# ‘The influence of condensed tannin extracts on gut health in chickens’

A thesis submitted to the University of Reading for the Degree of Doctor of Philosophy

School of Agriculture, Policy and Development
University of Reading

Written by

**Mohammed M. Dakheel**

Supervision by

**Dr Caroline Rymer**

**Prof Irene Mueller-Harvey**

**Prof Martin J Woodward**

## Declaration

I confirm that this is my own work and use of all material from other sources has been properly and fully acknowledged.

**Mohammed Munis Dakheel**

**__ __/__ __/ 2018**

## Acknowledgments

First and foremost, enormous gratitude is due to my supervisors, Dr Caroline Rymer, Professor Irene Mueller-Harvey and Professor Martin J. Woodward, who have been unstinting in their support and constructive critique. Appreciation due to workers and technicians in the Agriculture and Food & Nutritional Biosciences Schools as well as huge thanks to my colleagues and lab members Dr Chaweewan Klongsiriwet, Dr Honorata Ropiak, Dr Christos Frygans, Dr Sarah Jing Guo, Mss Parwin Majid (RIP), Dr Sokratis Stergiadis, Dr Prabhat Sakya, Mr Chris Humphry, Mr Chris Drake, Dr Gemma Walton, Dr Marina Mora Ortiz, Dr Fatemah A. Alkandari, Mrs Maha A. Aldeieg and Mss Afnan Al-Mnaseer. Further, I would like to express my sincere gratitude to all staff members at the University of Reading, who have helped and supported me during the PhD study.

Particular thank and appreciate must also be recorded to my sponsors (The Ministry of Higher Education and Scientific Research & Veterinary Medicine College at Baghdad university/ Iraq) and (Iraqi Cultural Attaché/ London), which provided generous supporting and encouragement to me since the beginning until the end.

Massive thanks and love to my family, Mum and Dad, Baidaa (my wife), Shams and Rawan (my daughters/ the beautiful roses), Dr Ali and his family (my brother), who covered my needs during this study and gave me the warmest feeling.

Finally, thank you to all my friends (in and outside Iraq), who had encouraged me to achieve this study successfully. Thank you for everything, love you all.

**Abstract**

Tannins belong to the group of polyphenols, which occur naturally in many plants. They exist in the form of two different groups: hydrolysable tannins and condensed tannins (CTs), the latter are also called proanthocyanidins. This thesis investigated the antimicrobial activities *in vitro* of CTs and studied their impacts on the fermentation and metabolism in chicken caecum.

An initial screening of a plant collection for CTs identified plant materials that contained different CT-types, e.g. high and low procyanidin/prodelphinidin or *cis*/*trans,* flavan-3-ol ratios and also of CTs with small or large mean degrees of polymerisation. After CT extraction and purification, the effects of these structural CT-features were studied and tested against two bacteria, avian pathogenic *E. coli* (APEC) as Gram negative bacteria and *Staphylococcus epidermidis* as Gram positive bacteria by the broth microdilution assays. Subsequent experiments investigated the effects of these different CT compositions on *in vitro* fermentation and metabolism, and determined the metabolic end products.

These CTs were effective at minimal bactericidal concentrations (MBCs) of 5 – 10 mg/ml against APEC and at 5 – 1.25 mg/ml against *S. epidermidis*. At these concentrations they significantly (P≤0.05) inhibited bacterial growth. Scanning electron microscopy (SEM) revealed antimicrobial activity of these extracts on the bacterial cells of both bacteria, but CTs were more potent against the Gram positive compared to the Gram negative bacteria, which required higher MBC values of 5 - 10 mg/ml. Interestingly, this study found that low concentrations (0.6 mg/ml) of tilia flower extracts, which contain mostly procyanidins (PC), showed slightly enhancement of APEC growth.

The anti-biofilm and anti-motility activity of the CTs were also evaluated. All CT extracts also affected significantly (P≤0.05) the biofilm formation of APEC depending on their concentrations and compositions. Significant (P≤0.05) anti-biofilm activity against APEC was observed for almost all CT extracts, especially at higher concentrations (10 – 2.5 mg/ml). More importantly, concentrations of 0.6 - 1.25 mg/ml, especially with extracts that contained low molecular weight of CTs, enhanced the bacterial cell attachment of APEC. This could be because surviving bacteria prefer to form the biofilm in stressful conditions, indicating further

investigations due to CT's high the anti-biofilm activity potential. In contrast, the CT extracts containing either high molar proportions of prodelphinidins (PD) or procyanidins (PC) interrupted and blocked swarming and swimming motility at 10h and 24h compared to the controls. These data support the theory that antimicrobial activity of the CTs, whether with high PC% and PD%, can elicit a positive relationship between anti-biofilm formation and anti-motility capacities. These findings are the first results that provide knowledge about the effect of different CTs on biofilm formation and motility of APEC.

The most potent CTs chosen from the above experiments were then tested *in vitro* using a fermentation culture over a 24h period. A nuclear magnetic resonance ($^{1}$H-NMR) experiment combined with multivariate statistics was used for the first time to investigate how CT compositions influenced either metabolic end products in a chicken contents or APEC metabolites in a nutrient medium. Both experiments showed interesting results of how CT compositions, in terms of PC% and PD%, impacted on metabolic compounds. Amino acids such as glutamate, leucine, lysine, pyroglutamate, phenylalanine, proline and sarcosine were significantly ($P \leq 0.05$/times) decreased. However, leucine and lysine were measured a highest significant difference ($P \leq 0.05$) by the interaction between treatments and times. Both CT compositions also influenced significantly ($P \leq 0.05$) on fermentation of some fatty acids such as acetate, butyrate and propionate were increased in the treatments. However, lactate was recorded statistically decreasing ($P \leq 0.05$) between the treatments. In addition, other metabolites such as some of carbohydrates (e.g. mannitol, glucose, fructose, lactose and galactose) were observed slightly fluctuations on their levels (but not significantly, $P \geq 0.05$) at different time-points.

Conversely, APEC culture responded metabolically and statistically to these CT compositions. Both CT compositions showed different effect on some amino acids such as lysine, leucine, glutamate phenylalanine and pyroglutamate, which illustrated a significant value ($P \leq 0.05$) in CT-groups, especially high PD% treatment, whilst the values of these amino acids showed very close results between PC treated and controls. Similarly, the fatty acids, such as butyrate, iso-butyrate, valerate and iso-valerate were increased in high PD of CTs compared to the high PC and controls. These values did not record significant difference at

P>0.05 between the integration of times and treatments, but they showed a significant different (P≤0.05) at different time-points. However, CTs with high PD% led to a significant decreasing (P≤0.05) of lactate, which should normally be increased by *E. coli* mixed acid fermentation. This is new finding that shows the effect of CTs, particularly high PD% group can influence on the biological process of mixed acid fermentation. The most important results were that CTs with high PD% showed different effects to CTs compared to high PC % in APEC cultures, and that the PC results were close to the controls. Therefore, more research is recommended to investigate the effects of the different CT compositions and concentrations separately by using other NMR techniques e.g. 2D-NMR.

In conclusion, this thesis identified the key structural features of CTs, which contain either high molar proportions of PD or PC that might be useful to improve the efficiency of feed utilisation and to reduce the incidence of pathogenic bacterial infections in chickens. This study was also the first investigation of the effects of different CT compositions on *in vitro* metabolism by $^{1}$H-NMR and provided new findings that could establish a powerful database for the future experiments by including the CT extracts in chicken diets.

## Table of contents

**Chapter 3:** Antimicrobial *In vitro* activities of condensed tannins

**Chapter 4:** Effect of condensed tannins on *in vitro* metabolic profile of caecal contents and APEC culture

## List of figures

## Abbreviations

| | |
|---|---|
| ABA | Acid-butanol-acetone assay |
| ANOVA | Analysis of variance |
| APEC | Avian pathogenic *E. coli* |
| BM | Benzyl mercaptan |
| C | Catechin |
| *C. jejuni* | *Campylobacter jejuni* |
| °C | Celsius degree |
| CFU | Colony forming units |
| CTs | Condensed tannins |
| *E. coli* | *Escherichia coli* |

| | |
|---|---|
| $R^2$ | Estimates goodness of fit of the model |
| $Q^2$ | Estimates of the predictive ability of the model |
| EC | Epicatechin |
| EGC | Epigallocatechin |
| F1 | First fraction of CT extracts (30:70 v/v) |
| F2 | Second fraction of CT extracts (80:20 v/v) |
| GC | Gallocatechin |
| g | Gram |
| HCL | Hydrochloric acid |
| kg | Kilogram |
| L | Litre |
| LB | Luria-bertani medium |
| MS | Mass spectrometry |
| mDP | Mean degree of polymerisations |
| µl | Microlitre |
| mg | Milligram |
| ml | Millilitre |
| mm | Millimetre |

| | |
|---|---|
| MBC | Minimum bactericidal concentration |
| nm | Nanometre |
| NMR | Nuclear magnetic resonance |
| OD | Optical density |
| OPLS | Orthogonal partial least squares |
| PBS | Phosphate buffer saline |
| pH | pH value |
| PC | Procyanidins |
| PCA | Principal component analysis |
| PD | Prodelphinidins |
| *S. epidermidis* | *Staphylococcus epidermidis* |
| SEM | Scanning electron microscopy |
| spp. | Bacterial species |
| TCA | Tricarboxylic acid cycle |
| TSP | 2,2′,3,3′ tetradeuteropropionic acid |
| UV-vis | Ultraviolet–visible |
| VL | Viade Levure |
| v/v | Volume/volume |
| w/v | Weight/volume |

# Chapter 1: General introduction

## 1.1 Health risks and consequences of pathogens in human and animal diets

Every year, humans are infected by many pathogens, which originate from animal sources. These pathogens can exist in nature and within the animal. Several of these pathogens are bacteria, which can survive some food production processes (Humphrey et al., 2007). Typical examples of infections include bowel ailments in consumers who eat undercooked or contaminated animal products. For instance, *Campylobacter* spp. can cause Campylobacteriosis that results in several pathogenic symptoms in humans such as acute diarrhoea, vomiting, high temperature and abdominal pain (Karikari et al., 2017). It has been suggested that the number of these bacteria in the intestine of broilers may be reduced through the use of medicinal plants or herbs that can act as probiotics or prebiotics, in order to reduce faecal contamination (Gharib Naseri et al., 2012).

In addition, *Salmonella* spp. can induce severe infection in humans who consume contaminated chicken meat. To limit and control the number of these bacteria in diets, several steps should be considered during food production and processing, which starts on the farm with flock hygiene and management, avoids pathogenic infection and minimises the colonisation of poultry (Heres et al., 2003). Researchers have also attempted to find a relationship between plants consumed by animals and the bacteria that infect these animals. Several plants contain acids that can act against these bacteria (Chichlowski et al., 2007). Heres et al. (2004) found that applying acidified and fermented feeds to broilers reduced the numbers of *Campylobacter* spp. and *Salmonella* spp. in *in vitro* experiments.

*Clostridium* spp. represents another pathogen that causes disorders in both humans and animals. These are spore-forming and anaerobic bacteria that exist in nature and within the animal. Humans can be infected with these bacteria if they consume infected birds, and patients can have gastrointestinal diseases such as food poisoning (Petit et al., 1999). In the animal it can cause necrotic enteritis in the gastrointestinal system of poultry, leading to changes in the caecum environment and microflora of poultry. However, these bacteria cannot induce necrotic enteritis in birds without other predisposing factors, such as other

infections or a change in the diet regime of the flock. Hence, good hygiene and appropriate food management can reduce the number of infections (Stanley et al., 2012).

Before the advent of modern drugs, animals have traditionally been treated with local knowledge or ethno-veterinary medicine, which covers methods for caring, healing, and managing animals (Moreki, 2012). Chinese scientists especially have examined many natural plant products as alternatives to modern medicines and reported benefits that include an increase of the immune response, an antimicrobial and anticancer effect, and less stress (Zhao et al., 2017; Liu et al., 2017b; Liu et al., 2017a; Xu et al., 2017).

After recent restrictions on the use of synthetic drugs to stimulate animal growth, the producers, particularly in Europe, stopped adding pharmacological products to animal diets, especially in the chicken industry (Castanon, 2007). Therefore, researchers have attempted to identify alternative products that can enhance bird health and productivity. One avenue that has been investigated is the use of medicinal plants and/or their extracts in numerous opportunities to improve the livestock production by adding these extracts as feed additives into their diets. In addition, feed supplements have also been explored as prebiotics, probiotics; typical examples are essential oils (Wallace et al., 2010). Some plants such as garlic or its bioactive compounds can positively affect intestinal ailments and abdominal distension, act as remedies against intestinal helminths, and can also be used to treat respiratory infections in chickens (Amagase et al., 2001). Further, Jorgensen et al. (2002) examined 241 chickens bought from retail outlets in England between 1998 and 2000, and 80% of chicken meat was contaminated with *Campylobacter* spp*.* and 25% was contaminated with *Salmonella* spp*.*, and the percentage of *Campylobacter* spp*.* in these chickens reached up to 98% for *Campylobacter jejuni.* Another study has pointed out that the prevalence of these bacteria could be between 6 to 50%, with *C. jejuni* being the dominant species in the caecum of birds rather than other *Campylobacter* spp. (El-Shibiny et al., 2007). The host's ability to defend itself may play a critical role in combatting these bacteria (Park, 2002; Gharib Naseri et al., 2012).

## 1.2 Management factors that impact on poultry health

It is not only pathogenic microorganisms that may influence bird health negatively, but several management and environmental factors can also impact unfavourably on animal condition. Litter materials are particularly relevant in the context of bacterial infections. These are used as bedding for poultry and poultry litter tends to be a mixture of bedding material (wood shavings), manure and feathers. However, both new and re-used bedding materials are also referred to as litter (Torok et al., 2009). Litter material with high levels of moisture can increase the risk of pathogen growth and ammonia production that can negatively affect poultry health (Carlile, 1984). Ammonia build-up can negatively affect body weight, feed conversion and mortality in poultry. Dust from litter materials that are too dry can make poultry more susceptible to diseases. The temperature and humidity profiles, management practices and equipment, geographical and environmental places that promote poultry welfare and other management issues need to be controlled and are considered critical to chicken welfare (Jones et al., 2005).

Feed constitutes the largest variable cost in poultry production and is a factor that is a crucial element in the poultry industry. Nutrition in particular can have a profound effect on the morphology and function of the caecum. Jensen and Maurice (1978) noticed that adding high levels of copper (120 – 250 mg/kg) to the chicken diet led to the caecum being distended and changing colour and they suggested that copper in the caecum could inhibit microflora activity. On the other hand, Bauer and Griminger (1980) pointed out that various concentrations (between 20 – 100 µg/kg) of biotin (or vitamin B7) in the diet of growing birds improved caecum condition, compared with other diets that contained different kinds of carbohydrates such as glucose, dextrin, sucrose, and sorbitol. Carneiro et al. (2008) reported that wine mixed with food in an external stomach model with pathogenic bacteria in an *in vitro* test reduced the number of *Campylobacter jejuni*. Similarly, (Ganan et al., 2009) studied the effect of phenolic compounds in wine on *C. jejuni*, and they found that red wine had more effect as an antimicrobial agent in broilers than white wine. It is thought that the mechanism behind this wine effect is the combination of ethanol and organic acids, e.g. malic and tartaric acids. These acids are known to be effective against pathogenic bacteria, particularly in low pH environment wines, as Just and Daeschel, (2003) demonstrated that the

antimicrobial properties of fermented grapes can be effective against *E. coli* and *Salmonella* spp.

### 1.3 Bacterial factors that impact on the digestive system of poultry

The caecum is an important part of the digestive system, because it plays a critical role in the microbial ecosystem of these animals. In poultry, it contains the greatest microbial population in the intestine, and provides a natural environment for the growth of different microflora (Guo et al., 2004), and these microflora can combat pathogenic bacterial colonisation. According to Barnes et al. (1972) who measured the total number of microflora in the chicken caecum during different periods of the chicken life stages, microbiota number was about $10^{11}$ per gram (wet weight), with about $10^{8}$ to $10^{9}$ per gram of anaerobic bacteria. It is well known that commensal bacteria can contribute to the health of the animal, and it is the pathogenic bacteria that can cause disorders in animals (Rinttila and Apajalahti, 2013). Thus, the main aim is to maintain the gastrointestinal tract so that levels of commensal bacteria rise, and harmful pathogens are inhibited. The microflora population of the caecum can vary in quantity, quality and activity, and can be affected by various factors, one of these is the animal diet (Apajalahti et al., 2001). A healthy intestinal tract condition is, sometimes, associated with active bacteria, which will help the animal to convert nutrients into body tissues (Rinttila and Apajalahti, 2013). However, characterising the caecal microflora community is more complex than other parts of the gastrointestinal tract of chickens (Guo et al., 2003) because the caecum has the highest population of the microbiota plus greatest diversity of bacteria species.

### 1.4 Effect of using antibiotics in animal diets

The first work with antibiotic compounds was started in the early twentieth century by a German physician called Paul Ehrlich (Burdon and Williams, 1968; cited Jones and Ricke, 2003). The first antimicrobial compound was named Salvarsan (De Kruif, 1926; cited in Jones and Ricke, 2003). In 1932, another German physician, Gerhard Domagk, tested prontosil rubrum on small laboratory animals to cure s*treptococcal* and *staphylococcal* infections. He succeeded and, at the same time, he used this to cure his child's illness. During this period, Alexander Fleming also discovered penicillin (Maurois, 1959; cited in Jones and Ricke, 2003).

The first use of antibiotics as growth promoters was in the 1940s due to an observation that the health of animals improved when they ate feeds mixed with dried mycelia (Castanon, 2007). This growth promoter has been found to affect intestinal microflora (Dibner and Richards, 2005). In the middle of the last century, due to increasing food demands, the US, European and some other countries allowed the use of antibiotics as feed additives. These were applied at sub-therapeutic levels to provide a benefit for improved growth and feed conversion (Groschke and Evans, 1950; cited in Jones and Ricke, 2003). However, in the last few decades, there were numerous reports that demonstrated the development of antibiotic resistance, which started to impact negatively on our ability to treat some human pathogens (Karikari et al., 2017). To avoid these concerns, it was necessary to stop using these antimicrobial agents as feed additives (Dibner and Richards, 2005). However, antibiotics were also used in the agricultural sector as therapeutic treatments for a long time to protect animals from pathogenic microorganisms and antimicrobial growth promoter such as avoparcin, which is a kind of antibiotic. These treatments were particularly suitable against some types of bacteria, especially Gram-positive (Aarestrup, 2003).

### 1.5 Plants and plant extracts for supporting human and animal health

As mentioned above, many plants have traditionally been used as herbal medicines for humans and animals across the world. Consequently, there are many studies on their phytochemical composition and biological activities that included antimicrobial properties (Goleniowski et al., 2006). Herbal medicines have been used as feed additives in order to utilise their antimicrobial properties (Gharib Naseri et al., 2012). These plants can reduce or inhibit the pathogenic effects of many undesirable microorganisms inside the gut and thus increase the performance of animals (Yan et al., 2012). Few examples shall serve to illustrate these points. When studying the effects of water plantain (*Alisma canaliculatum* A. Br. Et. *Bouche*) and mistletoe (*Viscum album* L.) as feed additives, these plants acted as growth promoters, in place of antibiotics, in broilers (Hossain et al., 2012). *Sanguinaria canadensis* has also been used to prevent the development of bacteria that cause digestive distress and improve the growth performance of birds (Mahady et al., 2003). Zhao et al. (2013) applied various dosages of *Portulaca*

*oleracea* extracts to broilers and suggested that these plants impacted on caecal microflora by increasing *Lactobacillus* and reducing *E. coli*. *Portulaca oleracea* has also been documented to support the intestinal environment in the chickens by restricting *E. coli* and *Salmonella* spp. development and promoting growth of *Lactobacillus* spp. and *Bifidobacterium* spp. (Wang et al, 1999; Ke et al, 2003; all cited in Zhao et al., 2013).

However, the effectiveness of medicinal plants or their extracts can be variable and can also depend on the method of administration, concentration of bioactive compounds in the diet (Windisch et al., 2008) and the composition of the 'bioactive compounds'. A research has also suggested that plant mixtures may have a greater effect on animal health and also may enhance the bioavailability of these compounds (Yan et al., 2012). Apart from *Campylobacter* spp., *Salmonella* spp. and *Clostridium* spp., there are also other microorganisms that can induce a disorder in the bird caecum, e.g. protozoa (McDougald, 1998).

The above examples suggest that there are opportunities for medicinal plants in animal health and for treating infected animals. Amongst the abundance of different secondary plant metabolites that can be used as feed additives or supplements, there are polyphenols and more specifically tannins, which are a sub-group of polyphenols.

