.14^{ab}	0.09^{a}	0.036	0.082	0.093
PCNA (ng/mL)	0.93	0.92	0.85	0.90	0.057	0.781	0.564
Day 12 post-weaning							
FD4 fluxes (10^{-7}cm/s）	9.27	11.01	11.96	8.31	2.610	0.754	0.675
Caspase (ng/mL)	3.56	3.14	2.67	2.73	0.408	0.403	0.182
OCC1 (ng/mL)	0.10	0.06	0.04	0.09	0.033	0.546	0.304
PCNA (ng/mL)	1.26	1.20	1.12	1.22	0.113	0.837	0.592
Average							
FD4 fluxes (10^{-7}cm/s）	9.65	9.89	10.45	7.89	1.529	0.668	0.891
Caspase (ng/mL)	3.72	3.65	3.41	3.23	0.321	0.684	0.444
OCC1 (ng/mL)	0.10	0.14	0.09	0.09	0.027	0.568	0.870
PCNA (ng/mL)	1.10	1.06	0.99	1.06	0.077	0.781	0.503

PCNA = Proliferating cell nuclear antigen; OCC1 = Occluding 1 protein, corresponding to synthetic peptide of occludin; CON = Control diet; QUE100 = quercetin is provided into the diet at 100 mg/kg feed; QUE300 = quercetin is provided into the weaner diet at 300 mg/kg feed; QUE900 = quercetin is provided into the weaner diet at 900 mg/kg feed; P-u = P value obtained by using the univariate test to test the treatment effect; P-c = P value obtained by using contrasts test; Values are presented as least square means with SEM (n=16 per treatment); Values with different superscripts within a row are significantly different (P<0.05; LSD post-hoc test).; P value smaller than 0.05 are in bold.

At day 5, a statistical trend has been observed on OCC1 content in distal small intestine (P = 0.082). The QUE100 has high OCC1 content than QUE900 (134.8%, P = 0.022) and CON (120.4%, P = 0.03). Also, when use the contrast test, a statistical trend has been shown in this parameter (P = 0.093) which suggests higher OCC1 content in quercetin groups than quercetin-free groups.

5.Discussion

In this study, the effect of dietary quercetin on GSH kinetics in small intestinal mucosa, blood and liver weaned piglets were examined, and the effective concentration of dietary quercetin was evaluated by using 3 different concentrations. The experimental piglets were weaned at 21 days of age and have been divided into 4 groups. During the 42 days of experiment, each groups has been taken a diet with quercetin concentration of either 0, 100, 300 or 900 mg/kg feed for first 14 days post-weaning and the same quercetin-free starter feed were taken by the 4 groups for the rest of experiment. The parameters that have been tested are performance and health, oxidative statues in different tissues and intestinal barrier integrity.

5.1 Effect of dietary quercetin on animal performance

Significant difference has been found in body weight at day 29 and day 42 post-weaning. The results showed that at both days, the group with quercetin of 900 mg/kg feed has significant higher body weight than the group with 300 mg/kg feed. However, there's no significant difference between quercetin treated groups and non-quercetin groups, which is in accordance with the study of Goliomytis et al (2014b) (1 g/kg feed, broilers), but in contrast with the study of Zhai & Liu (2013) (200 to 1600 mg/kg feed, fishes) and the study of Sohaib et al. (2015) (100, 200, 300 mg/kg feed, broilers).

Although no significant difference has been found in average daily gain, there are statistical trend of increase for QUE900 compared with QUE300 from day 14 to day 29 post-weaning ($P = 0.093$) and from day 0 to day 42 post-weaning ($P = 0.056$). This result is similar with the result of body weight in day 29 and day 42 post-weaning. Although the effect could resulted from the higher quercetin concentration in group QUE900, the difference may also be attributed by the unfavorable situation during the experimental period or unhealthy individuals in group QUE300, since the results of QUE300 stay the lowest among the 4 groups from day 5 to day 42 post-weaning. The test results from the contrasts test also support the assumption since no difference has been detected between the quercetin groups and quercetin -free group in average daily gain. The results differ from the result which reported higher growth rate in quercetin treated fishes and chicken (Sohaib et al., 2015; Zhai & Liu, 2013).

Results from average daily feed intake also shows no significant difference, but statistical trends has been shown from day 14 to day 42 post-weaning ($P = 0.069$) and from day 0 to day 42 post-weaning ($P = 0.059$), between the QUE900 group with the 3 other groups. The results could be biologically linked to the effect of quercetin, since QUE900 has the highest concentration of dietary quercetin, and QUE900 showed highest feed intake among the 4 groups during the whole experimental period. The

significantly higher feed intake would help the QUE900 group to deal with the weaning period easier by faster adjustment to solid feed and improve the digestion ability. The results also suggested that the effect of quercetin on animal performance in strongly linked to the concentration, since the results from the contrasts test showed that there is no significant difference when consider the 3 quercetin groups as a whole, and the only significant results came from the QUE900 group compared with the control group. But this results are in contrast with the study of Goliomytis, Tsoureki, et al. (2014b) since their results showed no effect of quercetin (1g/kg feed) on the feed intake of broilers.