### 1.6 Overview of tannins as bioactive compounds

Tannins are polyphenolic compounds that are synthesised by higher plants and there are two main groups: hydrolysable tannins (Mueller-Harvey, 2001) and condensed tannins (CT), which are also called proanthocyanidins (Schofield et al., 2001). The chemical structures of CT consist of flavan-3-ol subunits that are linked either via C-4 to C-8 or C-6 to C-8 bonds (Khanbabaee and van Ree, 2001) (Fig 1.1).

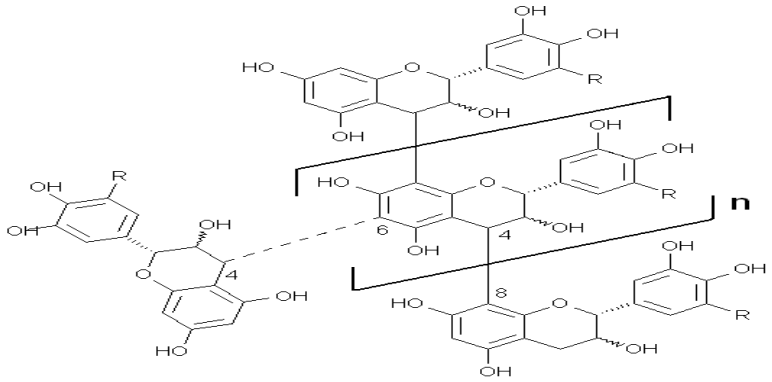


Figure 1.1: Chemical structure of condensed tannin polymer (Gea et al., 2011)

(n)= monomeric unit; (R) = H = PC; (R) = OH = PD

All tannins can bind with proteins, carbohydrates, lipids and other molecules, which can impact on the nutrient environment of intestinal microbiota (Brown et al 2017). Some of these compounds may also act on gastrointestinal bacteria (Espin et al., 2017). Several tannin-containing plants, such as tannin extracts from sweet chestnut wood, have also been studied as alternative remedies in mono-gastric animals, e.g. pigs, to inhibit the pathogenic effect of various *Salmonella* spp*.* and *Escherichia coli* both *in vitro* and *in vivo* (Van Parys et al., 2010; Reyes et al., 2017; Coddens et al., 2017).

In ruminant management systems, feeding tannin-containing plants, particularly condensed tannins, can promote weight gain, increase productivity and fertility by improving tolerance to intestinal parasites (Waghorn and McNabb, 2003). These tannins can also be added to the diet (Waghorn, 2008). For this reason, tannin-containing plants can also function as “neutraceuticals”, which means that these plants provide standardised nutrients and can also have pharmaceutical effects. A recent study reported the beneficial effects of good host nutrition as this provided useful options to control gastrointestinal parasites by feeding tannins as a component of an integrated strategy (Hoste et al., 2015 ; Hoste et al., 2016).

### 1.7 Tannin characterisation

Tannins commonly exist in many plants and are defined as water soluble polyphenolic compounds with molecular weights ranging from 500 – >20,000 Daltons) and have the ability to link and precipitate proteins in particular (Schofield

et al., 2001). CTs occur in a wide range of concentrations, e.g. from <0.5 to >30.0 g/100 g dry weight, in plants (Kraus et al., 2003). Most plants contain different types of CTs such as procyanidins and prodelphinidins that range from small oligomers to large polymers with *cis-trans*-stereo chemistries of the flavan-3-ol subunits (Fig 1.2).

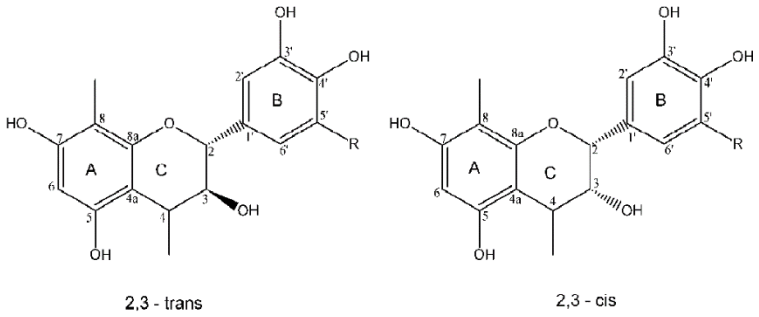



Figure 1.2: *Cis/trans* subunits of CT (Norris et al., 2011)

(R) = H = PC; (R) = OH = PD

In addition, there are considerable differences in the amounts of extractable and non-extractable tannins. Furthermore, there are often positive correlations between some of CT features such as mean degree of polymerisations (mDP) and molar percentages of prodelphinidins (PDs) (Mueller-Harvey et al., 2017).

Several techniques are available to quantify, characterise and isolate CTs. One of the most common method for CT quantification is the acid-butanol acetone assay (ABA) (Grabber et al., 2013). Although the ABA method is widely applied for CT quantitation, it does not provide information on CT composition. Therefore, CTs have also been subjected to thiolytic analysis (Gea et al., 2011) plus high performance liquid chromatography (HPLC) and mass spectrometry (MS) (Ropiak et al., 2016). This procedure is suitable for screening plants or extracts for the different CT types. Thiolysis provides information on CT composition such as mean degree of polymerisation (mDP), procyanidin/prodelphinidin (PC/PD) and *cis/trans* flavan-3-ol ratios.

Condensed tannins are generally isolated separating CTs from acetone/water extracts, and then purified on Sephadex LH-20 columns (Brown et al., 2017). This approach enables researchers to increase the tannin concentrations in their

samples for use in subsequent bioassays. These column fractions are then also analysed by thiolysis and HPLC/MS spectroscopy to determine their CT compositions. To sum up, these protocols provide options for preparing highly pure CT samples that can be used in microbial, fermentation and metabolism studies.

### 1.8 Antimicrobial activities of phenolic compounds and tannins in poultry

Numerous pathogenic bacteria can threaten poultry production and, for example, one of these undesirable microorganisms is *Escherichia coli,* which is also known as avian pathogenic *E. coli* (APEC) that causes *colibacillosis* (Maturana et al., 2011). Several medicinal plants, which contain bioactive compounds, have antimicrobial against these pathogens (Tan and Vanitha, 2004). However, these plants are not always a successful replacement for synthetic antibiotics. Nevertheless, such remedies can have favourable properties namely low cost, fewer side effects and are easy to apply. Tannins are a group of polyphenolic compounds that have shown efficacy against some microorganisms *in vitro* (Cueva et al., 2013) and *in vivo* (Choi et al., 2011).

Tannins can impact significantly on Gram negative and Gram positive pathogenic bacteria (Shao et al., 2015). Evidence exists that suggests that phenolic compounds act as anti-adhesion to *E. coli* and protect the epithelial layers of the intestine or other organs such as prevention of urinary tract infections (Ofek et al, 1996). Some phenolic compounds, including CTs, can be also used to inhibit the growth of pathogens by acting as bacteriostatic compounds (Viveros et al., 2011). Several studies focussed on phenolic acids, such as ferulic and gallic acids, and investigated their activities on different pathogenic bacteria, such as *Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli*, and the results from this study demonstrated different effects of these acids as antimicrobial activities such as inhibitory impact on the motility and prevention of biofilm formation of these pathogens (Borges et al., 2012; Borges et al., 2013). Relatively, Shao et al. (2015) found that a high concentration of gallic acid (8mg/ml) was the minimum concentration that induced significant inhibition on two strains of pathogenic bacteria, *Escherichia coli* and *Streptococcus mutans* at 25 °C and 37 °C. Thus, the researchers concluded that gallic acid was effective on agar that was used to test bacterial growth with two different temperatures and periods.

In general, CTs and other phenolic compounds showed various antimicrobial activities against different pathogens.

### 1.9 Microbial methods for investigating tannin activities

The literature indicates that many plants and their extracts have phytochemicals, such as polyphenols, that can be used for controlling pathogenic bacteria in chickens (Ganan et al., 2009). This thesis is focusing on one particular group of polyphenols, i.e. condensed tannins. Their activities were examined by testing different CT concentrations and compositions against selected pathogenic and non- pathogenic bacteria that can affect poultry gut health.

After completion of tannin extraction, fractionation and purification, the microbial assays were started. The first analysis determined the minimal bactericidal concentration (MBC) of CTs, which is the lowest concentration of an antibacterial agent required to kill bacteria. This procedure was applied by broth microdilution technique (Sheng et al., 2016), and provides a quantitative measurement to determine the *in vitro* antimicrobial activities of different tannin features. This method serves as a screen for identifying which CT extracts may be the most effective as antimicrobial compounds, and which doses will be most appropriate for subsequent laboratory experiments. Furthermore, biofilm formation and motility assays have been applied to investigate of tannin activities on adherent bacteria as mentioned before by Shao et al. (2015) and O'May et al. (2012). In addition, an electron microscopy (EM) study was conducted to investigate the effect of tannins, which extracted from *Ocimum basilicum* L. , as antibacterial agents against bacteria and then observed by EM for damage or malformation of the cells (Kaya et al., 2008).

In general, CT extracts were examined against avian pathogenic *Escherichia coli* (APEC), as a model for Gram negative, and *Staphylococcus epidermidis*, as a model for Gram positive bacteria. The data showed that CT features had significant influence on these pathogens.

### 1.10 Effects of tannins on gut health

Several publications have recently focused on fermentations in the gut, because of an increasing interest in the types of nutrients or bioactive compounds that might

improve animal health and enhance poultry production (Bederska-Łojewska et al., 2017). Moreover, bioactive compounds such as fatty acids and lactic acid can also have an impact on the gut flora, by increasing beneficial bacteria, which produce lactic acid and provide an acidic environment e.g. pH between 3.9 and 4.5 (Valenzuela-Grijalva et al., 2017). These acids are formed during the fermentation of plants by gut microflora (Missotten et al., 2013). A diet containing high concentrations of natural compounds can play a crucial role in providing a good environment for useful bacteria. At the same time, the presence of significant concentration of acids in fermented feeds can decrease pathogenic, foodborne bacteria in mono-gastric animals (Missotten et al., 2010). However, the mechanism of effect of acid on the entire digestive system is still unclear (Van Winsen et al., 2001).

When plant diets are fermented by microbiota and then fed to animals, this can lead to increased resistance against harmful organisms, which exist in the gastrointestinal tract (Missotten et al., 2013). It can also improve nutrient digestibility and intestinal morphology in the gut. However, fermented feeds are not used as an additive, but as a feeding strategy in weaning pigs (Missotten et al., 2010). For instance, Jeaurond et al. (2008) reported that feeding pigs on fermented sugar beet as a carbohydrate source and fermented chicken by-products, e.g. heads, feet, and viscera, as a protein source, led to a decrease in the pathogenic bacteria of the intestine. This led to improved nutrient digestion, better health and improved performance of gut microbiota. Several studies also examined the relationship between the acid environment in the stomach and the activity of gastric enzymes in piglets during the first weeks of their life (Montagne et al., 2003; Tagliazucchi et al., 2010; Heo et al., 2013). A low pH can also help to prevent pathogenic infections in animals. Additionally, it has been observed that acidic environment can play a critical role in the bird gut by enhancing the intestinal microflora (O'Keefe, 2008). On the other hand, fermentation of carbohydrates produces short chain fatty acids, which can be metabolised by gut microflora as Williams et al. (2005) described the benefits of fermentable carbohydrates that can be supplied in mono-gastric diets.

## 1.11 *In vitro* fermentation and metabolism approaches suitable for investigating the effects of CTs on metabolic end products

*In vitro* fermentation systems simulate events using chicken gut contents and provides a useful technique for predicting biological activity of CT compositions (Cilla et al., 2017). *In vitro* fermentation in the presence of CTs was carried out by a simple batch culture technique under strict anaerobic conditions for 24h. Afterwards, an *in vitro* chicken caecum model was utilised. This model simulates conditions that exist in the caecum, such as pH and temperature, and includes an appropriate medium and inoculum (Coles et al., 2005). It is a versatile and useful tool to study caecal microbiota and their metabolites *in vitro* (Gross et al., 2010).

After fermentation, nuclear magnetic resonance technique (NMR) can be used to provide information of the metabolism and degradation process and data on metabolic products such as amino acids, fatty acids and carbohydrates.

Metabolic analysis by NMR spectroscopy represents a rapid and high throughput technique, which requires minimal sample preparation (Bollard et al., 2005). NMR spectroscopy applies strong magnetic fields and radio frequency pulses to the nuclei of atoms. For these atoms with an odd atomic number ($^{1}$H), the presence of a magnetic field will lead the nucleus to spin, termed nuclear spin. Absorption of such pulses will, then, promote the nuclei from low energy to high energy spin states, and the subsequent emission of radiation during the relaxation process is detected (Dunn and Ellis, 2005). Metabolic analysis is very useful for detecting a large number of compounds simultaneously and also to detect quantitative changes in these compounds. Thus, it is possible to monitor how CT impact on the fermentation process and end-products.

## 1.12 The main objectives and hypotheses for this study

The objective of this work was to investigate how CT compositions could change the metabolism profile in the chicken caecum. It also aimed to improve the mechanistic understanding of the associations between microbial activity and the metabolism that could be regarded as a promising methodological approach for bird health. This study has been divided into three parts:

I. Chemical analyses:

   To identify and characterise a wide range of plant materials and their extracts for CT compositions; such information was needed in order to discover plant sources with structurally diverse CTs and where the CT compositions, such as mean degree of polymerisation and molar percentages of procyanidins and prodelphinidins, are not correlated. These features will differ and vary between these plant materials and their extracts.

II. *In vitro* microbial analyses

   The aim of this part of the study was to investigate the antimicrobial activities of different CT compositions by using purified CT samples. The hypothesis of this research was that structural features of CTs will show different effects against Avian pathogenic *Escherichia coli* (Gram-) and *Staphylococcus epidermidis* (Gram+). Based on the evidence in previous studies, the testable hypothesis will be that Gram-positive bacteria will be more sensitive to CTs than Gram-positive bacteria: In particular that the most important descriptor of CT characteristics is the PC/PD ratio in terms of effects on the growth curve, biofilm formation and motility of pathogenic bacteria.

III. *In vitro* fermentation and metabolism analyses

   The final testable hypothesis is that differences in CT composition have a measurable impact on the metabolism of caecal contents or selected pathogenic bacteria (APEC) from chickens by modifying the concentrations of important end products, which result from altered biochemical pathways.

## 1.13 References:


Aarestrup, F. M. (2003). Effects of termination of AGP use on antimicrobial resistance in food animals. *in Working papers for the WHO international review panels evaluation, Document WHO/CDS/CPE/ZFK/2003.1 a. World Health Organization, Geneva, Switzerland*, 6-11.

Amagase, H., Petesch, B. L., Matsuura, H., Kasuga, S., and Itakura, Y. (2001). Intake of Garlic and Its Bioactive Components. *J. Nutr* 131, 955S-962S.

Apajalahti, J. H., Kettunen, A., Bedford, M. R., and Holben, W. E. (2001). Percent G+C profiling accurately reveals diet-related differences in the gastrointestinal microbial community of broiler chickens. *Appl Environ Microbiol* 67, 5656-67.

Barnes, E. M., Mead, G. C., Barnum, D. A., and Harry, E. G. (1972). The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. *Br Poult Sci* 13, 311-26.

Bauer, K. D., and Griminger, P. (1980). Effect of dietary carbohydrates and biotin level on cecal size and biotin concentration of growing chickens. *Poult Sci* 59, 1493-8.

Bederska-Łojewska, D., Świątkiewicz, S., and Muszyńska, B. (2017). The use of Basidiomycota mushrooms in poultry nutrition–a review. *Anim Feed Sci Technol* 230, 59-69.

Bollard, M. E., Stanley, E. G., Lindon, J. C., Nicholson, J. K., and Holmes, E. (2005). NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. *NMR Biomed* 18, 143-62.

Borges, A., Ferreira, C., Saavedra, M. J., and Simoes, M. (2013). Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microb Drug Resist* 19, 256-65.

Borges, A., Saavedra, M. J., and Simoes, M. (2012). The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling* 28, 755-67.

Brown, R. H., Mueller-Harvey, I., Zeller, W. E., Reinhardt, L., Stringano, E., Gea, A., Drake, C., Ropiak, H. M., Fryganas, C., Ramsay, A., and Hardcastle, E. E. (2017). Facile Purification of Milligram to Gram Quantities of Condensed

Tannins According to Mean Degree of Polymerization and Flavan-3-ol Subunit Composition. *J Agric Food Chem*.

Carlile, F. S. (1984). Ammonia in Poultry Houses - a Literature-Review. *Worlds Poult Sci J.* 40, 99-113.

Carneiro, A., Couto, J. A., Mena, C., QueiroZ, J., and Hogg, T. (2008). Activity of wine against Campylobacter jejuni. *Food Control* 19, 800-805.

Castanon, J. I. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poult Sci* 86, 2466-71.

Chichlowski, M., Croom, W. J., Edens, F. W., McBride, B. W., Qiu, R., Chiang, C. C., Daniel, L. R., Havenstein, G. B., and Koci, M. D. (2007). Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poult Sci* 86, 1121-1132.

Choi, J. G., Kang, O. H., Lee, Y. S., Chae, H. S., Oh, Y. C., Brice, O. O., Kim, M. S., Sohn, D. H., Kim, H. S., Park, H., Shin, D. W., Rho, J. R., and Kwon, D. Y. (2011). *In Vitro* and *In vivo* Antibacterial Activity of *Punica granatum Peel* Ethanol Extract against *Salmonella*. *Evid Based Complement Alternat Med* 2011, 690518.

Cilla, A., Barberá, R., and Alegría, A. (2017). Overview of *In vivo* and *In vitro* Methods for Food Assessing Bioavailability of Bioactive Compounds. *In* "Frontiers in Bioactive Compounds", Vol. 2, pp. 54-98. Bentham Science Publishers.

Coddens, A., Loos, M., Vanrompay, D., Remon, J. P., and Cox, E. (2017). Cranberry extract inhibits *in vitro* adhesion of F4 and F18+*Escherichia coli* to pig intestinal epithelium and reduces *in vivo* excretion of pigs orally challenged with F18+ verotoxigenic *E. coli*. *Vet Microbiol* 202, 64-71.

Coles, L. T., Moughan, P. J., and Darragh, A. J. (2005). *In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Anim Feed Sci Technol* 123, 421-444.

Cueva, C., Sánchez-Patán, F., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, P. J., Bartolomé, B., and Moreno-Arribas, M. V. (2013). *In vitro* fermentation of grape seed flavan-3-ol fractions by human faecal microbiota:

changes in microbial groups and phenolic metabolites. *FEMS microbiology ecology* 83, 792-805.

Dibner, J. J., and Richards, J. D. (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* 84, 634-43.

Dunn, W. B., and Ellis, D. I. (2005). Metabolomics: Current analytical platforms and methodologies. *Trac-Trends in Analytical Chemistry* 24, 285-294.

El-Shibiny, A., Connerton, P. L., and Connerton, I. F. (2007). *Campylobacter* succession in broiler chickens. *Vet Microbiol* 125, 323-32.

Espin, J. C., Gonzalez-Sarrias, A., and Tomas-Barberan, F. A. (2017). The gut microbiota: A key factor in the therapeutic effects of (poly)phenols. *Biochem Pharmacol* 139, 82-93.

Ganan, M., Martinez-Rodriguez, A. J., and Carrascosa, A. V. (2009). Antimicrobial activity of phenolic compounds of wine against *Campylobacter jejuni*. *Food Control* 20, 739-742.

Gea, A., Stringano, E., Brown, R. H., and Mueller-Harvey, I. (2011). In situ analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high-throughput germplasm screening. *J Agric Food Chem* 59, 495-503.

Gharib Naseri, K., Rahimi, S., and Khaki, P. (2012). Comparison of the effects of probiotic, organic acid and medicinal plant on *Campylobacter jejuni* challenged broiler chickens. *JAST* 14, 1485-1496.

Goleniowski, M. E., Bongiovanni, G. A., Palacio, L., Nunez, C. O., and Cantero, J. J. (2006). Medicinal plants from the "Sierra de Comechingones", Argentina. *J ethnopharmacol* 107, 324-341.

Gross, G., Jacobs, D. M., Peters, S., Possemiers, S., van Duynhoven, J., Vaughan, E. E., and van de Wiele, T. (2010). *In vitro* bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. *J Agric Food Chem* 58, 10236-46.

Guo, F., Williams, B., Kwakkel, R., and Verstegen, M. (2003). *In vitro* fermentation characteristics of two mushroom species, an herb, and their polysaccharide fractions, using chicken cecal contents as inoculum. *Poult Sci* 82, 1608-1615.

Guo, F. C., Kwakkel, R. P., Williams, B. A., Li, W. K., Li, H. S., Luo, J. Y., Li, X. P., Wei, Y. X., Yan, Z. T., and Verstegen, M. W. (2004). Effects of mushroom

and herb polysaccharides, as alternatives for an antibiotic, on growth performance of broilers. *Br Poult Sci* 45, 684-94.

Heo, J. M., Opapeju, F. O., Pluske, J. R., Kim, J. C., Hampson, D. J., and Nyachoti, C. M. (2013). Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *J Anim Physiol Anim Nutr (Berl)* 97, 207-37.

Heres, L., Engel, B., Urlings, H. A., Wagenaar, J. A., and van Knapen, F. (2004). Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by *Campylobacter* and *Salmonella*. *Vet Microbiol* 99, 259-67.

Heres, L., Engel, B., Van Knapen, F., Wagenaar, J. A., and Urlings, B. A. P. (2003). Effect of fermented feed on the susceptibility for *Campylobacter jejuni* colonisation in broiler chickens with and without concurrent inoculation of *Salmonella enteritidis*. *Int J Food Microbiol* 87, 75-86.

Hossain, M. E., Kim, G. M., Sun, S. S., Firman, J. D., and Yang, C. J. (2012). Evaluation of water plantain (*Alisma canaliculatum* A. Br. et Bouche) and mistletoe (*Viscum album* L.) effects on broiler growth performance, meat composition and serum biochemical parameters. *J. Med. Plants Res.* 6, 2160-2169.

Hoste, H., Torres-Acosta, J., Quijada, J., Chan-Perez, I., Dakheel, M. M., Kommuru, D., Mueller-Harvey, I., and Terrill, T. (2016). Chapter Seven-Interactions Between Nutrition and Infections With *Haemonchus contortus* and Related Gastrointestinal *Nematodes* in Small Ruminants. *Advances in parasitolog* 93, 239-351.

Hoste, H., Torres-Acosta, J. F. J., Sandoval-Castro, C. A., Mueller-Harvey, I., Sotirakie, S., Louvandini, H., Thamsborg, S. M., and Terrill, T. H. (2015). Tannin containing legumes as a model for nutraceuticals against digestive parasites in livestock. *Vet Parasitol* 212, 5-17.

Humphrey, T., O'Brien, S., and Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: A food production perspective. *Int J Food Microbiol* 117, 237-257.

Jeaurond, E. A., Rademacher, M., Pluske, J. R., Zhu, C. H., and de Lange, C. F. M. (2008). Impact of feeding fermentable proteins and carbohydrates on

growth performance, gut health and gastrointestinal function of newly weaned pigs. *CJAS* 88, 271-281.

Jensen, L. S., and Maurice, D. V. (1978). Effect of high dietary copper on the ceca of chicks. *Poult Sci* 57, 166-70.

Jones, F., and Ricke, S. (2003). Observations on the history of the development of antimicrobials and their use in poultry feeds. *Poult Sci* 82, 613-617.

Jones, T. A., Donnelly, C. A., and Dawkins, M. S. (2005). Environmental and management factors affecting the welfare of chickens on commercial farms in the United Kingdom and Denmark stocked at five densities. *Poult Sci* 84, 1155-1165.

Jorgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D. R., Bolton, F. J., Frost, J. A., Ward, L., and Humphrey, T. J. (2002). Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. *Int J Food Microbiol* 76, 151-64.

Just, J. R., and Daeschel, M. A. (2003). Antimicrobial Effects of Wine on *Escherichia coli* O157:H7 and *Salmonella typhimurium* in a Model Stomach System. *J. Food Sci.* 68, 285-290.

Karikari, A. B., Obiri-Danso, K., Frimpong, E. H., and Krogfelt, K. A. (2017). Antibiotic Resistance in *Campylobacter* Isolated from Patients with Gastroenteritis in a Teaching Hospital in Ghana. *Open J. Med. Microbiol* 7, 1.

Kaya, I., Yigit, N., and Benli, M. (2008). Antimicrobial activity of various extracts of *Ocimum basilicum* L. and observation of the inhibition effect on bacterial cells by use of scanning electron microscopy. *AJTCAM* 5, 363-369.

Khanbabaee, K., and van Ree, T. (2001). Tannins: classification and definition. *Natural Product Reports* 18, 641-649.

Kraus, T. E. C., Dahlgren, R. A., and Zasoski, R. J. (2003). Tannins in nutrient dynamics of forest ecosystems - a review. *Plant and Soil* 256, 41-66.

Le Roy, C. I., Mappley, L. J., La Ragione, R. M., Woodward, M. J., and Claus, S. P. (2016). NMR-based metabolic characterization of chicken tissues and biofluids: a model for avian research. *Metabolomics* 12, 157.

Liu, J., Peng, L., Huang, W., Li, Z., Pan, J., Sang, L., Lu, S., Zhang, J., Li, W., and Luo, Y. (2017a). Balancing Between Aging and Cancer: Molecular Genetics Meets Traditional Chinese Medicine. *J Cell Biochem* 118, 2581-2586.

Liu, M., Huang, P., Wang, Q., Ren, B., Oyeleye, A., Liu, M., Zhang, J., Li, X., Zhang, X., Zhang, L., and Liu, X. (2017b). Synergistic antifungal indolecarbazoles from *Streptomyces* sp. CNS-42 associated with traditional Chinese medicine *Alisma orientale*. *J Antibiot (Tokyo)* 70, 715-717.

Mahady, G. B., Pendland, S. L., Stoia, A., and Chadwick, L. R. (2003). *In vitro* susceptibility of Helicobacter pylori to isoquinoline alkaloids from *Sanguinaria canadensis* and *Hydrastis canadensis*. *Phytother Res* 17, 217-21.

Maturana, V. G., de Pace, F., Carlos, C., Mistretta Pires, M., Amabile de Campos, T., Nakazato, G., Guedes Stheling, E., Logue, C. M., Nolan, L. K., and Dias da Silveira, W. (2011). Subpathotypes of Avian Pathogenic *Escherichia coli* (APEC) Exist as Defined by their Syndromes and Virulence Traits. *Open Microbiol J.* 5, 55-64.

McDougald, L. R. (1998). Intestinal protozoa important to poultry. *Poult Sci* 77, 1156-1158.

Missotten, J. A., Michiels, J., Dierick, N., Ovyn, A., Akbarian, A., and De Smet, S. (2013). Effect of fermented moist feed on performance, gut bacteria and gut histo-morphology in broilers. *Br Poult Sci* 54, 627-34.

Missotten, J. A., Michiels, J., Ovyn, A., De Smet, S., and Dierick, N. A. (2010). Fermented liquid feed for pigs. *Arch Anim Nutr* 64, 437-66.

Montagne, L., Pluske, J. R., and Hampson, D. J. (2003). A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Anim Feed Sci Technol* 108, 95-117.

Moreki, J. (2012). Use of Ethnoveterinary Medicine in family poultry health management in Botswana: A review. *J. Vet. Adv* 2, 254-260.

Mueller-Harvey, I. (2001). Analysis of hydrolysable tannins. *Anim Feed Sci Technol* 91, 3-20.

Mueller-Harvey, I., Bee, G., Dohme-Meier, F., Hoste, H., Karonen, M., Kölliker, R., Lüscher, A., Niderkorn, V., Pellikaan, W. F., and Salminen, J.-P. (2017). Benefits of condensed tannins in forage legumes fed to ruminants: importance of structure, concentration and diet composition. *Crop Science*.

Ofek I., Goldhar J., Sharon N. (1996) Anti-*Escherichia Coli* Adhesin Activity of Cranberry and Blueberry Juices. In: Kahane I., Ofek I. (eds) Toward Anti-

Adhesion Therapy for Microbial Diseases. Advances in Experimental Medicine and Biology, vol 408. *Springer*, Boston, MA

O'Keefe, S. J. (2008). Nutrition and colonic health: the critical role of the microbiota. *Curr Opin Gastroenterol* 24, 51-8.

O'May, C., Ciobanu, A., Lam, H., and Tufenkji, N. (2012). Tannin derived materials can block swarming motility and enhance biofilm formation in *Pseudomonas aeruginosa*. *Biofouling* 28, 1063-1076.

Park, S. F. (2002). The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int J Food Microbiol* 74, 177-188.

Petit, L., Gibert, M., and Popoff, M. R. (1999). *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol* 7, 104-10.

Reyes, A. W. B., Hong, T. G., Hop, H. T., Arayan, L. T., Huy, T. X. N., Min, W., Lee, H. J., Lee, K. S., and Kim, S. (2017). The *in vitro* and *in vivo* protective effects of tannin derivatives against *Salmonella enterica* serovar Typhimurium infection. *Microb Pathog* 109, 86-93.

Rinttila, T., and Apajalahti, J. (2013). Intestinal microbiota and metabolites--Implications for broiler chicken health and performance1. *J Appl Poult Res* 22, 647-658.

Ropiak, H. M., Ramsay, A., and Mueller-Harvey, I. (2016). Condensed tannins in extracts from European medicinal plants and herbal products. *J Pharm Biomed Anal* 121, 225-231.

Schofield, P., Mbugua, D. M., and Pell, A. N. (2001). Analysis of condensed tannins: a review. *Anim Feed Sci Technol* 91, 21-40.

Shao, D., Li, J., Li, J., Tang, R., Liu, L., Shi, J., Huang, Q., and Yang, H. (2015). Inhibition of Gallic Acid on the Growth and Biofilm Formation of *Escherichia coli* and *Streptococcus mutans*. *J Food Sci* 80, M1299-305.

Sheng, L., Olsen, S. A., Hu, J., Yue, W., Means, W. J., and Zhu, M. J. (2016). Inhibitory effects of grape seed extract on growth, quorum sensing, and virulence factors of CDC "top-six" non-O157 Shiga toxin producing *E. coli*. *Int J Food Microbiol* 229, 24-32.

Stanley, D., Keyburn, A. L., Denman, S. E., and Moore, R. J. (2012). Changes in the caecal microflora of chickens following *Clostridium perfringens* challenge to induce necrotic enteritis. *Vet Microbiol* 159, 155-62.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., and Conte, A. (2010). *In vitro* bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry* 120, 599-606.

Tan, B. K. H., and Vanitha, J. (2004). Immunomodulatory and antimicrobial effects of some traditional Chinese medicinal herbs: A review. *Curr. Med. Chem*. 11, 1423-1430.

Torok, V. A., Hughes, R. J., Ophel-Keller, K., Ali, M., and Macalpine, R. (2009). Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poult Sci* 88, 2474-81.

Valenzuela-Grijalva, N. V., Pinelli-Saavedra, A., Muhlia-Almazan, A., Domínguez-Díaz, D., and González-Ríos, H. (2017). Dietary inclusion effects of phytochemicals as growth promoters in animal production. *JAST* 59, 8.

Van Parys, A., Boyen, F., Dewulf, J., Haesebrouck, F., and Pasmans, F. (2010). The use of tannins to control *Salmonella typhimurium* infections in pigs. *Zoonoses Public Health* 57, 423-8.

Van Winsen, R. L., Urlings, B. A., Lipman, L. J., Snijders, J. M., Keuzenkamp, D., Verheijden, J. H., and van Knapen, F. (2001). Effect of fermented feed on the microbial population of the gastrointestinal tracts of pigs. *Appl Environ Microbiol* 67, 3071-6.

Viveros, A., Chamorro, S., Pizarro, M., Arija, I., Centeno, C., and Brenes, A. (2011). Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. *Poult Sci* 90, 566-78.

Waghorn, G. (2008). Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-Progress and challenges. *Anim Feed Sci Technol* 147, 116-139.

Waghorn, G. C., and McNabb, W. C. (2003). Consequences of plant phenolic compounds for productivity and health of ruminants. *Proc Nutr Soc* 62, 383-392.

Wallace, R. J., Oleszek, W., Franz, C., Hahn, I., Baser, K. H., Mathe, A., and Teichmann, K. (2010). Dietary plant bioactives for poultry health and productivity. *Br Poult Sci* 51, 461-87.

Williams, B. A., Bosch, M. W., Boer, H., Verstegen, M. W. A., and Tamminga, S. (2005). An *in vitro* batch culture method to assess potential fermentability of

feed ingredients for monogastric diets. *Anim Feed Sci Technol* 123-124, 445-462.

Windisch, W., Schedle, K., Plitzner, C., and Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *J Anim Sci* 86, E140-8.

Xu, L.-Q., Yu, X.-T., Gui, S.-H., Xie, J.-h., Wang, X.-F., Su, Z.-Q., Li, Y.-C., Lai, X.-P., Zhan, J. Y.-X., and Xie, Y.-L. (2017). Protective Effects of Li-Fei-Xiao-Yan Prescription on Lipopolysaccharide-Induced Acute Lung Injury via Inhibition of Oxidative Stress and the TLR4/NF-κB Pathway. *Evid-Based Complementary Altern Med.* 2017.

Yan, L., Meng, Q. W., and Kim, I. H. (2012). Effect of an herb extract mixture on growth performance, nutrient digestibility, blood characteristics, and fecal microbial shedding in weanling pigs. *Livest Sci* 145, 189-195.

Zhao, S., Yang, L., Liu, H., and Gao, F. (2017). *Stenotrophomonas maltophilia* in a university hospital of traditional Chinese medicine: molecular epidemiology and antimicrobial resistance. *J Hosp Infect* 96, 286-289.

Zhao, X. H., He, X., Yang, X. F., and Zhong, X. H. (2013). Effect of *Portulaca oleracea* extracts on growth performance and microbial populations in ceca of broilers. *Poult Sci* 92, 1343-7.

# Chapter 2: Analysis, characterisation and purification of condensed tannins from a wide range of herbs and plants

## 2.1. Introduction

Plants are important sources of bioactive compounds and have traditionally been used by the pharmaceutical industry as starting materials in the search for new medicines (Amagase et al., 2001). Tannins represent one group of natural plant products; they are polyphenolic compounds that are produced as secondary metabolites in many higher plants, which have been used for centuries as alternative medicines (Santos-Buelga and Scalbert, 2000). These compounds have several important biological activities and medicinal properties; e.g. they possess antioxidant, anti-inflammatory, antibacterial, antiviral and antitumor properties (Haslam, 1996). However, there is lack of information on tannin contents and composition in many medicinal plants, especially in Europe (Ropiak et al., 2016).

There are two main groups of tannins: i) hydrolysable tannins (Mueller-Harvey, 2001) include gallotannins and ellagitannins; and ii) condensed tannins (CTs) or proanthocyanidins (Schofield et al., 2001). The CT structures contain flavan-3-ol subunits that are linked either via C-4 to C-8 or C-4 to C-6 bonds to form oligomers and polymers (Khanbabaee and van Ree, 2001). There are several different types of CT, which are based on the number of OH or H groups in the B ring (Schofield et al., 2001). It is thought that the different CT types possess different biological activities (Hagerman et al., 1992). This thesis focuses solely on CT.

During the last century, several analytical assays have been developed to analyse CT; methods are based on their anti-oxidant properties, the depolymerisation of CTs with acid, precipitation reactions, enzyme and inhibition of microbes (Djipa et al., 2000). For instance, colorimetric reactions with the ABA method have been used to detect CTs in plant materials by generating coloured anthocyanidins; in this reaction CTs are depolymerised in the presence of acid. The acid-butanol method is the most commonly used assay to detect and quantify CT in plant materials or plant extracts (Schofield et al., 2001; Grabber et al., 2013).

Thiolysis is another depolymerisation reaction, where the reaction products are subsequently quantified by High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS) analysis. Thiolysis can also be used to determine CT compositions. In this method CT react with benzyl mercaptan (BM) under acidic conditions at an elevated temperature. The polymers are broken into terminal and extension flavan-3-ol subunits. Thus, terminal units are released as flavan-3-ols and extension units as their BM adducts (-BM adducts). These subunits can be quantified by HPLC/MS and this provides information on the mean degree of polymerisation (mDP) and procyanidin/prodelphinidin (PC/PD) and *cis/ trans* ratios, which are useful for describing CT compositions (Karonen et al., 2007; Gea et al., 2011).

CTs are often also characterised by their ability to bind with proteins, carbohydrates and other molecules, and such interactions can impact on the nutrient environment of intestinal microbiota. A report have shown that CT compounds may be active against gastrointestinal bacteria (Patra and Saxena, 2011). Thus, several tannin-containing plants have been studied for their potential to act as alternative remedies for various ailments using *in vitro* or *in vivo* studies (Van Parys et al., 2010). The present study screened several plants and herbal products that could potentially be used for herbal treatments and as sources for the different CT types. Raw materials and plant extracts were assessed in detail with different analytical methods to investigate CT concentrations and compositions.

## 2.2 Materials and methods

### 2.2.1 Approach to selection of plant materials

To start with approximately 300 dried plant materials were screened, which have provided from a previous project (EU REPLACE). These plants were analysed for CTs using the HCl-butanol-acetone assay (Grabber et al., 2013). Afterwards, a total of 16 plants with the highest CT contents were subjected to thiolysis and HPLC/MS analysis, which provided information of CT compositions such as mean degree of polymerisation (mDP), procyanidin and prodelphinidin (PC/PD) and *cis/trans* ratios, and CT concentrations. It also provided data on the molar composition of terminal and extension flavan-3-ol subunits. From these CT-containing materials 12 different CT fractions were prepared that contained high,

medium and low mDP values and a wide range of different PC/PD ratio of flavan-3-ol. These extracts were used for *in vitro* analyses in subsequent experiments.

### 2.2.2 Sample collection and preparation

The plants and herbs (whole plant materials or leaves; Table A - Appendix 1) were dried, ground in an impeller SM1 cutting mill (Retsch, Haan, Germany) to pass a <1 mm screen, and stored in containers at room temperature.

These samples were analysed for CT with the HCl-butanol-acetone acid (ABA) assay. The reagent was prepared as previously described by Grabber et al. (2013) but contained a slightly higher concentration of ammonium iron (III) sulphate dodecahydrate (see Section 2.2.4 below) as recommended subsequently by John Grabber (2014, personal communication). Some samples of fresh leaves were also collected and three were purchased (for further information see Table C-Appendix-1). These samples were used to extract CT for subsequent *in vitro* experiments.

### 2.2.3 Chemicals

Ammonium chloride (10%), hydrochloric acid (37%), butan-1-ol, acetone, dichloromethane, hexane, and methanol (all of laboratory reagent grade) were obtained from Fisher Scientific (Loughborough, UK). Taxifolin (98%) and benzyl mercaptan (BM; 99%) were obtained from Sigma-Aldrich. Ammonium iron (III) sulphate dodecahydrate (99%) and formic acid (98%) were from Acros-Organics, Sephadex LH-20 was from GE Healthcare, and ultrapure water was sourced from a Milli-Q $H_2O$ system (Millipore, UK).

### 2.2.4 Tannin analysis by HCl-butanol-acetone assay (ABA)

Fresh ABA reagent (100 ml) was prepared on a daily basis by mixing ammonium iron (III) sulphate (40 mg) with 3.3 ml water and 5.0 ml of HCl (12 M), and then 42 ml of butan-1-ol was added followed by 50 ml of acetone. Dried plant material (10 mg) was weighed into a test tube followed by the ABA reagent (10 ml) and a small magnetic stirrer bar (Grabber et al, 2013). Experimental treatments were replicated at least twice by carrying out assays on separate days with freshly prepared ABA reagent. The tubes were heated in a stirred block heater at 70 °C for 2.5 hours in

the dark. They were then cooled to room temperature, centrifuged, transferred to quartz cuvettes and scanned between 400 to 600 nm with a spectrophotometer (Jasco V530, Gross-Umstadt, Germany). The absorption intensity and the wavelength of the maximum were recorded. The same reagents were also used as blanks and used as diluent for samples that had absorbance readings outside the range of the standards, i.e. >1.5 absorbance units.

### 2.2.5 Tannin analysis by thiolysis and HPLC-MS (*in situ* analysis)

Thiolysis was performed according to Gea et al. (2011) and Williams et al. (2014a). The thiolytic degradation of CT was applied to the 16 selected plant samples that had high CT concentrations. The dried plant materials (200 mg) were accurately weighed into screw top glass test tubes (16 mm). A magnetic stirrer was added to each tube. Then, the reagent was prepared as follows: methanol (50 µl) acidified with concentrated 3.3% v/v HCl, (i.e., 3.3 ml of 37% HCl and 100 ml of methanol), and 100 µl of BM was added. After that 3.1 ml of this mixture was added to each tube. These tubes were capped with rubber plugs and vigorously stirred at approximately 800 rpm for 60 min at 40 °C. After one hour, the tubes were transferred to room temperature and 9 ml of 1% of formic acid (1 ml of formic acid in 100 ml of water) was added into the tubes to stop the reaction. The tubes were vortexed for a few seconds, and then centrifuged for 5 min at 4000 rpm. Afterwards, the supernatant (1 ml) was pipetted into an HPLC glass vial and closed with a crimp top. The HPLC vials were loaded into the HPLC/MS auto sampler and a taxifolin standard was run at the start and end of each batch. Samples (5 µl) were injected into the HPLC system.