No significant difference has been found in feed conversion rate among the 4 groups, which suggest that the concentrations of quercetin we tested has no positive or negative effect on feed conversion rate in weaned piglets. Our results are in agreement with Zhai & Liu (2013) (200 to 1600 mg/kg feed), but differ from the results of Goliomytis, Tsoureki, et al. (2014b) since they reported negative effect of quercetin (1 g/kg feed) on feed conversion ratio in broilers.

To conclude, in this study, quercetin treatment groups showed no significant difference in body weight, average daily gain and feed conversion rate when compared with quercetin-free group. Statistical trend has been observed between QUE900 groups and other groups in average daily feed intake which could help the piglets to adjust to the solid feed easier and deal with the weaning stress. The results suggest that the effect of quercetin may be affected by the supplemental dose as well as the species.

5.2 Effect of dietary quercetin on enzyme activities related to glutathione redox cycle

Results of GPx from day 5 post-weaning shows significant differences between quercetin treatment groups and quercetin-free group ($P = 0.047$). In plasma, the highest GPx activity appears in the control group, and the QUE100 group has the lowest activity. But this difference has been altered in day 12 post-weaning. In day 12 post-weaning, the 3 quercetin treatment groups all showed higher GPx activity than the control group in plasma, and the QUE300 and QUE100 are 24.9% ($P = 0.052$) and 22.0% ($P = 0.083$) higher than CON. This suggests that the QUE100, QUE300 and QUE900 groups has improved their anti-oxidative capacity from day 5 to day 12 post-weaning, which could be resulted from the dietary quercetin. Thus, quercetin changed the unfavorable situations in day 5 post-weaning and improved the antioxidant system in weaned piglets. The results are in agreement with the study which reported the protective effect of quercetin on animals by upregulating impaired GPx level (Coskun et al., 2005; Sanders et al., 2001). Also, GPx results from day 12 post-weaning showed significant treatment effects in distal small intestinal mucosa ($P = 0.045$). The QUE900 shows 42.7% higher activity than CON ($P =0.027$). This result is in accordance with Dias et al. (2005) and Molina et al. (2003). The results suggested that quercetin has an effect on improve GPx activity in distal small intestine.

Results of GST from day 5 post-weaning shows significant difference between the control group and the 3 quercetin treatment groups in liver (P = 0.009). The QUE100, QUE300 and QUE900 are 46.4% (P = 0.004), 50.9% (P = 0.002) and 42.4% (P = 0.008) higher than CON. Also, results from day 12 post-weaning showed a statistical trend in liver (P = 0.079). The QUE100 has 17.8% increase when compared with CON (P = 0.016). The results are in agreement with Odbayar et al. (2009) and Olayinka et al. (2014). Since GST is the enzyme to catalyze the conjugation of GSH with a wide range of harmful compounds (Hayes & Pulford, 1995; Nordberg & Arner, 2001), the higher activity from the quercetin treatment groups suggested higher capacity of liver to bind and excrete harmful substances through GSH-conjungation. Also, since a lot of lipid peroxidation products are excreted by this pathway, avoiding them to take part in further oxidation chain reactions, it can be speculated that the results of GST in liver at day 5 and day 12 post-weaning suggests that dietary quercetin has a positive effect on antioxidant system in weaned piglets. However, since there are studies which reported quercetin as an oxidant (Miles et al., 2014), another possible explanation could be the increased hepatic GST level is up-regulated to metabolize the quercetin to water soluble compounds in which way it could be excreted. Next to the results in liver on day 5 post-weaning, also the GST activity in the distal small intestinal mucosa showed a statistical trend (P = 0.086). The QUE300 shows 37.0% lower GST activity than CON (P = 0.013) and the QUE900 shows 18.3% lower GST activity than CON (P = 0.192). But at day 12 post-weaning, the trend in the same tissue has been alleviated that the GST activity of 4 groups showed no significant difference. This suggests that the quercetin treatments has increased the GST level of QUE300 and QUE900 in distal SI mucosa from day 5 to day14 post-weaning, and helped the 2 groups to build up better antioxidant system. This result is in accordance with the study which reported the quercetin treatment (20 mg/kg BW) has shown a protective effect against oxidative stress through up-regulating GST level (Olayinka et al., 2014).