The CT degradation products after thiolysis were analysed by HPLC/MS (Agilent 1100 series HPLS system consisting of a G1379A degasser, G1312A binary pump, a G1313A ALS auto-injector, a G1315A diode array detector and a G1316A column oven; Agilent Technologies, Waldbronn, Germany). The MS was run in the negative ion mode (Ropiak et al., 2016). The column used in this assay was an ACE super C18 column that was fitted with an ACE guard column (5 µm, 150x3.0 mm, Hichrom Ltd, Theale, UK). The sample table temperature was 60 $^{0}$C, with UV detection at 280 nm. The solvent A for the HPLC was prepared and inserted in a solvent pump bottle (720 ml from acid/ acetone in water for 30 samples). This

solvent was premixed from 10 ml of formic acid 1%, which was added to 1000 ml water, followed by the addition of 100 mg of ammonium chloride. This mobile phase was called solvent A. The second mobile phase (solvent B) was 1% formic acid in acetonitrile. The following gradient programme was used with a flow rate of 0.4 ml/min from 0–7 min 2.5% of solvent B was used; from 8-15 min it increased to 5%; from 16-22 min it was 10% and from 23-40 min this was increased to 40%. Then solvent B was increased to 100% from 40-45 min, and returned to 2.5% between 45 to 60 min (Williams et al., 2014b).

Tannin concentrations were calculated from the concentration of terminal units namely catechin (C), epicatechin (EC), gallocatechin (GC) or epigallocatechin (EGC) and extension units, which were detected as the BM-adducts of C, EC, GC or EGC (Fig 1.3). This analysis provided information on molar percentages of these flavan-3-ols and enabled calculation of the mean degree of polymerisation (mDP), procyanidin/prodelphinidin and *cis/trans* ratios within these CT. These compounds were identified based on their retention times; their uv-vis spectra between 220 and 595 nm, and their MS spectra in the negative ionisation mode (Ramirez-Coronel et al., 2004).

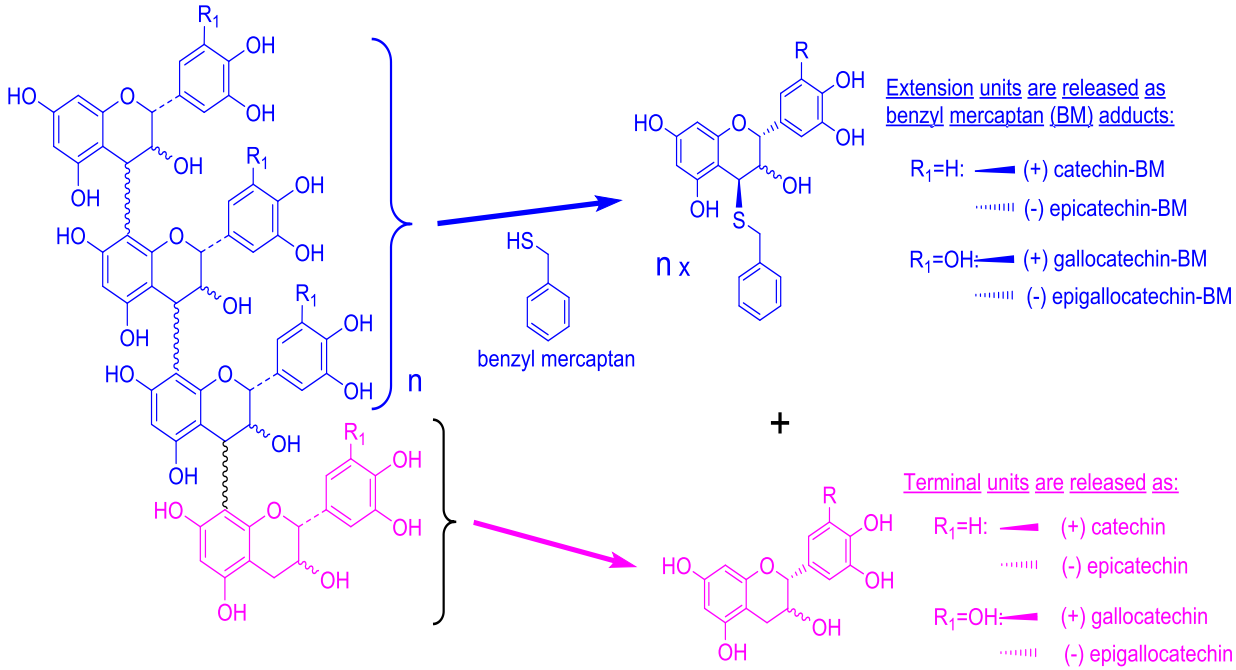



Figure 2.1: Thiolytic degradation of condensed tannin polymers. Extension subunits are released as flavan-3-ol benzyl mercaptan (BM) adducts, terminal subunits are released as underivatised flavan-3-ols (Gea et al., 2011).

### 2.2.6 Tannin extraction

Following the initial screening of the EU REPLACE samples, leaves with highest CT concentrations (Table B – Appendix 1) were identified and larger sample quantities were either collected from trees around Reading University, or purchased. Samples were freeze-dried for 4-5 days. Ten CTs were extracted and purified (see below). Samples were ground to pass through a 1 mm sieve and stored in plastic bags in the dark (Dai and Mumper, 2010).

Ground plant samples (40 g) were extracted with aqueous acetone (acetone/water, 70:30, v/v; 500 ml) in a conical flask and stirred for 1 hour at room temperature. The extract was filtered through a Buchner flask fitted with a ceramic funnel and filter paper (Whatman No. 1, 150 mm diameter) under vacuum. The filtrate was then poured into a separating funnel; dichloromethane (250 ml) was added to remove non-phenolic substances such as chlorophyll. The upper aqueous layer was collected, evaporated on a rotary evaporator to remove acetone and the remaining aqueous solution was frozen at –20 °C for subsequent analysis and CT purification as these extracts also other compounds such as carbohydrates, lipids and chlorophyll (Cheynier and Fulcrand, 2003).

### 2.2.7 Tannin purification and fractionation

Extracts with high CT concentrations were subjected to CT purification by column chromatography (Brown, et al 2017). The column material was prepared 1-2 days in advance by weighing Sephadex LH-20 (50 g) into a beaker and adding distilled water (200 ml). The mixture was gently stirred on an orbital shaker and left overnight to settle. Then the mixture was poured into the column (185 mm length x 30 mm i.d.) and allowed to settle. The resin bed was washed with distilled water (1L) and the water level was kept to 1 cm above the resin; now the column is ready for CT fractionation (Williams et al., 2014b).

First, the aqueous sample extract was allowed to defrost at room temperature. Then distilled water (1.5 L) was added. This mixture was poured into a separating funnel through a Whatman filter paper No. 1 to remove insoluble materials. The funnel with the sample was placed on top of the column and the funnel tap was adjusted to control the flow rate (40 ml/min) so that the Sephadex LH-20 resin bed

was not disturbed. After all of the aqueous sample had passed into the resin, the column was thoroughly washed with distilled water (1 L) to remove water soluble constituents until the eluent was clear (Asquith et al., 1983).

Acetone/water (30/70, v/v; 1 L) was then poured into the column to obtain the first fraction (F1-fraction). The liquid was collected (15 ml/min) and acetone evaporated with a rotary evaporator <40 °C, and the final aqueous fractions were frozen at –20 C°. The column was then subjected to another elution to obtain a second fraction (F2-fraction). This fractionation step involved pouring acetone/water (80/20, v/v; 1L) into the column (25 ml/min). The resulting eluent was evaporated as above to remove acetone. The column flow was stopped when the solvent was just 1 cm above the resin bed to keep it free of microorganisms. Both the aqueous (F1) and (F2) fractions were frozen and lyophilised, and stored at –20 C° for subsequent thiolysis analysis.

### 2.3 Statistical analyses

The data were analysed statistically by applying different methods depending on the relationship between the variables and factors. All data were analysed statistically using Minitab (version 18.0; Minitab, Inc, PA, USA). The nonparametric correlation (Spearman's rank correlation coefficient), the regression analysis (linear regression model) and t-test (comparisons of means) and one-way analysis of variance (ANOVA) were applied.

### 2.4 Results and discussion

The following text describes the results from the initial screening of plants for high CT concentrations and for contrasting CT compositions.

#### 2.4.1 Condensed tannin analysis by HCl-butanol-acetone assay (ABA)

The plant samples (Table A –Appendix 1) were screened with the ABA assay to determine CT concentrations (Terrill et al., 1992). To stabilise the anthocyanidins, which are derived from the CT during the reaction, iron salt ($Fe^{3+}$) was added (Porter et al., 1985). A more recent modification reported that the presence of acetone greatly enhanced the colour yield in this assay (Grabber et al 2013). From this analysis, it was concluded that 60 samples contained sufficiently high CT

concentrations (Table 2.1). Then 16 plant materials, which contained the highest CT concentrations, as seen from their colour intensity, were selected for detailed structural analysis by thiolytic degradation with BM to determine flavan-3-ol subunit compositions (Table 2.2).

Fig. 2.2 are illustrated the plant materials that contained different chemical structures of CT types (i.e. PC/PD) and their colours are shown in Fig. 2.3**.** The absorption spectrum obtained from plants that contained procyanidins, i.e. the absorption maximum is below 550 nm, is demonstrated in Fig. 2.4, while Fig. 2.5 shows the spectrum from plants with prodelphinidins, which have an absorption maximum above 555 nm. In other words, procyanidins give cyanidin chloride and absorbance peaks between 550 to 555 nm, whereas prodelphinidins give delphinidin chloride and absorbance peaks between 555 to 600 nm (Grabber et al., 2013) as demonstrated in Fig 2.4 and Fig 2.5. This screening led to the selection of 60 plant materials based on the presence of CTs. Plants were classified into four different groups according to their CT concentrations as measured by the ABA reaction. Based on Table 2.2, 16 samples with high CT concentrations were selected for subsequent analysis.

Although, the ABA assay can determine CT concentrations in these plant samples, CT compositions are complex and unique in each plant; hence thiolysis reaction was applied (Schofield et al., 2001).

**Figure 2.2:** Example of a condensed tannin dimer

Procyanidins (PC): R = H

Prodelphinidins (PD): R = OH

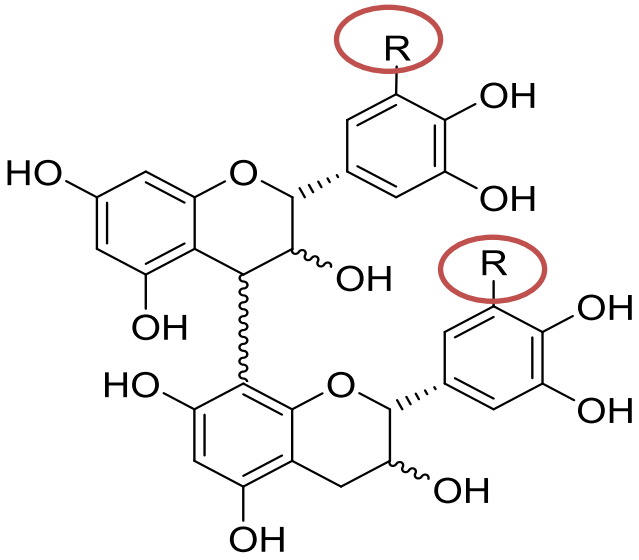
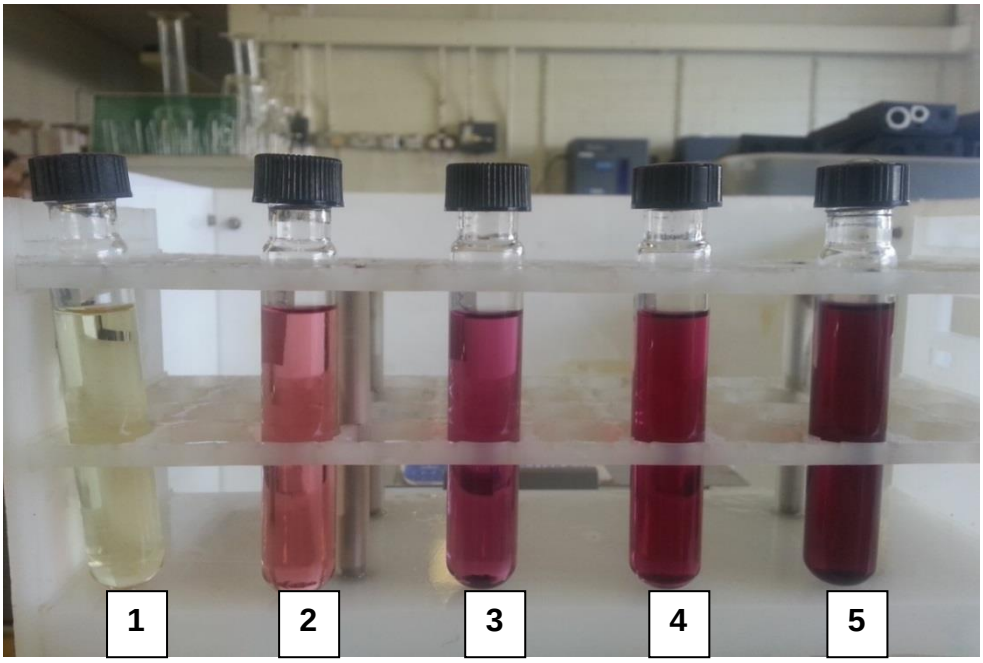



**Figure 2.3:** Colour generated by ABA assay with condensed tannin-containing plants and a negative control




1= negative control; 2= light CT; 3= + CT; 4= ++ CT; 5= +++ CT (this classification system is used in Table 2.1)

**Figure 2.4:** Vis spectrum of anthocyanidins released in the HCl-butanol acetone assay from procyanidin-rich tannins (absorption maximum is below 550 nm)

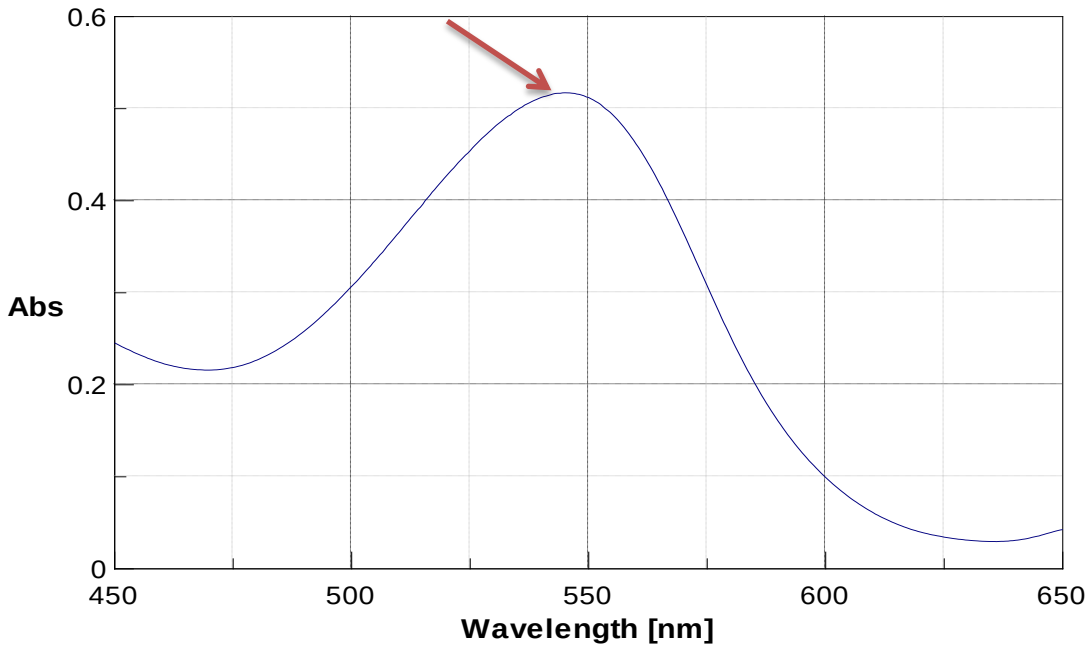



**Figure 2.5:** Vis spectrum of anthocyanidins released in the HCl-butanol acetone assay from prodelphinidin-rich rich tannins (absorption maximum is above 550 nm)

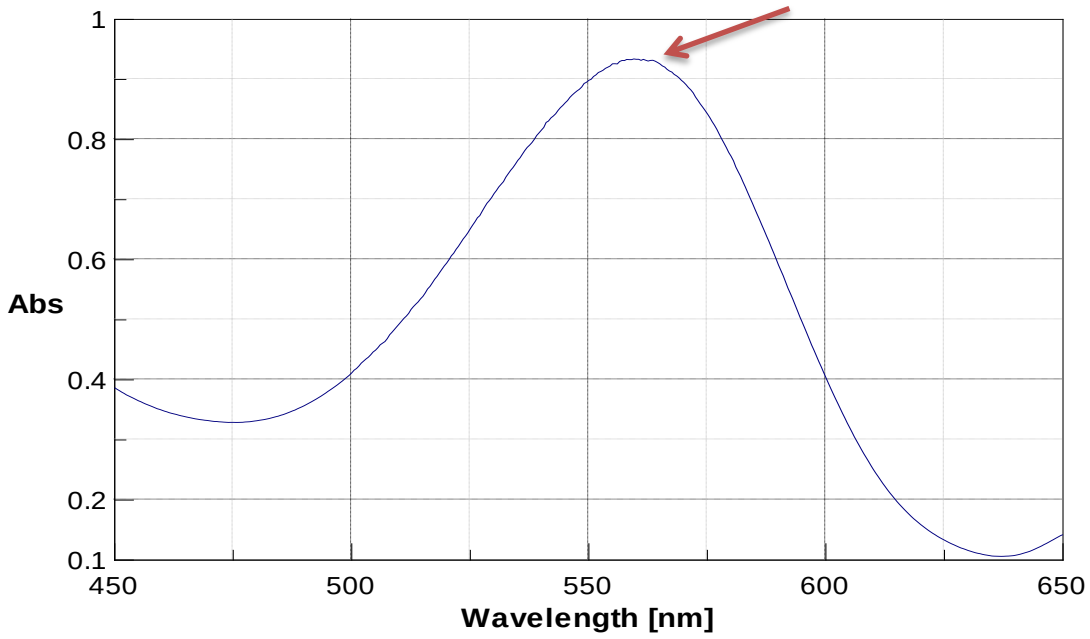


**Table 2.1:** Classification of plant materials depending on their condensed tannin contents according to the HCl-butanol-acetone assay

*(Light) = negligible CT; + = low CT; (++) = medium CT; (+++) = high CT contents; this table is ordered according to Absorption Units.

| No. | Botanical name | Common name | CT % | Abs (nm) |
|---|---|---|---|---|
| 1 | *Myristica fragrans* | Nutmeg | *LIGHT | 0.121 ±0.01 |
| 2 | *Potentilla aurea* | Dwarf yellow | LIGHT | 0.155 ±0.01 |
| 3 | *Juglans regia* | Walnut | LIGHT | 0.292 ±0.02 |
| 4 | *Chenopodium album* | White goosefoot | LIGHT | 0.324 ±0.01 |
| 5 | *Filipendula vulgaris* | Dropwort | LIGHT | 0.353 ±0.01 |
| 6 | *Magnolia officinalis* | Magnolia-bark | LIGHT | 0.363 ±0.01 |
| 7 | *Malus sylvestris* | Forest apple | LIGHT | 0.403 ±0.03 |
| 8 | *Arctostaphylos uva-ursi* | Bearberry | LIGHT | 0.425 ±0.02 |
| 9 | *Castanea sativa* | Sweet chestnut | LIGHT | 0.457 ±0.01 |
| 10 | *Impatiens parviflora* | Small balsam | LIGHT | 0.473 ±0.01 |
| 11 | *Fagopyrum esculentum* | Buckwheat | + | 0.510 ±0.03 |
| 12 | *Salix caprea* | Goat willow | + | 0.520 ±0.04 |
| 13 | *Alnus glutinosa* | Alder | + | 0.583 ±0.01 |
| 14 | *Peltiphyllum peltatum* | Indian rhubarb | + | 0.615 ±0.02 |
| 15 | *Quercus robur* | Oak | + | 0.616 ±0.01 |
| 16 | *Humulus lupulus* | Hop | + | 0.664 ±0.01 |
| 17 | *Primula florindae* | Tibetan cowslip | + | 0.695 ±0.02 |
| 18 | *Crataegus monogyna* | Hawthorn | + | 0.728 ±0.01 |
| 19 | *Fagus sylvatica* | Beech | + | 0.753 ±0.04 |
| 20 | *Prunus avium* | Wild cherry | + | 0.772 ±0.02 |
| 21 | *Sorbus aucuparia* | Rowan | + | 0.815 ±0.05 |
| 22 | *Dryopteris filix-mas* | Male fern | + | 0.819 ±0.02 |
| 23 | *Houttuynia cordata* | Chameleon plant | + | 0.832 ±0.06 |
| 24 | *Salix fragilis* | Crack willow | + | 0.860 ±0.02 |
| 25 | *Polygonum japonicum* | Knotweed | + | 0.886 ±0.05 |
| 26 | *Laurus virosa* | Bay laurel | + | 0.929 ±0.01 |
| 27 | *Acer platnoides* | Norway maple | + | 0.976 ±0.01 |

| | | | | |
|---|---|---|---|---|
| 28 | *Jasminum grandiflorum* | Jasmin | + | 1.016 ±0.03 |
| 29 | *Rhododendron ponticum* | Rhododendron | + | 1.029 ±0.03 |
| 30 | *Lotus corniculatus* | Bird's foot trefoil | + | 1.113 ±0.02 |
| 31 | *Juglans nigra* | Black walnut | + | 1.178 ±0.01 |
| 32 | *Thuja plicata* | Pacific redcedar | + | 1.344 ±0.03 |
| 33 | *Cinnamonum cassia* | Chinese cassia | + | 1.358 ±0.02 |
| 34 | *Fagus purpurea* | Copper Beech | + | 1.435 ±0.04 |
| 35 | *Gleditsia japonica* | Gleditsia | + | 1.450 ±0.01 |
| 36 | *Aesculus hippocastanum* | Horse chestnut | + | 1.451 ±0.04 |
| 37 | *Vaccinium vitis-idaea* | Cowberry | + | 1.451 ±0.04 |
| 38 | *Abies nordmannia* | Nordmann fir | + + | 0.512 ±0.01 |
| 39 | *Sequoiadendron giganteum* | Giant sequoia | + + | 0.519 ±0.04 |
| 40 | *Vaccinium myrtillus* | Bilberry | + + | 0.554 ±0.01 |
| 41 | *Rosa Gallica* | Gallic rose | + + | 0.560 ±0.01 |
| 42 | *Gaultheria procumbens* | Checkerberry | + + | 0.561 ±0.04 |
| 43 | *Tilia europea* | Lime | + + | 0.572 ±0.02 |
| 44 | *Tilia cordate* | Small leaved lime | + + | 0.574 ±0.03 |
| 45 | *Taxus baccata L.* | Yew | + + + | 0.814 ±0.02 |
| 46 | *Polygonum bistorta* | Bistort | + + + | 0.817 ±0.01 |
| 47 | *Hypericum perforatum* | St John's wort | + + + | 0.837 ±0.02 |
| 48 | *Robinia pseudoacacia* | Black locust | + + + | 0.855 ±0.04 |
| 49 | *Hydrangea petiolaris* | Climbing hydrangea | + + + | 0.860 ±0.03 |
| 50 | *Tilia L.* | Tilia | + + + | 0.883 ±0.02 |
| 51 | *Pimenta officinalis* | Allspice | + + + | 0.927 ±0.02 |
| 52 | *Corylus avellana* | Hazel | + + + | 0.934 ±0.06 |
| 53 | *Pinus L.* | Pine | + + + | 1.015 ±0.01 |
| 54 | *Picea abies* | Norway spruce | + + + | 1.036 ±0.01 |
| 55 | *Arbutus unedo* | Strawberry tree | + + + | 1.177 ±0.05 |
| 56 | *Cistus laurifolius* | Laurel-leaf cistus | + + + | 1.178 ±0.02 |
| 57 | *Parrotia persica* | Persian ironwood | + + + | 1.207 ±0.02 |
| 58 | *Euonymus alatus* | Winged spindle | + + + | 1.217 ±0.06 |

| | | | | |
|---|---|---|---|---|
| 59 | *Ribes nigrum* | Blackcurrant | + + + | 1.275 ±0.03 |
| 60 | *Platanus X hybrid* | London plane | + + + | 1.340 ±0.03 |

All values are means of triplicate analysis (n=3) ± standard deviation (SD); * Samples with high colour yields from CTs (absorption values) were diluted by 3 to keep all samples within the same range after dilution.

### 2.4.2 Condensed tannin analysis by thiolysis and HPLC assay (*in situ* method)

After screening the plants by the ABA assay, 16 samples (Table 2.2) were selected for characterising the CT features further by thiolysis and HPLC. These 16 plant materials were then subjected for detailed structural analysis by thiolytic degradation with BM. This method enabled measurement and calculation of the mean degree of polymerisation (mDP), PC/PD and *cis/trans* ratios, and CT concentrations (Gea et al., 2001). During the thiolysis reaction CTs are degraded into their monomeric flavan-3-ol units. The terminal units are released as GC, EGC, C or EC. Extension units are released as the corresponding BM derivatives. These flavan-3-ols and their BM derivatives can be analysed and measured by HPLC/ MS. Thiolysis combined with HPLC/MS is suitable for quantifying and for characterising CTs (Guyot et al., 2001). The chosen plant materials had between 3.2 to 14 % CT (1 g CT/100 g dried plant material). Table 2.2 illustrated the ratios between mDP, PC/PD and *cis/trans* ratios. Thiolytic analysis showed statistically significant differences between the CT compositions of the chosen plant materials (*P<0.05*).

The CT compositions were calculated according to the equations that are derived from a formula of CT monomer, and refer to molar ratios of terminal and extension flavan-3-ol units (Ropiak et al., 2016):

$$mDP = \frac{\text{amount of terminal units} + \text{amount of extension units (mol)}}{\text{amount of terminal units (mol)}}$$

$$\frac{PC}{PD}\% = \frac{\text{percentage of C units} + \text{percentage of EC units}}{\text{percentage of GC unit} + \text{percentage of EGC units}}$$

Table 2.3 shows that the molar percentage of PD within the CT is correlated with CT concentration. The same was also found for mDP values and molar percentages of *trans*-flavan-3-ols (Karonen et al., 2006). There was considerable variation among these values and this stems from the large concentration differences of the individual flavan-3-ols, i.e. C, GC, EC and EGC within the terminal and extension units. C and GC are the major components in PC and GC and EGC are the main components in PD (Zhang and Lin, 2008).

Analysis of the CT degradation products by thiolysis reaction and reversed-phase HPLC/MS showed that these plants could be classified into three distinct classes based on their CT compositions and the relationship between the mDP values on one hand and molar percentages of PC/PD on the other hand (Table 2.4). This correlation was analysed statistically by using a nonparametric correlation in Table 2.3 and demonstrated high correlations between mDP and CT concentration, then mDP and PD, also PD showed good correlation with CT concentrations.

**Table 2.2:** Concentration and composition of CT in the crude acetone/water extracts of plant materials: mean degree of polymerisation (mDP), molar percentages of procyanidins (PC %), prodelphinidins (PD %), and *trans* flavan-3-ols. * This table is ordered according to mDP values.

| No | Botanical names | Common names | mDP | PC %* | PD %* | *trans %** | Tannin %** |
|---|---|---|---|---|---|---|---|
| 1 | *Tilia L.* | Tilia flowers | 4.0 ±0.33 a | 100.0 ±1.25 c | 0.0 ±1.00 a | 2.3 ±1.20 a | 3.4 ±0.14 a |
| 2 | *Hydrangea petiolaris* | Climbing hydrangea | 4.1 ±0.28 a | 5.0 ±1.76 a | 95.0 ±1.76 c | 10.7 ±1.00 a | 8.2 ±0.35 b |
| 3 | *Taxus baccata L.* | Yew | 4.3 ±0.06 a | 50.9 ±1.51 b | 49.1 ±1.51 b | 30.0 ±0.71 b | 5.8 ±0.16 b |
| 4 | *Arbutus unedo* | Strawberry tree | 4.5 ±0.81 a | 59.5 ±1.74 b | 40.5 ±1.74 b | 21.7 ±0.32 b | 5.0 ±0.48 b |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5 | *Picea abies* | Norway spruce | 4.5 ±0.38 a | 85.6 ±2.75 c | 14.4 ±2.75 a | 22.6 ±1.21 b | 4.3 ±0.22 a |
| 6 | *Pinus L.* | Pine buds | 5.0 ±0.51 ab | 54.6 ±1.05 c | 31.6 ±1.29 b | 31.1 ±1.00 b | 7.8 ±0.40 b |
| 7 | *Hypericum perforatum* | St John's Wort | 7.2 ±1.41 ab | 89.3 ±2.51 c | 10.7 ±2.51 a | 3.6 ±2.08 a | 4.0 ±0.06 a |
| 8 | *Euonymus alatus* | Winged spindle | 7.4 ±0.34 ab | 72.8 ±1.25 c | 27.2 ±1.25 b | 14.5 ±2.52 a | 5.8 ±0.29 b |
| 9 | *Polygonum bistorta* | Bistort | 7.7 ±0.34 ab | 100.0 ±0.75 c | 0.0 ±0.75 a | 5.1 ±1.53 a | 5.9 ±0.17 b |
| 10 | *Ribes nigrum* | Black currant | 8.2 ±1.79 ab | 9.3 ±1.29 a | 90.7 ±1.29 c | 76.4 ±1.75 c | 10.1 ±0.84 c |
| 11 | *Corylus avellana* | Hazel | 8.8 ±1.42 ab | 25.6 ±1.05 b | 74.4 ±1.05 c | 29.1 ±2.0F b | 5.2 ±0.33 b |
| 12 | *Platanus X hybrid* | London plane | 12.0 ±1.54 b | 31.3 ±0.36 b | 68.7 ±0.36 c | 20.1 ±0.61 b | 10.3 ±0.32 c |
| 13 | *Pimenta officinalis* | Allspice | 12.9 ±1.6 b | 37.0 ±1.20 b | 63.0 ±1.20 c | 42.2 ±1.00 b | 3.2 ±0.49 a |
| 14 | *Cistus laurifolius* | Laurel-leaf cistus | 13.9 ±1.56 b | 57.5 ±1.51 b | 42.5 ±1.51 b | 0.0 ±1.75 a | 7.3 ±0.58 b |
| 15 | *Parrotia persica* | Persian ironwood | 26.8 ±1.24 c | 12.0 ±1.53 a | 88.0 ±1.53 c | 8.3 ±2.75 a | 6.1 ±0.12 b |
| 16 | *Robinia pseudoacacia* | Black locust | 27.6 ±1.55 c | 7.0 ±0.51 a | 93.0 ±0.51 c | 6.3 ±1.26 a | 14.0 ±1.0 c |

All values are means calculated from triplicate analyses (n=3) ± standard deviation (SD). Different letters in the same column in each parameter of each plant material indicate a significant difference (P≤0.05) between them. %* molar percentage; %** 1 g CT /100 g plant material

**Table 2.3:** Correlations between the CT compositions in the plants extracts

| | **mDP** | **PD** %[a] | ***trans*** %[a] | **Tannin** %[b] |
|---|---|---|---|---|
| **mDP** | 1 | | | |
| **PD** %[a] | 0.499 * | 1 | | |
| ***trans*** %[a] | 0.131 | 0.323 | 1 | |
| **Tannin** %[b] | 0.540 * | 0.605 * | 0.096 | 1 |

(*) indicates the best correlation between these compositions; %[a] = molar percentage; %[b] = 1g CT /100 g samples.

**Table 2.4:** Overview of *in situ* thiolysis results of selected plants for their contrasting tannin compositions, which were grouped into three categories (low, medium and high mDP values and molar percentage of PD).

| **PD%*** / **mDP** | **Low ≤ 30** | **Medium 31 - 69** | **High ≥ 70** |
|---|---|---|---|
| **≤ 5** | Tilia flowers<br>(mDP 4.0, PD 0)<br>Norway spruce<br>(mDP 4.5, PD 14.4) | Yew<br>(mDP 4.3, PD 49.1)<br>Strawberry tree<br>(mDP 4.5, PD 40.5) | Climbing hydrangea<br>(mDP 4.1, PD 95.0) |
| **~ 6 - 9** | Bistort<br>(mDP 7.7, PD 0)<br>St John's Wort<br>(mDP 7.2, PD 10.7)<br>Winged spindle<br>(mDP 7.4, PD 27.2) | Pine buds<br>(mDP 5.0, PD 54.6) | Blackcurrant<br>(mDP 8, PD 90.7)<br>Hazel<br>(mDP 8.8, PD 29.1) |
| **≥ 10** | N/A | Allspice<br>(mDP 12.9, PD 63.0)<br>Laurel-leaf cistus<br>(mDP 14, PC 42.5) | London plane<br>(mDP 12, PD 68.7)<br>Persian ironwood<br>(mDP 26.8, PD 88.0)<br>Black locust<br>(mDP 33.6, PD 93.0) |

N/A = not applicable; %* = molar percentage of prodelphinidins

### 2.4.3 Purification and fractionation of condensed tannins

Organic solvents are widely used for the extraction and isolation of bioactive compounds such as CTs. However, the resulting crude plant extracts tend to contain not only tannins but also soluble carbohydrates, lipids, monomeric flavonoids and many other organic compounds (Okuda, 2011). These non-tannin contaminants need to be removed, in order to assess the bioactivity of CTs (Le Bourvellec, 2007). However, a few plants specialise in the synthesis of either PCs or PDs of CT types. Therefore, these plants can be valuable sources for CT compositions, and as research tools for probing structure–activity (Klongsiriwet et al., 2015).

To increase the CT concentration in these samples, extracts were purified on a Sephadex LH-20 column (Asquith et al., 1983). In general, contaminants can be eliminated by rinsing with selected solvents; in the case of CT various concentrations of aqueous acetone are commonly used (Brown et al., 2017).

A Sephadex LH-20 column has been employed for the purification and fractionation of CTs in crude extracts (Williams et al., 2014b). Purification with 30% acetone in water yielded (Fraction – F1, which contained low molecular weight CT) and 80% acetone in water (Fraction - F2, which contained high molecular weight CT). This method applied to increase the CT concentrations and purified the CT compositions (Brown et al., 2017).

This enabled selection of 12 CT fractions, which covered a CT matrix with low, medium and high mDP and PC values and generated two fractions from each extract. The results showed higher CT concentrations compared to their plant origins or extracts. Fractions that were chosen for the microbial and metabolic experiments had CT concentrations between 87 to 95 g CT/100 g fractions (Table 2.5).

**Table 2.5:** Relationship between mean degree polymerisation (mDP) and molar percentage of prodelphinidins in CT fractions

* This table is ordered according to mDP values.

| | Common name | Fractions | mDP | PD %[a] | CT %[b] | mDP X PD% |
|---|---|---|---|---|---|---|
| 1 | London plane | F1 | 2.1 | 29.2 | 90 | L L |
| 2 | Yew | F1 | 2.1 | 48.4 | 93 | L M |
| 3 | Yew | F2 | 2.3 | 40.6 | 91 | L M |
| 4 | Pine buds | F1 | 2.9 | 54.6 | 88 | L M |
| 5 | Tilia flowers | F1 | 3.2 | 7.7 | 95 | L L |
| 6 | Black locust | F1 | 4.4 | 74.9 | 94 | M H |
| 7 | Black current | F1 | 4.5 | 73.4 | 90 | M H |
| 8 | Pine buds | F2 | 7.0 | 56.6 | 87 | M M |
| 9 | London plane | F2 | 7.7 | 45.0 | 89 | H M |
| 10 | Tilia flowers | F2 | 8.9 | 3.9 | 92 | H L |
| 11 | Black current | F2 | 8.9 | 75.1 | 95 | H H |
| 12 | Black locust | F2 | 9.8 | 74.6 | 95 | H H |

L = low; M = medium; H = high; all extracts originated from leaves; %[a] = molar percentage; %[b] = 1 g CT /100 g samples

Tables 2.2 showed that the CT purification did not achieve 100% purities. It is well known that aqueous acetone plant extracts contain many different compounds such sugars, phenolic acids, flavonoids, proteins and lipids (Hagerman and Butler, 1980). Therefore, to complete purification would require many more purification steps by Sephadex LH-20 column. The problem is that each purification step preferentially removes the large molecular weight CT and this makes investigation into mDP effects especially challenging (Stringano et al., 2012). For this reason, the microbiological experiments used the F1 and F2 fractions with further purifications. The isolated CT included homo- and heterogeneous mixtures, which contained a wide range of molecular weights. Further, this study focused on CT compounds that contain only free PC or PD ratio of flavan-3-ol (Table 2.5), and not include samples with galloylated flavan-3-ol subunits. Bindon et al. (2010) mentioned previously that galloylated CTs could interfere with the calculation of CT concentrations and lead to interesting effect as antibacterial agents (Santos-

Buelga and Scalbert, 2000). Thus, these CT extracts hypothesized that CTs can influence on different kind of bacteria, and this will be examined in the next chapters. Fig 2.6 summarised the whole processes that have been used in chapter 2 to extract and purify of CTs.

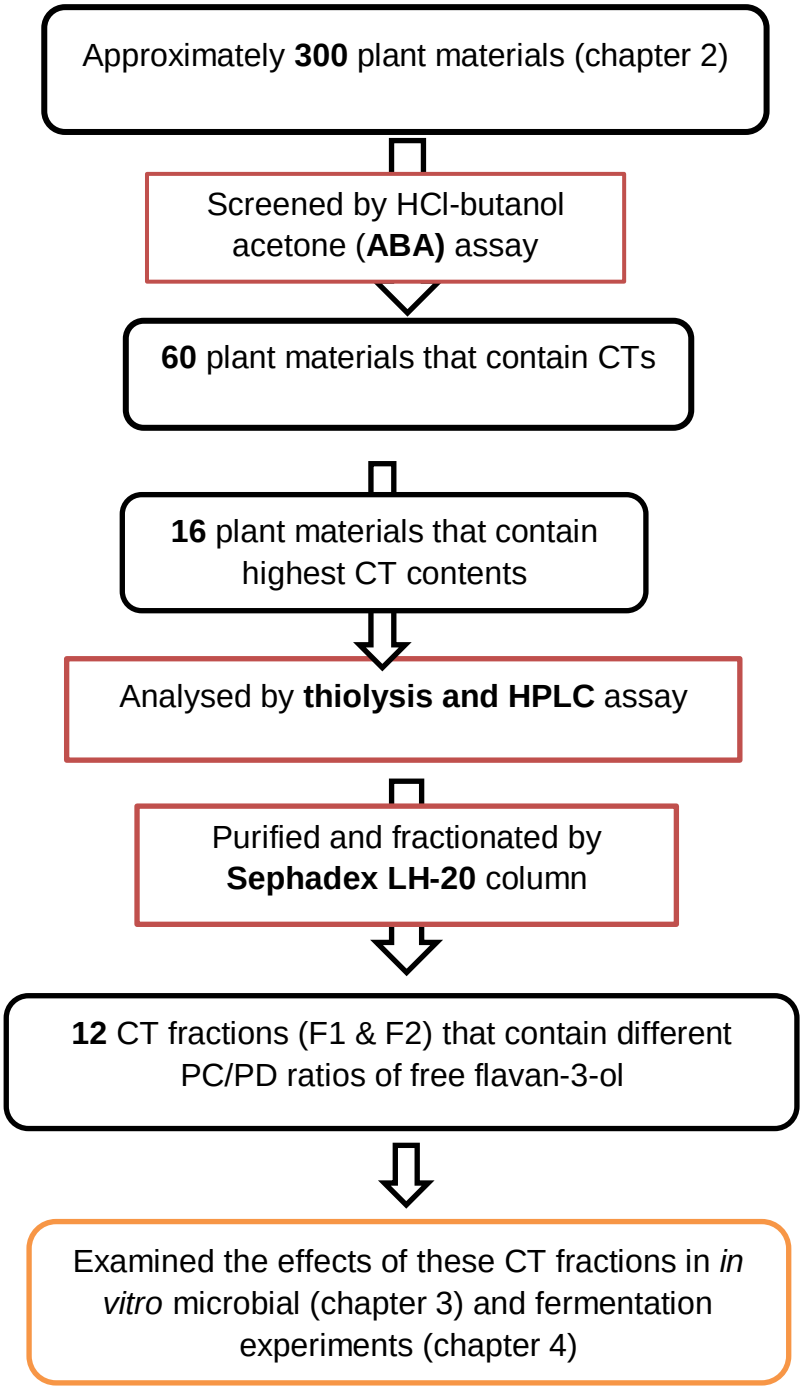



**Figure 2.6:** Flow diagram showing the overall strategy that was used for analysing, extracting and testing antimicrobial effects of CTs in this thesis.