Also, significant effect on GST has been shown at day 12 post-weaning in plasma (P = 0.031). The QUE900 group has higher GST activity than CON (P = 0.059) and QUE100 (P = 0.005). Also, the QUE 300 group has higher activity than QUE100 (P = 0.04). This suggests that quercetin has improved the GST activity in QUE300 and QUE900 groups, but not in QUE100 group.

No significant effect on GR has been shown in day 5 post-weaning, but a statistical trend has been observed in distal small intestine mucosa (P = 0.053). The data suggested that the quercetin groups has higher GR activity than the quercetin-free group, and the dietary quercetin has an effect to increase GR level. This is similar with the results of Molina et al(Molina et al., 2003). In contrast, the result in liver at day 12 post-weaning has shown significant differences between treatments (P = 0.038). The group of QUE300 and QUE100 has 36.5% (P = 0.005) and 21.4% (P = 0.084) higher GR activity than CON. Since GR is responsible for catalyzing the reduction of GSSG into GSH, which maintains the oxidative and redox status and helps to minimize oxidative damage in cells, the increased GR activity suggests that dietary quercetin has helped the QUE300 and QUE100 group to improve their antioxidative capacity. This result is in agreement with Molina et al. (25, 50 and 75mg/kg body weight)

and Liu et al. (Liu et al., 2014; Molina et al., 2003).

In conclusion, the results from day 5 post-weaning shows less significance than day 12 post-weaning, which could be resulted from the appetite loss in the first several days after weaning and very little feed has been taken. Also, the results shows a tendency that the lower GPx and GST activity of quercetin treatments groups from day 5 post-weaning tend to be altered at day 12 post-weaning, which suggests that with the quercetin treatments, the animals are able to build up a better antioxidatvie system between day 5 and day 12 post-weaning. This suggest the anti-oxidative function of the dietary quercetin. Although the quercetin groups showed good enzyme activities, the appropriate quercetin concentration remains uncertain. From the results of the enzyme activities, some highest activities has been found in QUE100 or QUE300 groups instead of in QUE900 groups. Therefore, future studies may be required to find out the most effective concentration of dietary quercetin in weaned piglets.

5.3 Effect of dietary quercetin on glutathione redox cycle and malondialdehyde content

Results from day 5 post-weaning shows no significant effect of treatments. A statistical trend has been observed in the GSSG content in liver ($P = 0.08$). The result showed that the QUE100 group has 58.2% GSSG higher content when compared with CON ($P = 0.023$), which suggests that QUE100 group suffers from more severe oxidative stress. When using the contrast test, the hepatic GSSG/GSH ratio showed a tendency to increase in quercetin groups in day 5 post-weaning ($P = 0.082$), which could be resulted from the higher hepatic GSSG content of the quercetin groups. These results are in contrast with the study of Molina et al., Meyers et al. and Galvez et al(Galvez et al., 1994; Meyers et al., 2008; Molina et al., 2003). But when compared with the same parameter results obtained at day 12 post-weaning, the GSSG content of all the 3 quercetin treatment groups showed no significant difference with the control group, which suggest that at day 12 post-weaning the oxidative stress in the 3 quercetin treatment groups are similar with the control group. This suggests that dietary quercetin has helped the weaned piglets to improve their antioxidative capacity from day 5 to day 12 post-weaning, which is in agreement with our findings of the enzyme activities.

However, the hepatic MDA content in day 12 post-weaning showed a trend in the contrast test that the quercetin groups has higher MDA content than the quercetin-free groups. This suggests that there are likely more oxidative damage in the liver of quercetin groups than in the quercetin-free groups. This result is in contrast with the study of Molina et al. (2003), Galvez et al. (1994), Olayinka et al. (2014), and Van Le Thanh et al. (2016).

The results from day 12 post-weaning has significant difference at the GSSG/GSH ratio in red blood cells ($P = 0.027$) and the MDA content in distal small intestine mucosa ($P = 0.049$). In red blood cells, the QUE300 group has significantly higher GSSG/GSH ratio when compared with the control group

and the QUE100 group, which suggests higher oxidative stress. This result has been supported by the contrast test (P = 0.056). The results are different from the study of Meyers et al. who reported that the glutathione redox ratio in red blood cells was not affected by the quercetin treatment. In distal SI mucosa, the QUE300 has significantly higher MDA content when compared with the CON and QUE100 group, which also suggests higher oxidative stress. Similar results has been given by the contrast test.

But when compare this result with the animal performance result where the QUE300 showed significant lower body weight, similar situations are that the significant difference are all caused by the abnormally high oxidative stress in group QUE300, while no evidence of higher stress in the other 2 quercetin treatment groups been observed. Possible explanation could be that the higher stress in QUE300 group is not caused by dietary quercetin but by other factors such as unfavorable situations, limitation of the number of experimental units, individuals with genetic drawbacks or diseases transmitted inside QUE300.