## 2.5 Overall conclusion

The ABA method enabled screening of approximately 300 plants and herbs to rank the samples according to their CT concentrations. The CTs were then extracted from plant materials with the highest concentrations and their compositions analysed by thiolytic degradation and HPLC-MS analysis. Sixteen plant materials with high CT concentrations and a wide range of CT compositions were chosen. These CT groups were analysed statistically and confirmed positive correlations between mDP values, molar percentages of PD and CT concentrations. Finally, six CT extracts were chosen (with low and high molecular weight of CT fractions, F1 and F2 respectively) in order to generate purified CT samples for subsequent bioassays (Chapters 3 and 4). The resulting six CT extracts contained different CT types in terms of procyanidin and prodelphinidin compositions.

To our knowledge, this is the first detailed analysis of CT in extracts from black locust, yew and London plane. Information on CT composition tends to be hard to find as plants are not generally screened for CT concentrations or compositions. In fact such information on CT extracts could present opportunities for studying their health effects and could add useful information to natural compound databases. Such databases could prove valuable as a tool for further research on CT bioactivities. In addition, it could contribute to the standardisation and quality control of herbal products (Ropiak et al., 2016). The first objective of this study was achieved by identification and characterisation of a wide range of plant CT concentrations and compositions. Further analysis of extracts showed that these could be used as a source of contrasting CTs which differed in terms of mDP values and PC/PD ratios (Table 2.5). As a result, the hypothesis for this study was accepted, because CT compositions differed and varied between the plant materials and their extracts. Moreover, CT compositions such as mDP and molar percentages of PC were not correlated but mDP and molar percentages of PD were correlated. The next testable hypothesis is that some of these extracted and purified CTs may contribute to the control and management of the selected pathogens. The CT extracts from Chapter 2 were grouped according to CT compositions and concentrations and were used for investigations into the antibacterial effects of CTs against the selected pathogens. This is to be addressed in the next chapter.

## 2.6 References


Amagase, H., Petesch, B. L., Matsuura, H., Kasuga, S., and Itakura, Y. (2001). Intake of Garlic and Its Bioactive Components. *J Nutr* 131, 955S-962S.

Asquith, T. N., Izuno, C. C., and Butler, L. G. (1983). Characterization of the Condensed Tannin (*Proant hocyanidin*) from a Group II Sorghum. *J. Agric. Food Chem.*, 1299-1303.

Bindon, K. A., Smith, P. A., Holt, H., and Kennedy, J. A. (2010). Interaction between Grape-Derived Proanthocyanidins and Cell Wall Material. 2. Implications for Vinification. *J Agric Food Chem* 58, 10736-10746.

Brown, R. H., Mueller-Harvey, I., Zeller, W. E., Reinhardt, L., Stringano, E., Gea, A., Drake, C., Ropiak, H. M., Fryganas, C., Ramsay, A., and Hardcastle, E. E. (2017). Facile Purification of Milligram to Gram Quantities of Condensed Tannins According to Mean Degree of Polymerization and Flavan-3-ol Subunit Composition. *J Agric Food Chem*.

Cheynier, V., and Fulcrand, H. (2003). Analysis of polyphenolic proanthocyanidins and complex polyphenols. *RSC Adv* 284−313.

Dai, J., and Mumper, R. J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules* 15, 7313-7352.

Djipa, C. D., Delmee, M., and Quetin-Leclercq, J. (2000). Antimicrobial activity of bark extracts of *Syzygium jambos* (L.) alston (*Myrtaceae*). *J Ethnopharmacol* 71, 307-13.

Gea, A., Stringano, E., Brown, R. H., and Mueller-Harvey, I. (2011). *In situ* analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high-throughput germplasm screening. *J Agric Food Chem* 59, 495-503.

Grabber, J. H., Zeller, W. E., and Mueller-Harvey, I. (2013). Acetone Enhances the Direct Analysis of Procyanidin- and Prodelphinidin-Based Condensed Tannins in Lotus Species by the Butanol-HCl-Iron Assay. *J Agric. Food Chem*. 61, 2669-2678

Guyot, S., Marnet, N., Sanoner, P., and Drilleau, J. F. (2001). Direct thiolysis on crude apple materials for high-performance liquid chromatography characterization and quantification of polyphenols in cider apple tissues and juices. *Flavonoids and Other Polyphenols* 335, 57-70.

Hagerman, A. E., and Butler, L. G. (1980). Condensed Tannin Purification and Characterization of Tannin-Associated Proteins. *J. Agric. Food Chem*. 28, 947-952.

Hagerman, A. E., Robbins, C. T., Weerasuriya, Y., Wilson, T. C., and Mcarthur, C. (1992). Tannin Chemistry in Relation to Digestion. *JRM* 45, 57-62.

Haslam, E. (1996). Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J. Nat. Prod* 59, 205-215.

Karonen, M., Leikas, A., Loponen, J., Sinkkonen, J., Ossipov, V., and Pihlaja, K. (2007). Reversed-phase HPLC-ESI/MS analysis of birch leaf proanthocyanidins after their acidic degradation in the presence of nucleophiles. *Phytochem. Anal.* 18, 378-386.

Karonen, M., Ossipov, V., Sinkkonen, J., Loponen, J., Haukioja, E., and Pihlaja, K. (2006). Quantitative analysis of polymeric proanthocyanidins in birch leaves with normal-phase HPLC. *Phytochem Anal* 17, 149-56.

Khanbabaee, K., and van Ree, T. (2001). Tannins: classification and definition. *Natural Product Reports* 18, 641-649.

Klongsiriwet, C., Quijada, J., Williams, A. R., Mueller-Harvey, I., Williamson, E. M., and Hoste, H. (2015). Synergistic inhibition of *Haemonchus contortus* exsheathment by flavonoid monomers and condensed tannins. *Int J Parasitol Drugs Drug Resist* 5, 127-34.

Le Bourvellec, C., Le Quere, J.-M., and Renard, C. M. (2007). Impact of noncovalent interactions between apple condensed tannins and cell walls on their transfer from fruit to juice: Studies in model suspensions and application. *J Agric Food Chem* 55, 7896-7904.

Mueller-Harvey, I. (2001). Analysis of hydrolysable tannins. *Anim Feed Sci Technol* 91, 3-20.

Okuda, T., and Ito, H. (2011). Tannins of Constant Structure in Medicinal and Food Plants—Hydrolyzable Tannins and Polyphenols Related to Tannins. *Molecules* 16, 2191-2217.

Patra, A. K., and Saxena, J. (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *J Sci Food Agric* 91, 24-37.

Porter, L. J., Hrstich, L. N., and Chan, B. G. (1985). The Conversion of Procyanidins and Prodelphinidins to Cyanidin and Delphinidin. *Phytochemistry* 25, 223-230.

Ramirez-Coronel, M. A., Marnet, N., Kolli, V. S., Roussos, S., Guyot, S., and Augur, C. (2004). Characterization and estimation of proanthocyanidins and other phenolics in coffee pulp (*Coffea arabica*) by thiolysis-high-performance liquid chromatography. *J Agric Food Chem* 52, 1344-9.

Ropiak, H. M., Ramsay, A., and Mueller-Harvey, I. (2016). Condensed tannins in extracts from European medicinal plants and herbal products. *J Pharm Biomed Anal* 121, 225-231.

Santos-Buelga, C., and Scalbert, A. (2000). Proanthocyanidins and tannin like compounds–nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* 80, 1094-1117.

Schofield, P., Mbugua, D. M., and Pell, A. N. (2001). Analysis of condensed tannins: a review. *Anim Feed Sci Technol* 91, 21-40.

Stringano, E., Hayot Carbonero, C., Smith, L. M., Brown, R. H., and Mueller-Harvey, I. (2012). Proanthocyanidin diversity in the EU 'HealthyHay' sainfoin (*Onobrychis viciifolia*) germplasm collection. *Phytochemistry* 77, 197-208.

Terrill, T. H., Rowan, A. M., Douglas, G. B., and Barry, T. N. (1992). Determination of Extractable and Bound Condensed Tannin Concentrations in Forage Plants, Protein-Concentrate Meals and Cereal-Grains. *J. Agric. Food Chem.* 58, 321-329.

Van Parys, A., Boyen, F., Dewulf, J., Haesebrouck, F., and Pasmans, F. (2010). The use of tannins to control *Salmonella typhimurium* infections in pigs. *Zoonoses Public Health* 57, 423-8.

Williams, A., Christos, F., Ramsay, A., Mueller-Harvey, I., and Thamsborg, S. (2014a). Direct Anthelmintic Effects of Condensed Tannins from Diverse Plant Sources against *Ascaris suum*. *PLOS ONE* 9, e97053.

Williams, A. R., Ropiak, H. M., Fryganas, C., Desrues, O., Mueller-Harvey, I., and Thamsborg, S. M. (2014b). Assessment of the anthelmintic activity of medicinal plant extracts and purified condensed tannins against free-living and parasitic stages of *Oesophagostomum dentatum*. *Parasit Vectors* 7, 518.

Zhang, L. L., and Lin, Y. M. (2008). HPLC, NMR and MALDI-TOF MS analysis of condensed tannins from *Lithocarpus glaber* leaves with potent free radical scavenging activity. *Molecules* 13, 2986-97.

# Chapter 3: Antimicrobial *in vitro* activities of condensed tannins

## 3.1 Introduction

The first use of antibiotics as a growth promoter was in the 1940s due to an observation that the health of animals improved when they ate feed mixed with dried mycelia of *Streptomyces* or certain fungi (Castanon, 2007). In the middle of the last century, when the demand for food was increasing, the US, European and some other countries allowed the use of antibiotics as feed additives. However, these were applied at sub-therapeutic levels to provide a benefit for improved growth and feed conversion (Groschke and Evans, 1950). In the 1980s, there were numerous reports regarding the emergence of antibiotic resistance and how to avoid the use of antimicrobial agents as feed additives (Dibner and Richards, 2005). In addition growth promotion by antibiotic addition to feeds has been found to affect intestinal microflora (Niewold, 2007). In response to these issues, European countries banned the use of the antibiotics as growth promoters since 2006, which led to increased demands to find effective substances that can reduce the pathogenic bacteria and improve animal health (Kroismayr et al., 2008). Thus, medicinal plants and herbs are becoming recognised as a potential solution to promote animal performance without fostering antibiotic resistances (Baurhoo et al., 2007). Some natural products are bioactive compounds with multiple roles including medicinal action such as antioxidant and antimicrobial activities (Windisch et al., 2008).

In fact, Liu et al. (2011) investigated several Chinese herbal products, which contain numerous bioactive compounds such as essential oils, alkaloids and phenols that are often incorporated into animal diets. Thus, some medicinal plants and herbs may serve as alternative supplements instead of synthetic medicines. Many plants are good sources of natural products that may be suitable as feed additives, which can add commercial value to these plants. One example are tannins, which occur in the form of a complex of chemicals that are produced as part of the secondary metabolism of several higher plants, especially leguminous plants (Frutos et al., 2004). Plants which contain tannin have been used by human beings for centuries because of their many interesting biological activities and medicinal properties. ‘Tannin’ is an industrial term associated with the use of bark

in the conversion of skin, particularly the hides of animals, to produce leather in the presence of tannin-containing plant extracts. In addition, it has contributed to making many popular drinks, e.g. tea and wine (Waghorn, 2008). Tannins can possess antimicrobial properties (Scalbert, 1991), but when used in diets can also be anti-nutritional (Butler, 1992).

In this chapter the research is focused on the anti-bacterial properties of the CTs described earlier in this thesis. Microorganisms, especially bacteria, can adhere to biotic and abiotic surfaces to create dense communities known as biofilms. Bacteria can attach to one another and to host surfaces; adhesion is demonstrated to be important for the primary step of biofilm formation that contributes to the pathogenicity of microorganisms (Schluter et al., 2015). Thus, adhesion is considered a vital factor that promotes the evolution of the collaboration between cells to form the biofilm (Sachs, 2008). Moreover, bacteria can contaminate and form biofilms on inert surfaces such as floors and walls of food processing plants and hospital wards. Tannin extracts have been used experimentally to reduce such biofilm formation of pathogens. For instance, cranberry tannin extracts have been applied to reduce and control *Porphyromonas gingivalis* effectively by impacting on their attachment (Labrecque et al., 2006). This research suggested that CT extracts could prevent the attachment of this pathogen by influencing its capacity to colonise on infected sites.

*E. coli* have flagella that contribute to motility dependent upon the environment and can be an essential part of the induction of adhesion of microbes on a host surface enabling biofilm formation (Verstraeten et al., 2008). *E. coli* is also a diverse species that causes diarrheal disorders and a variety of gastro-intestinal infections (Kaper et al., 2004). Some of these strains have demonstrated an ability to penetrate the mucus layer and efficiently colonise the mucosa of the large intestine (Torres et al., 2005). Therefore, *E. coli* has been one of the most important Gram negative bacteria for *in vitro* experiments to form the biofilm on host surfaces (O'Toole et al., 2000 ; Van Houdt and Michiels, 2005). Interestingly there are studies that demonstrate inhibition of *E. coli* O157:H7 in both *in vitro* and *in vivo* studies by using probiotic effects (Medellin-Pena et al., 2007). These effects showed the mechanisms of interference by probiotics include direct action against pathogens through adherence competition at colonisation sites and antibacterial

effects. In addition there is evidence that tannins can inhibit pathogenic bacteria as well (Akiyama et al., 2001).

Bacteria, e.g. APEC, can move and swim in different environments and induce infection to a host. Motility can also play a critical role in primary interference with a surface and help these bacteria to promote biofilm development (Kearns, 2010). Grape seed extracts, which contain tannins, possess antimicrobial properties; they can decrease the swarming motility of some kinds of *E. coli* according to dosage (Lee et al., 2011; Zhu et al., 2015). These studies concluded that the tannin extracts effectively blocked the movement of pathogens, such as *E. coli* and other bacteria. *E. coli* have been used as the best model for bacterial motility and they have shown biofilm formation when they were unable to generate their normal motility (Pratt and Kolter, 1998). Therefore, there is evidence that bacteria can use various strategies to initiate biofilm formation, and it is not surprising that bacteria commonly utilise their cell structures such as flagella in motile stages.

Concerning the trend towards the evolution of antibiotic-resistant strains, the antimicrobial activity of CTs could be of interest in the form of feed additives for the management of chicken pathogens. In this chapter, thus, the testable hypotheses will be that different compositions of CTs are needed as antimicrobial agents against Gram negative bacteria, as avian pathogenic *Escherichia coli* (APEC) and Gram positive bacteria, as *Staphylococcus epidermidis.* Further, this chapter determined the effect of CTs on the minimum bactericidal concentration (MBC) and monitored the effects on these pathogens by scanning electron microscopy (SEM) on these pathogens. It also investigated the ability of various CTs to interfere with APEC microbial activities such as its growth curve, biofilm formation and motile activity in *in vitro* experiments.

## 3.2 Material and methods

### 3.2.1 Bacterial strains

Two bacterial strains were provided for this study. The first one was provided by a PhD student (Fatemah Al-Kandari) of the microbial laboratory in the Department for Food and Nutritional Sciences at the University of Reading. This was an Avian Pathogenic *Escherichia coli* (APEC/ Gram -), which was isolated from chickens

during 2015 – 2016. The second bacterial strain (LAB strain) was *Staphylococcus epidermidis* (Gram +), which was provided from another PhD student (Amjed Al-Soltan) in the microbiological laboratory of the School of Biological Sciences, University of Reading. These bacterial strains were stored in 87% sterile glycerol that was mixed with Luria-Bertani broth (LB) and maintained at - 80 °C.

### 3.2.2 Minimal bactericidal concentration (MBCs) for both pathogens

This assay was performed by a microtiter broth dilution method, as described by (Sheng et al., 2016). A total 130 µl from each concentration of the CTs (see chapter 2 for CT preparation and concentrations), which were diluted into sterile LB broth, which was added to each well of a sterile 96 well microtiter plate (U-bottom; Sigma-Aldrich) containing 130 µl of the LB broth medium. A 20 µl volume of freshly prepared standard numbers of cells were added in different plates for each experiment, respectively. This suspension was inoculated into the wells of 96-well plates in the presence of CTs of different concentrations (0, 10, 5, 2.5, 1.25 and 0.63 mg/ml) and their incubation was at 37 °C overnight before being read. For every experiment, sterile LB broth was used as a negative control and bacterial suspension with the LB broth was included as a positive control.

In order to test for the survival of bacteria after overnight growth, a 10 µl sample from each well was inoculated aseptically onto LB agar and the Petri dishes were incubated at 37 °C overnight. Bacterial colonies were counted. For the purpose of this study the MBC is defined as the lowest concentration of an antibacterial agent required to kill and prevent bacterial growth over a somewhat extended period. All experiments were carried out in triplicate and this experiment has been repeated three times.

### 3.2.3 Determining the growth curve for APEC

This method has been described by (Sheng et al., 2016). Overnight APEC cultures were diluted in LB medium supplemented with a range of concentrations of CTs to give $1 \times 10^7$ CFU/ml, and 200 µl of this mixture which were added to 96 well microtiter plates. The plates were then incubated aerobically at 37 °C overnight with shaking at 100 rpm. One row of wells was used per treatment, 6 inner wells of each column was inoculated with bacteria, while the two outside wells of each

column were loaded with the positive and negative controls. OD values were read hourly at 600 nm using a FluoStar spectrometer (Molecular Device, BMG, Offenburg, Germany). The experiments were repeated three times plus three replicates with fresh culture.

### 3.2.4 Biofilm formation and cell adhesion of APEC

The determination of the effect of CT on biofilm formation used the method described by (Shao et al., 2015). The same 96 well plates that included the same aliquots from the previous growth curve analysis and contained APEC with various CTs were stored for 5 days in the incubator room at 25 °C without shaking after the readings were taken for the growth curve data. The LB broth only served as the negative control and there were two sorts of positive controls: one was LB broth plus CTs, to detect tannin adhesion on the surface, and the second was only the bacterial suspension.

After the 5th day of incubation, the content of each well was gently removed, and the wells were washed twice with 150 µl of phosphate buffered saline (PBS) to remove planktonic bacteria. These plates were dried at room temperature for 15 minutes, and adherent bacteria were stained with 150 µl of 0.1% of crystal violet (w/v) for 15 minutes. The wells were then rinsed twice with distilled water to remove any residues. After the plates were dried at room temperature, stained adherent cells were detached from the plates using 150 µl of 9:1 ethanol/acetone for 10 min. Then the optical density of stained adherent bacteria was determined with the FlurStar spectrophotometer. The OD was read at a wavelength of 600 nm and the mean OD value obtained from the medium control wells was subtracted from all the samples of OD values. The formation of biofilm was determined according to the final biofilm formation formulae:

Total OD600 observed – control positive with tannins = Final biofilm formation
Three independent experiments were performed in triplicate.

### 3.2.5 Motility tests for APEC

This assay was performed with different concentrations of CTs that were tested in *in vitro* against APEC using the method previously described by (O'May et al., 2012).

Briefly, swarming and swimming methods were undertaken in petri dishes containing swarm agar (nutrient broth 8 g and 1.0 % agar w/v, as mentioned (Kearns, 2010) and swim agar supplemented with the same nutrient broth above plus 0.3% agar w/v as described by (Zhu et al., 2015). These plates were left to dry at room temperature and they were, then, inoculated with 5 µl aliquots of broth culture that contained different concentrations of CTs plus bacterial suspension as also the treated groups or broth culture without CTs as control. The inoculum was placed on the centre of the agar surface to enable the visualisation of bacterial motility across the agar surface. Afterwards, these plates were inoculated and taken for growth phase measurements at 37 °C for 10h and 24h. The diameters of the motility zones were recorded.

### 3.2.6 Scanning electron microscope (SEM)

Bacterial suspensions of APEC and *S. epidermidis* were prepared after overnight incubation in LB medium at 37˚C. This suspension was mixed with different concentrations of CT fractionations (10.0 and 1.25 mg/ml) added to the bacterial suspension and incubated in a rotary shaker set at 100 rpm and 37°C for 5 hours for APEC or without shaking at 37°C for 5 hours as well for *S. epidermidis*. Untreated bacterial controls were grown in standard LB medium without CTs. This protocol to observe the bacteria by SEM followed the methods of Kaya et al. (2008).

Afterwards, the bacterial cells were harvested by centrifugation at 13000x g for 2 minutes and washed twice by re-suspending in phosphate buffer saline (PBS). Then 200 µl of each suspension were applied to a poly-L-lysine-coated glass cover slip for 15 minutes. The adhered cells were fixed with 2.5% glutaraldehyde (Sigma–Aldrich) at pH 7.2 for 20 minutes with gentle agitation. These specimens were then rinsed with distilled water for 10 minutes, then the specimens were taken through an ethanol dehydration cycle consisting of 10 min in each of the following solutions, 30%, 50%; 70%, 90% and 100% (v/v, ethanol/water) and incubated for 10 minutes, except for samples in 100% ethanol, which were left for an hour. These specimens were, finally, dried in the critical point dryer (high vacuum environment) and coated with metal gold that made the specimens surfaces electrically conductive for SEM analysis. Then these specimens were observed with a field emission equipped SEM.

### 3.3 Statistical analyses

Data obtained from the analysis were processed with Minitab (version 18.0; Minitab software, Inc., PA, USA), which was used to analyse the data via Student's t-tests, ANOVA (one way) and Tukey adjusted comparisons.

For statistical analysis the impact of different CT concentrations and compositions on both microbes were assessed separately. The significant differences (*P-values*; the statistical significance was set at $P \leq 0.05$) between the control and treated groups were compared. This generated the values for each CT treatment that had an influence on the microbes in MBC and/or growth curve tests, and on APEC biofilm formations and motility by ANOVA analysis. All values were based on three replicates (n=3) including control values plus standard error of the means (±SEM), and they were assessed depending on the factors such as CT compositions and concentrations, and their interaction on bacterial activities as illustrated in Tables 3.1, 3.2 and 3.3.

### 3.4 Results and discussion

#### 3.4.1 Determination of MBCs and impacts of CTs on bacterial growth

A microtiter broth dilution and plate counting method was used to determine the MBC effects of CTs on APEC (as Gram negative) and *S. epidermidis* (as Gram positive). These results (Fig 3.2) showed that CTs inhibited both APEC and *S. epidermidis* at different concentrations: MBCs were between 10 to 1.25 mg/ml, respectively. Thus, the observations showed that MBCs of APEC were higher than for *S. epidermidis,* which could be explained to some extent by the presence of negatively charged lipopolysaccharide in APEC (Smith et al., 2007) (Fig 3.1).

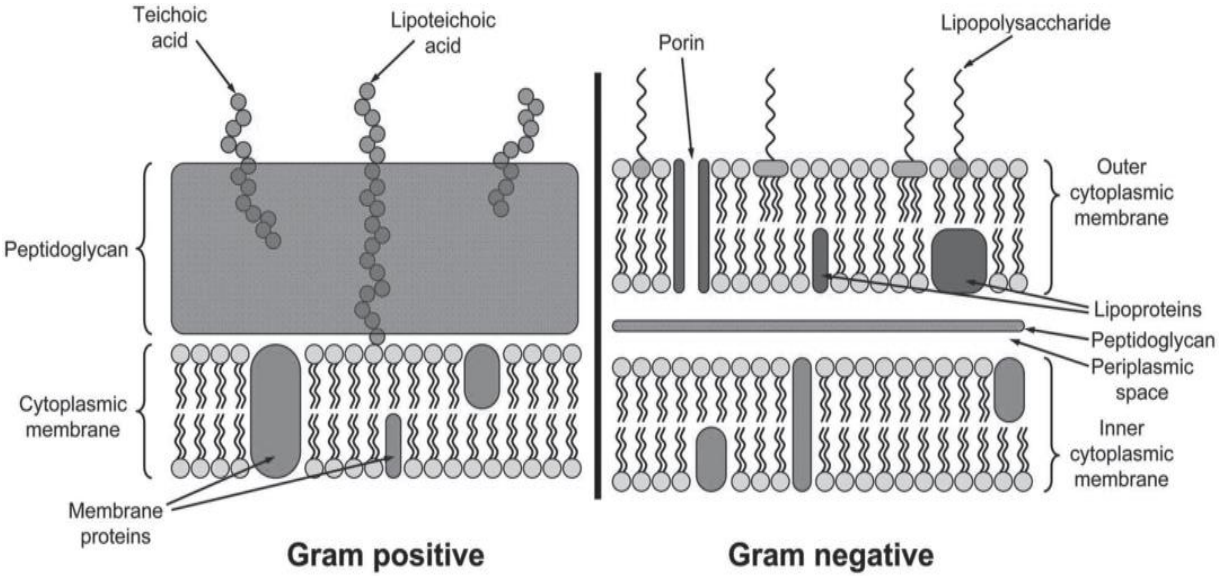



Figure 3.1: The differences in cell walls between Gram-positive and Gram-negative bacteria (Djurisic et al., 2015).

The data generated by following the cell density (growth curve for APEC at OD600) after inoculating LB broth with bacteria and CTs of various concentrations generally supported the findings of the MBC method. For instance, CTs in yew (F2/ medium PC-PD %), black locust (F2/ high PD %) and tilia flowers (F2/ high PC%) gave different MBC values (5 - 10 mg/ml) by the microdilution and plating method. However, with different concentrations of CTs, they showed different effects on substantial growth in the growth curve experiments.

Generally speaking the growth curves show a dependency on CT concentration, with the higher the CT concentration the lower the growth. However, it is interesting to observe that in the three graphs shown (Fig. 3.3) there is evidence for some enhancement of APEC growth by low concentrations (0.6 mg/ml) of CTs, especially for yew (F2/ medium PC-PD %), and tilia flowers (F2/ high PC %). This is intriguing and suggests that PC have less effect than PD compositions on bacteria, probably because the number of active hydroxyl groups is lower in the PC type than in the PD. Thus, the proportion of PDs within CT was the most important parameter that influenced the biological activities of microorganisms. However, it is also possible that the growth was similar but that the bacterial cell sizes were different; as this is actually the parameter that is measured (light refraction) by the spectrophotometer used in these assays. This can be assessed by Electron

Microscopy studies. To determine whether that was the case, the data from the electron microscopy studies then need to be considered.

The antimicrobial activity of CT extracts on a number of bacteria has been reported in several plants which are rich in tannins (Scalbert, 1991; Doss et al., 2009). There are probably several mechanisms involved in tannin toxicity to different microorganisms (Wang et al., 2008). A study indicated that the ability of tannin-containing plants to interact and make a complex with various compounds appears to be the basis of the CT inhibitory effect on several bacterial pathogens (Maisak et al., 2013) . CT can cause direct inhibition by interacting with membranes, cell walls and/or extracellular proteins (Scalbert, 1991). The data generated in this thesis show inhibition but do not give any firm identification of the involved mechanism. However, a study reported by Holloway et al., (2015) concluded that catechin, and other monomeric CTs, which combined with inorganic compounds such as copper sulphate to generate hydrogen peroxide that would have an antimicrobial effect on pathogens.

Growth curves of APEC gave the same results that matched the MBC values for all the three CT types (F2/ PC-PD ratio). This mean that this study revealed the antimicrobial efficacy of these CT extracts at relative concentrations against APEC in terms of MBCs and growth activity. Interestingly, the concentrations of CTs at 0.63 mg/ml, especially those from yew (F2/ medium PC-PD %) and tilia flowers (F2/ high PC %), showed an enhancement of bacterial growth of APEC. Moreover, tilia flowers (F2/ high PC %) was statistically significant $P \leq 0.05$ at this concentration compared to control. This finding showed that CTs with lower concentrations may fail to show any inhibitory impact on Gram-negative growth such as APEC, and this matches the findings of Sheng et al. (2016) for their studies with *E. coli* O103:H2 and *E. coli* O111:H2. As this is a repeatable trend observed in different *E. coli* isolates, this is certainly worthy of further investigation. A similar finding for the tested Gram-positive organism tested was not found and furthermore the MBC was much lower 0.6 - 1.25 mg/ml of these CTs that inhibited the bacterial growth of *S. epidermidis*.

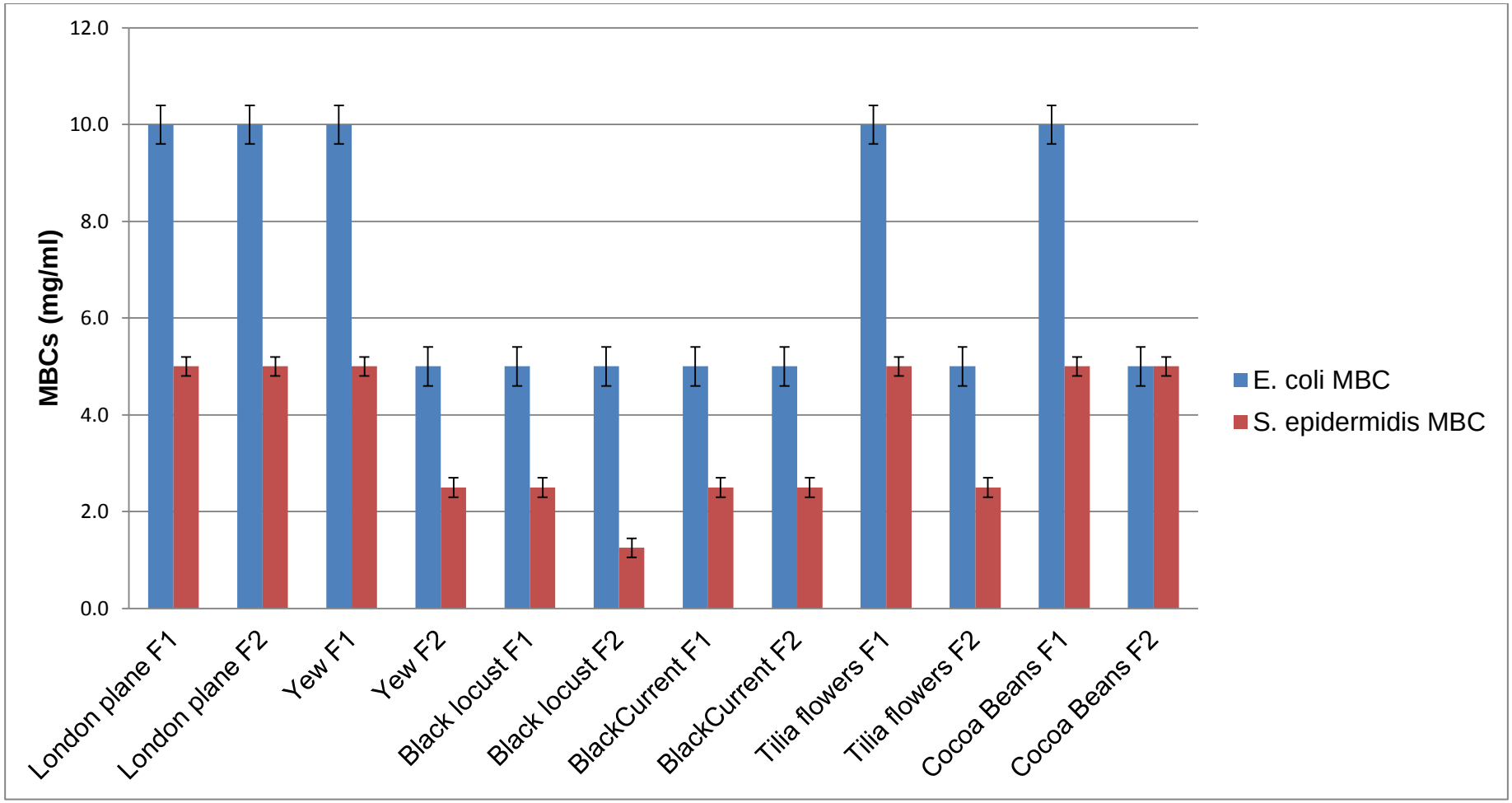
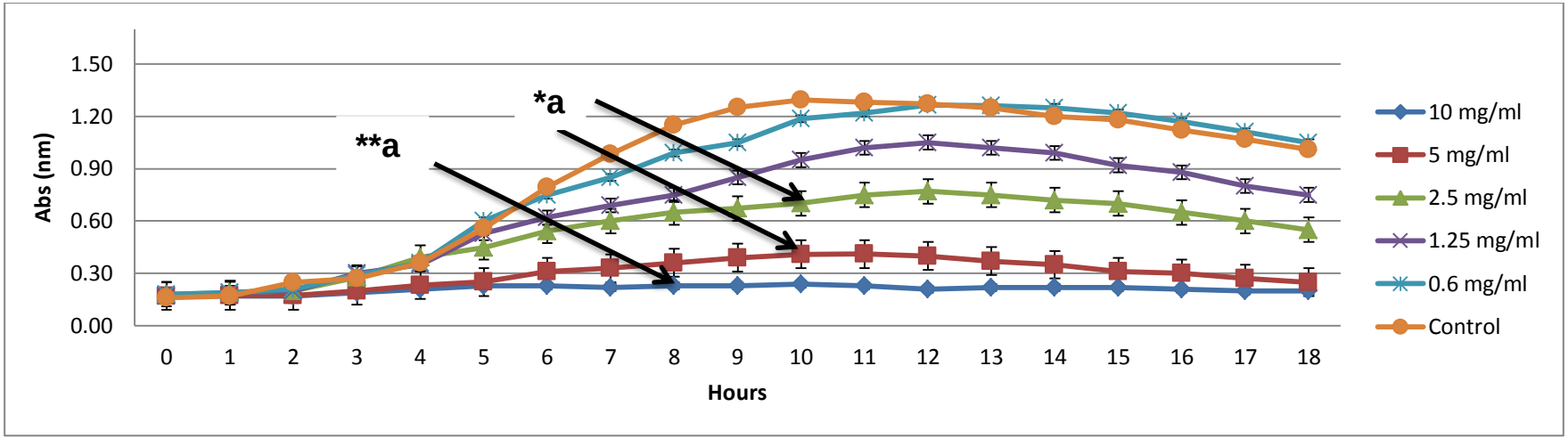


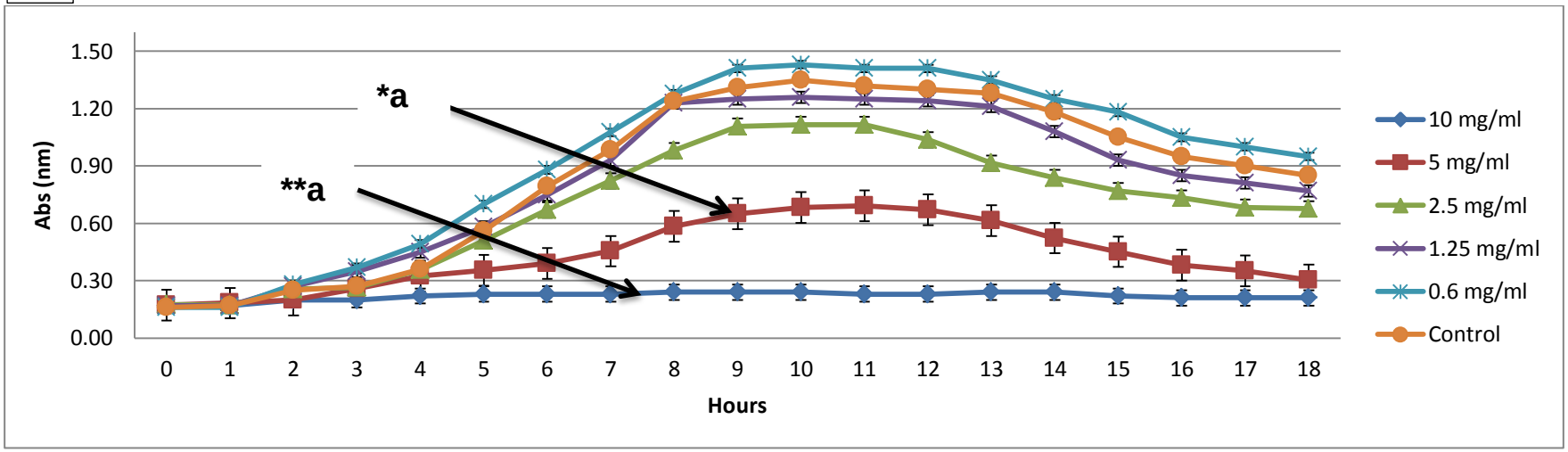

**Figure 3.2:** Effect of extracted CT fractions (PC/PD types) on MBCs of both pathogens (APEC and *S. epidermidis*).

Mean (n=3); Error bars indicate standard error (± SE); (F1) indicates CTs that contain low molecular weight; (F2) indicates CTs that contain high molecular weight.

i


1.50
1.20
0.90
0.60
0.30
0.00
Abs (nm)
*a
**a
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Hours
10 mg/ml
5 mg/ml
2.5 mg/ml
1.25 mg/ml
0.6 mg/ml
Control

ii


1.50
1.20
0.90
0.60
0.30
0.00
Abs (nm)
*a
**a
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Hours
10 mg/ml
5 mg/ml
2.5 mg/ml
1.25 mg/ml
0.6 mg/ml
Control

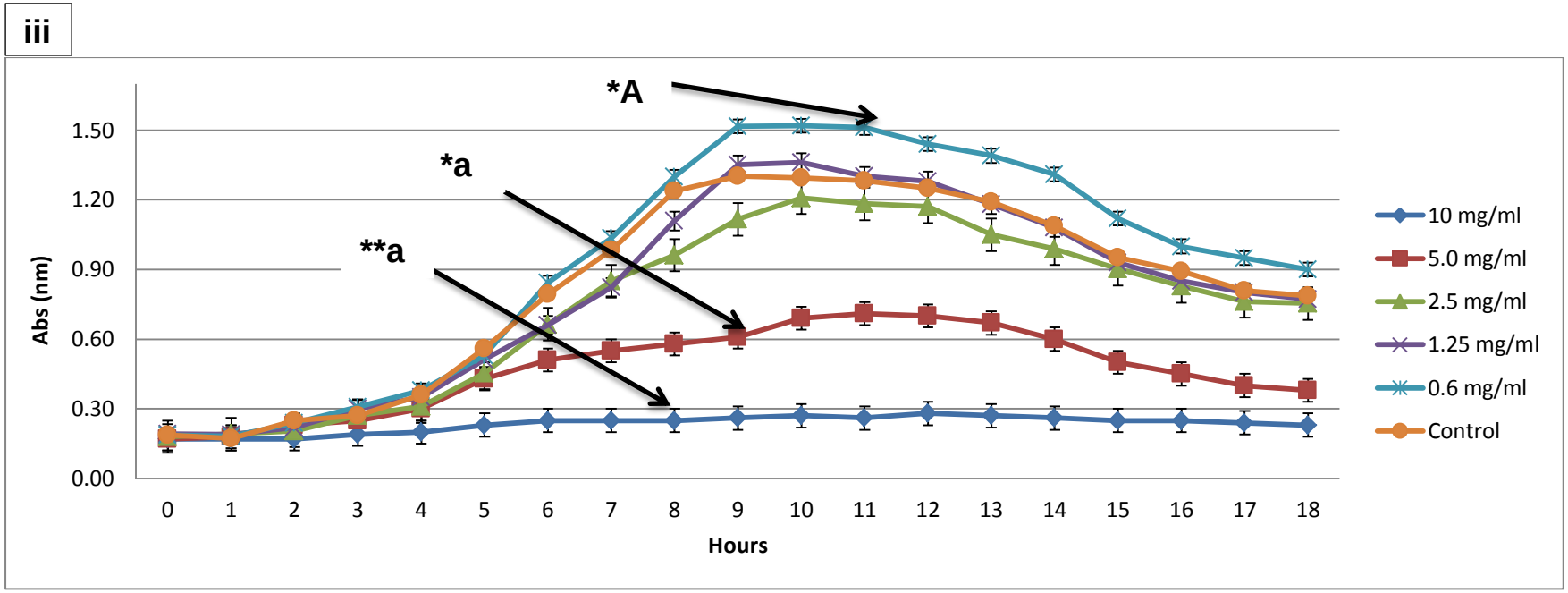



**Figure 3.3:** Effect of different concentrations of CTs on growth curves of APEC: **(i)** black locust (F2/ high PD), **(ii)** yew (F2/ medium PC/PD), **(iii)** tilia flowers (F2/ high PC); n=3 ± SE; (a) indicates decreased growth curve; (A) indicates increased growth curve compared to control; (*) indicates $P \leq 0.05$; (**) indicates $P \leq 0.01$

### 3.4.2 Biofilm formation of APEC

The effect of CT concentration and composition on biofilm formation by APEC is illustrated in Figure 3.4. The results indicated that high concentrations of different CT compositions could completely inhibit bacterial cell attachment of APEC (2.5 - 10 mg/ml) for all CTs and this significantly affected APEC ($P \leq 0.01$) because this concentration was at the same level of MBCs, while sub-MBC concentrations (1.25 – 0.6 mg/ml) displayed various values of inhibition with significant differences ($P \leq 0.05$). This interesting finding could be explained that when the bacteria tried to survive, they adhered on the surfaces and formed the biofilm (Donlan and Costerton, 2002).