Another possible explanation could be that the relation between dose and effect of quercetin in weaned piglets is not liner, and the dose of QUE300 is one of the unfavorable treatment for the animals. This could explain the significantly higher oxidative stress which is suggested by the results.

To conclude, when test the redox cycle and MDA content in plasma, liver and intestinal mucosa, most of the results suggests that quercetin treatment may have increased oxidative stress in weaned piglets, which is in contrast with our hypothesis. Possible explanation could be that the relation between dose and effect of quercetin remains unclear due to lack of *in vivo* study in piglets, the effect of quercetin may be affected by species, dose and the health status of the animal. Although between day 5 and day 12 post-weaning, the quercetin groups managed to recover from heavier oxidative stress, the total effect of quercetin on the redox status in different organs is still inconsistent.

5.4 Effect of dietary quercetin on small intestine functionality

Intestinal mucosal permeability was assessed *ex vivo* by measuring the trans-location of FD4 using the Ussing chamber. Higher permeability of FD4 would suggest damage to integrity of the small intestine. No significant difference has been observed in day 5 or day 12 post-weaning. This result differs from the study of Suzuki & Hara (2011), Amasheh et al. (2008) and Mercado et al. (2013) that indicated lower permeability with quercetin treatment.

Small intestinal epithelial cells show apoptosis in turnover of cells, and a number of studies have indicated that the final stage of apoptosis involves activation of caspases (Hyoh et al., 2002). Thus, the content of caspases could serve as an indicator for the regeneration capacity in small intestine mucosa.

No significant effect has been observed at both day 5 and day 12 post-weaning.

OCC1 corresponding to synthetic peptide of occludin which is the tight junction integral membrane protein (Wong & Gumbiner, 1997). OCC1 could increase the trans-epithelial electrical resistance of Caco-2 cell mono-layers thus improve the integrity of intestine. No significant effect has been observed, but a statistical trend has been shown in day 5 post-weaning ($P = 0.082$). The QUE100 has higher OCC1 content than CON (120.4%, $P = 0.03$). Also, the contrast test showed a statistical trend in this parameter ($P = 0.093$). This suggests possible effect of quercetin on improvement of the small intestine integrity. The result is in agreement with the study of Amasheh et al (2008).

Proliferating cell nuclear antigen (PCNA) is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle, the level of PCNA in small intestine represents the rate of intestinal cell turnover (Tazuke et al., 2011). No significant effect has been observed in both day 5 and day 12 post-weaning.

To conclude, no significant effect of dietary quercetin has been found on the parameters regarding to the functionality of small intestine, although a statistical trend has been observed on OCC1 content which suggests possible effect.

5.5 Future perspectives

Although the current study showed some encouraging results, future research may be required in order to have a better understanding of the topic.

First, future studies should explore the effect using different, especially higher dose of quercetin. In other studies, significant effect of quercetin has been reported when use higher doses, such like 2g/kg feed in the study on lambs (Andres et al., 2014; Andres et al., 2013). Also, in current study, the most effective results are often found in the group who been feed quercetin at a dose of 900 mg/kg feed, which indicates there are possibilities that higher dose than 900 mg/kg will lead to more significant effects.

Second, the current study tested the activity of GSH related enzymes, but the effect at mRNA level is unknown. Future researches should explore the mechanism of how the quercetin has affected the anti-oxidant system, and more tests should be processed to study the molecular mechanisms that related to the effect of quercetin.

6.Conclusion

In summary, this study has examined the effects of dietary quercetin on the GSH kinetics in small intestinal mucosa, blood and liver in weaned piglets. Also, the effect on small intestinal barrier function and animal performance have been investigated.

No significant effect of quercetin has been observed on animal performance, although moderate increases have been found in average daily gain and average daily feed intake by QUE900 compared with the control group. We found out that dietary quercetin significantly increased the GPx activity in plasma at day 5 post-weaning, increased GST activity in liver at day 5 post-weaning and in plasma at day 12 post-weaning. Also, quercetin significantly increased the GPx activity in distal small intestine mucosa and increased the GR activity in liver at day 12 post-weaning. Dietary quercetin significantly increased oxidative stress in different organs through higher GSSG/GSH ratio in red blood cells and liver and higher MDA content in liver and distal small intestine mucosa. Also, no significant difference has been found on the small intestine functionality although moderate increase has been observed in the OCC1 content by QUE100 group at day 5 post-weaning.

In conclusion, while the results of GSH related enzymes suggest that quercetin could help to improve the anti-oxidative capacity by up-regulating the enzymes, the results from the redox cycle and MDA content in blood, liver and small intestine suggest the opposite. Thus, the effect of quercetin on GSH kinetics in weaned piglets is inconsistent and future studies may be required.

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