Interestingly and, following on from this, the point made in the previous paragraph, that the low molecular weight of all CT types (which predominated in F1) seemed to give a significant ($P \leq 0.05$) enhancement at 0.6 mg/ml of biofilm formation. In contrast to this, different high molecular weight CTs (which presented in F2) showed no significant differences ($P > 0.05$) in enhancement of APEC. However, CT from tilia flowers (high PC %) showed significantly ($P \leq 0.05$) different results at the low concentrations compared to the control. This result indicates that APEC was more resistant to F1 than F2 tannins, as was also demonstrated in the previous part in this chapter that reported its lower inhibition values. Importantly, CT/ F2 fractions from black locust (high PD %) showed strong anti-biofilm activity, and no enhancement at the lowest concentrations compared to other CT extracts. Thus, low concentrations that are not inhibitory to APEC growth may actually contribute physically to increasing binding and biofilm formation. This is a novel finding that has not been reported before. Nonetheless, it should be pointed out that tannins bound to the walls of the 96 well plate and bound to the crystal violet. For all the measurements, the extent of biofilm formation was calculated by subtracting the value of tannin crystal violet binding.

Based on the above results, the inhibitory effect of CT on the growing of APEC is sensitive to both of the compositions and concentrations. Similar results were reported on biofilm inhibition using tea extract that also contains different polyphenolics and flavonoids such as tannins against *E. coli* (Zhang et al., 2014). Although CTs inhibited growth, which is an antimicrobial effect, the biofilm

formation still protects bacterial cells from stressful factors such as antimicrobial agents (Bendaoud et al., 2011). Therefore, the antimicrobial effect of these CT extracts combined with low nutrients in the medium may stimulate biofilm formation as a survival strategy (Borges et al., 2012).

In total, these results illustrated that CT compositions and concentrations influenced the biofilms of APEC. Further, these results showed a very close similarity to APEC anti-biofilm formation and growth inhibition.

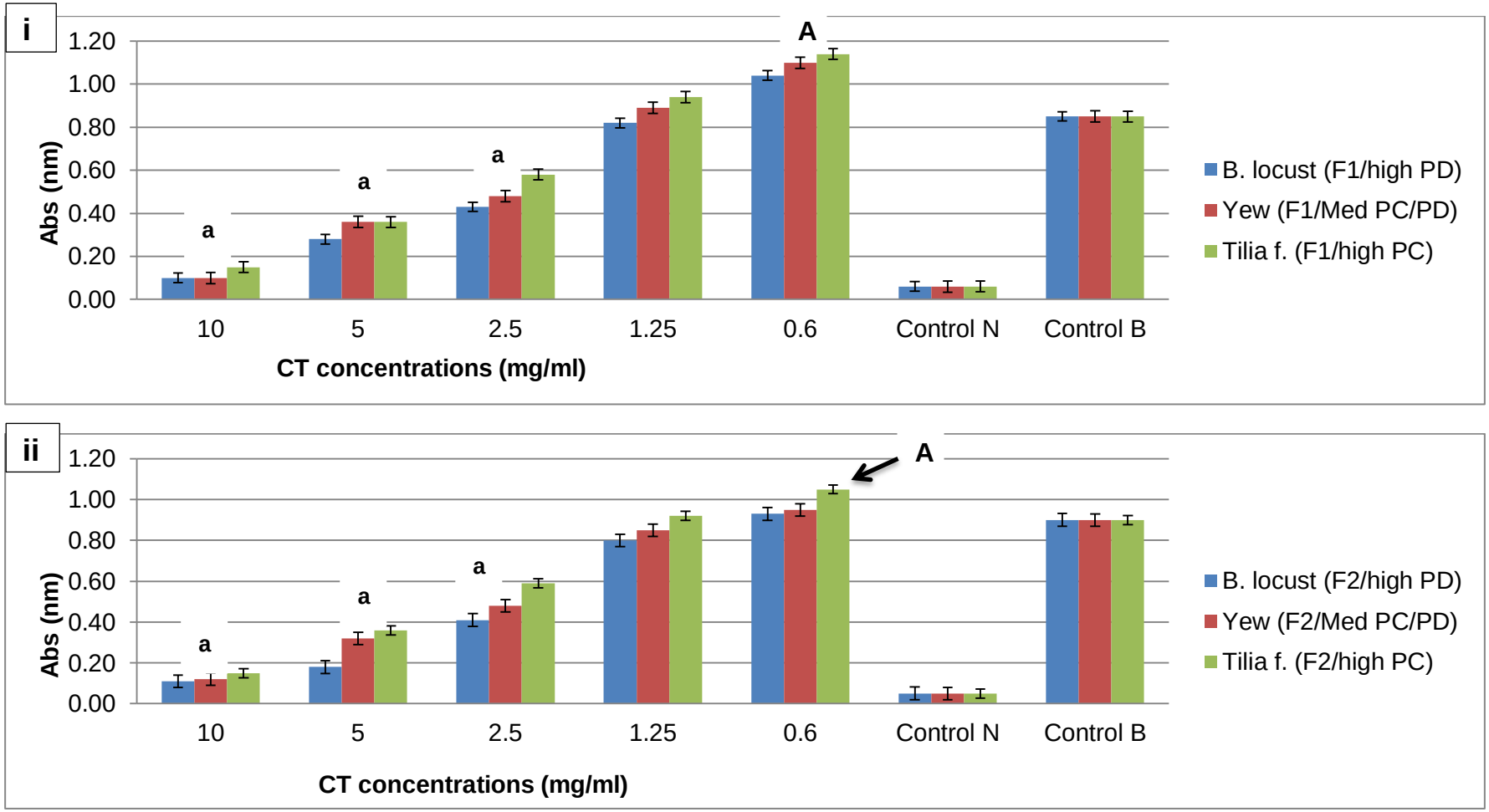



**Figure 3.4:** Effect of different concentrations of CTs on APEC of the biofilm formation: **(i)** F1 = CTs extracted with low molecular weight; **(ii)** F2 = CTs extracted with high molecular weight. Control N= control negative (LB medium); Control B= bacterial suspension, control positive; Note, treated CTs, which made colour, that subtracted from the total biofilm. (n=3 ± SE); Significant differences at *P≤0.05*; Capital letters indicate (increases) and small letters indicate (decreases) compared to positive control (B).

### 3.4.3 Motile activities of APEC

In the absence of CT extracts, APEC displayed proficient swimming and swarming motility and formed growth that migrated outwards from the point of bacterial inoculation. However, CTs could act on and affect bacterial motility. The observation that APEC were more resistant to PC% than PD% could probably be ascribed to some impact on their motile structures, e.g. flagella, as suggested previously (Pratt and Kolter, 1998).

Figure 3.5 shows significant differences (P≤0.05) between APEC motility and different concentrations of CT extracts. A study reported that different tannin-containing plants can block the motility of bacteria (O'May and Tufenkji, 2011). Therefore, our finding has been expanded to demonstrate that not only the concentration of CTs can influence motility but also CT compositions can impact the motility of APEC as well. However, these results can be linked to anti-biofilm effectors that were mentioned in the previous chapter (3.4.2). Bacterial motility plays an important role in adherence to surfaces and on the induction of biofilm formation and bacterial colonisation (Verstraeten et al., 2008).

This is the first study that demonstrates the effect of different concentrations and compositions of CT on blocking APEC motility in terms of swimming and swarming, which can cause the migrating bacteria to change direction. CTs showed different significant values (P≤0.05) on swimming and swarming activities. CTs were more effective against swimming than swarming. The controls showed that the normal ability of APEC was to remain motile and to form a diameter of 30 mm at 10h and of 40 mm at 24h in swimming tests. Conversely, controls in the swarming zone were recorded as 28 mm at 10h and 35 mm at 24h.

In general, CT compositions, PD or PC types, seemed to be the most important parameter that impacted the motility of APEC. The same CT parameters also showed the effects on other microbial properties in this chapter. However, our results showed that sub-MBC (0.6 mg/ml) of these CTs blocked swimming and swarming activities without impairing APEC growth capacities. Further, high molecular weight (F2) of all CTs showed a significant impact (P≤0.05) on swimming, whereas low molecular weight (F1) did not show significant differences (P≥0.05) except for the CT extract from black locust (high PD). On swarming

activity, black locust (F2/ high PD) was the only extracts that recorded a significant value ($P \leq 0.05$) compared to control.

This present study strongly concluded that different CT extracts blocked the motility of APEC. Given that CT compounds can bind to and precipitate many different types of proteins such as proteins in flagella (O'May and Tufenkji, 2011). However, in swimming activities, the cells move more independently, but swarming requires the bacteria to effectively work together which involves bacteria sensing the extracellular signals produced by other bacteria (Sheng et al., 2016). Further, these findings supported by the suggestion mentioned by O'May et al. (2012) about the relationship between motility and biofilm, are proved by this study.

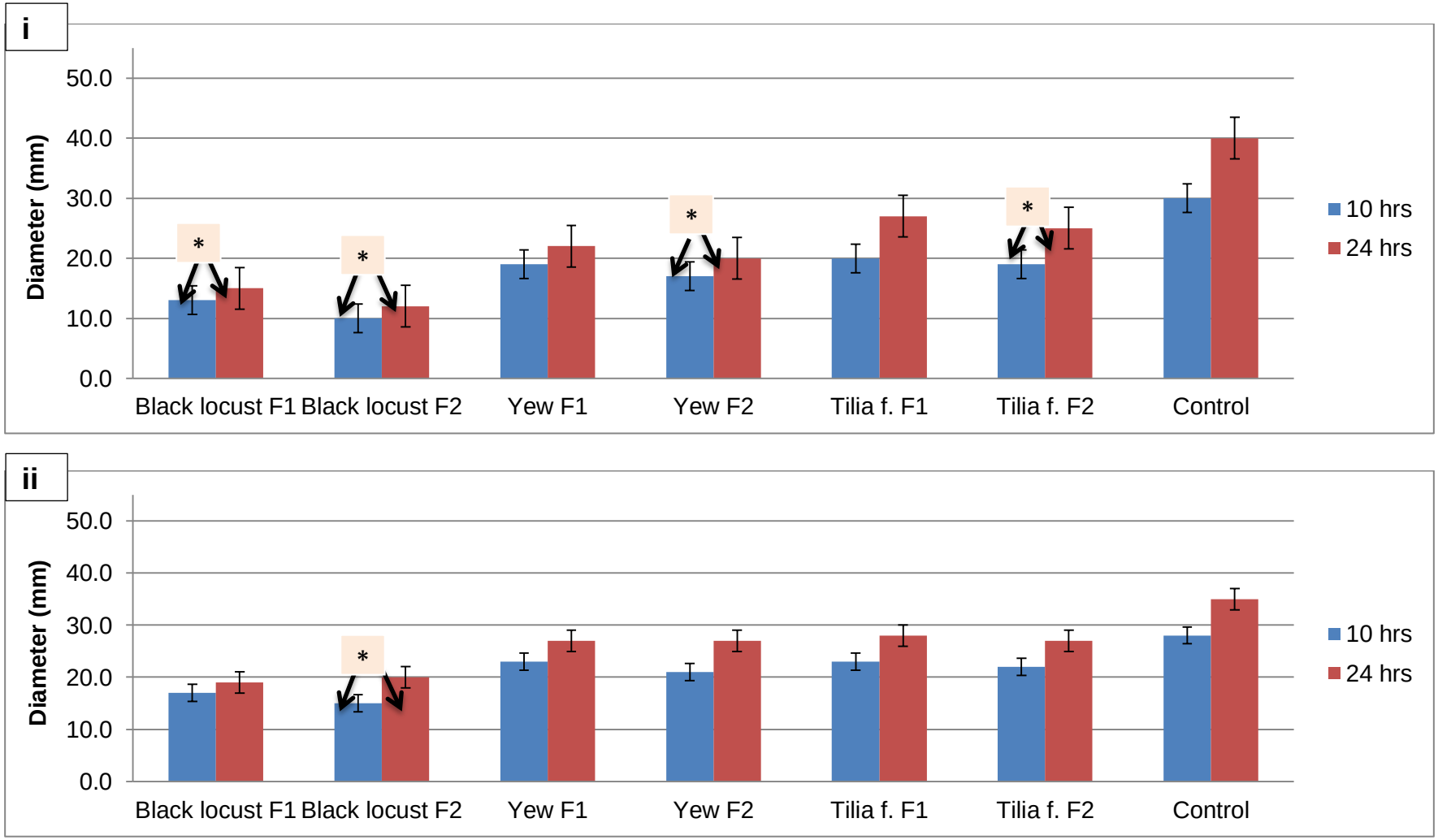



**Figure 3.5:** Effect of different CT extracts on motility of APEC at 10h and 24h: **(i)** swimming activity and **(ii)** swarming activity.

Black locust = high PD, yew = medium PC/PD and tilia flowers = high PC; (n=3 ± SE); **(*)** = significant differences at *P≤0.05*

### 3.4.4 Scanning electron microscope (SEM) for both pathogens

The SEM study showed that CTs could disrupt cell structures and destabilise them. The bacterial cells of both microbes had undergone some distinct morphological alterations. In order to further our understanding of the mode of action of CT on bacterial growth, SEM was performed for APEC and *S. epidermidis* that had been grown in LB medium. Changes in SEM characteristics were observed when both microbes were grown in the presence of CTs.

Figures from 3.6 to 3.9 below show different SEM micrographs of the untreated (as control) and treated cells of both microbes at different concentrations of CT compositions; i.e. PD extracted from black locust and PC from tilia flowers. Controls, named letter (A) in each figure, showed the smooth surfaces of typical cells of these bacteria. However, letters (B, C, D, E and F/ in each figure) illustrate the SEM micrographs of bacterial cells that were treated with either black locust CT (high PD) or treated with tilia flowers CT (high PC). The same system was applied to the bacterial cells of *S. epidermidis.*

Based on this result, the appearance of the specimens at various CT concentrations was compared with controls. In addition, the figures revealed the normal cell structures and shapes on typical cells and smooth surfaces compared to treated groups. However, the APEC cells seemed to have totally disappeared or collapsed when the cells were actually visible, especially at high CT concentrations of 10 – 5 mg/ml. It was observed that the treated cells were damaged. The effects of CTs on the morphology of these bacteria also implicate the cell wall probably as a target of tannin toxicity (Jones et al., 1994). In contrast, larger number of surviving cells were observed when medium or low concentrations (1.25 and 0.6 mg/ml) of CT were added, but these cells had become stuck together leading to alteration and distortion, and had lost their original shape as compared to the control cells. One unanticipated finding was that CTs showed impact on the binary fission of these cells and preventing the division of single cells into two daughter cells. It is possible to hypothesise that these conditions are less likely to occur in controls.

Conversely, the *S. epidermidis* cells started to show multiple defects and shrunken cells. These bacterial cells seemed to be totally deformed and many collapsed

cells were seen at all concentrations. These bacterial cells had, thus, also lost their original shape as compared to the control cells. In the present investigation, *S. epidermidis* cells were killed by a high CT concentration, whereas medium and low concentrations caused severe damage and whole cell lysis for some cells. It has been suggested previously that any interactions with the hydrophobic cell surface of microbes can play a critical role in the antimicrobial effect on *Staphylococci* strains (Carson et al., 2002). These findings matched previous results (Darah et al., 2013) that also reported morphological changes when antimicrobial agents such as flavonoids and tannins attacked the cell membrane. It is likely that the CTs adhered to the cell surface. Clearly, any disruption of cell walls will lead to a great impact on bacterial growth (Sheng et al., 2016). Given these findings it may be postulated that CTs disrupt cell structure and function including septum formation which is essential for cellular division and population growth.

However, it is thought that CTs have a greater antimicrobial effect on Gram-positive than on Gram-negative bacteria, because the latter contain an extra outer membrane, which includes lipids, phospholipids and lipopolysaccharide that are located in the inner and outer layers. These membranes can act as a barrier that hinders the movement of foreign substances into the bacterial cell (Lewu et al., 2006; Brown et al., 2017).

In general, these SEM results confirm that different CT extracts in high concentrations possess antibacterial activity and this supports the MBC results. However, the lowest concentrations of the active CT compositions showed less activity but interesting effects on APEC. Thus, it will be possible to determine the effect of these compositions on metabolic end products in the next chapter. This study will be a base for our further investigations on the CT effect on the metabolites of caecal contents and APEC.

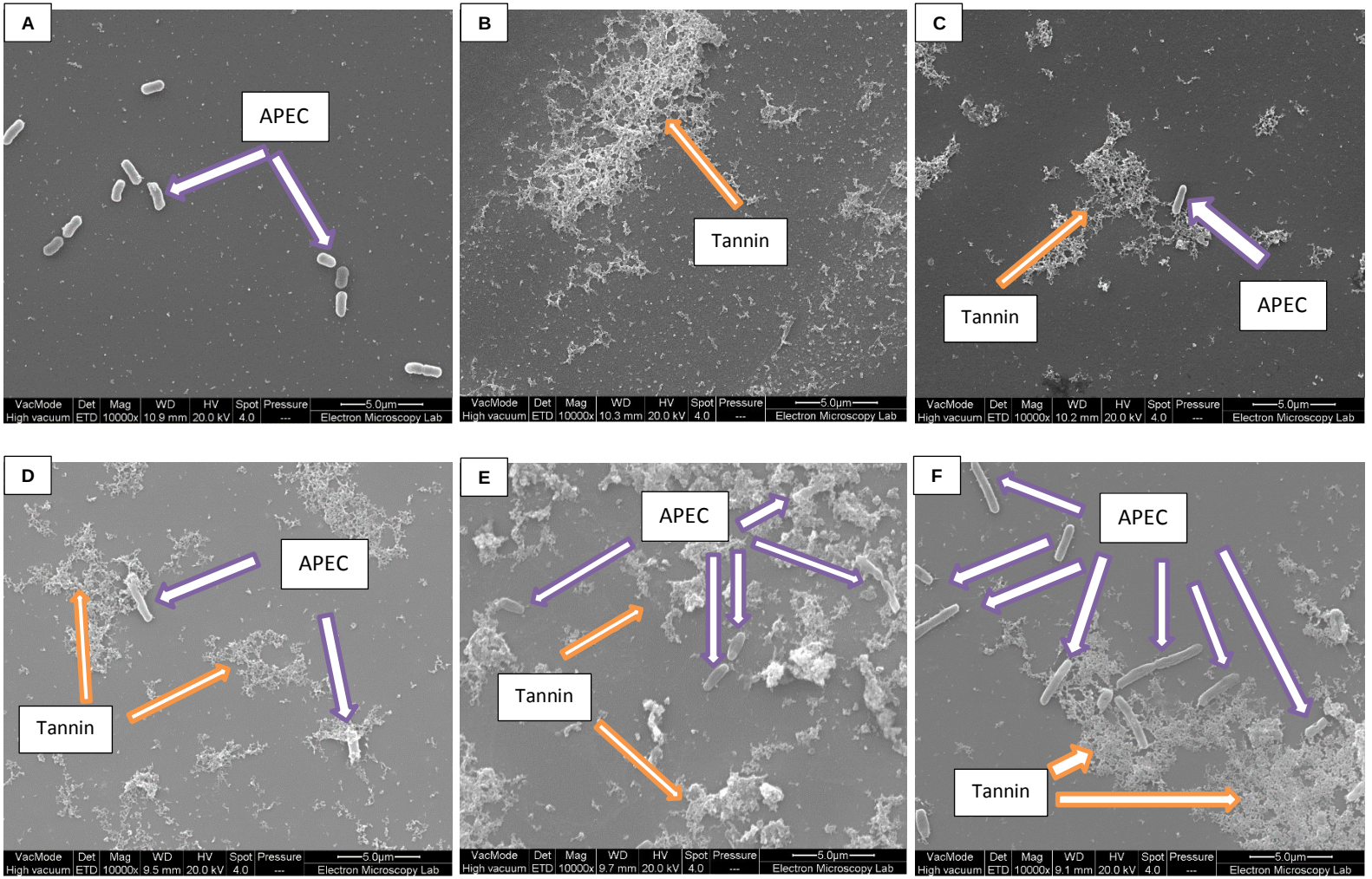



**Figure 3.6:** SEM images of APEC treated with or without CTs from black locust, PD type: **(A)** control; **(B)** 10 mg/ml; **(C)** 5 mg/ml; **(D)** 2.5 mg/ml; **(E)** 1.25 mg/ml; **(F)** 0.6 mg/ml.

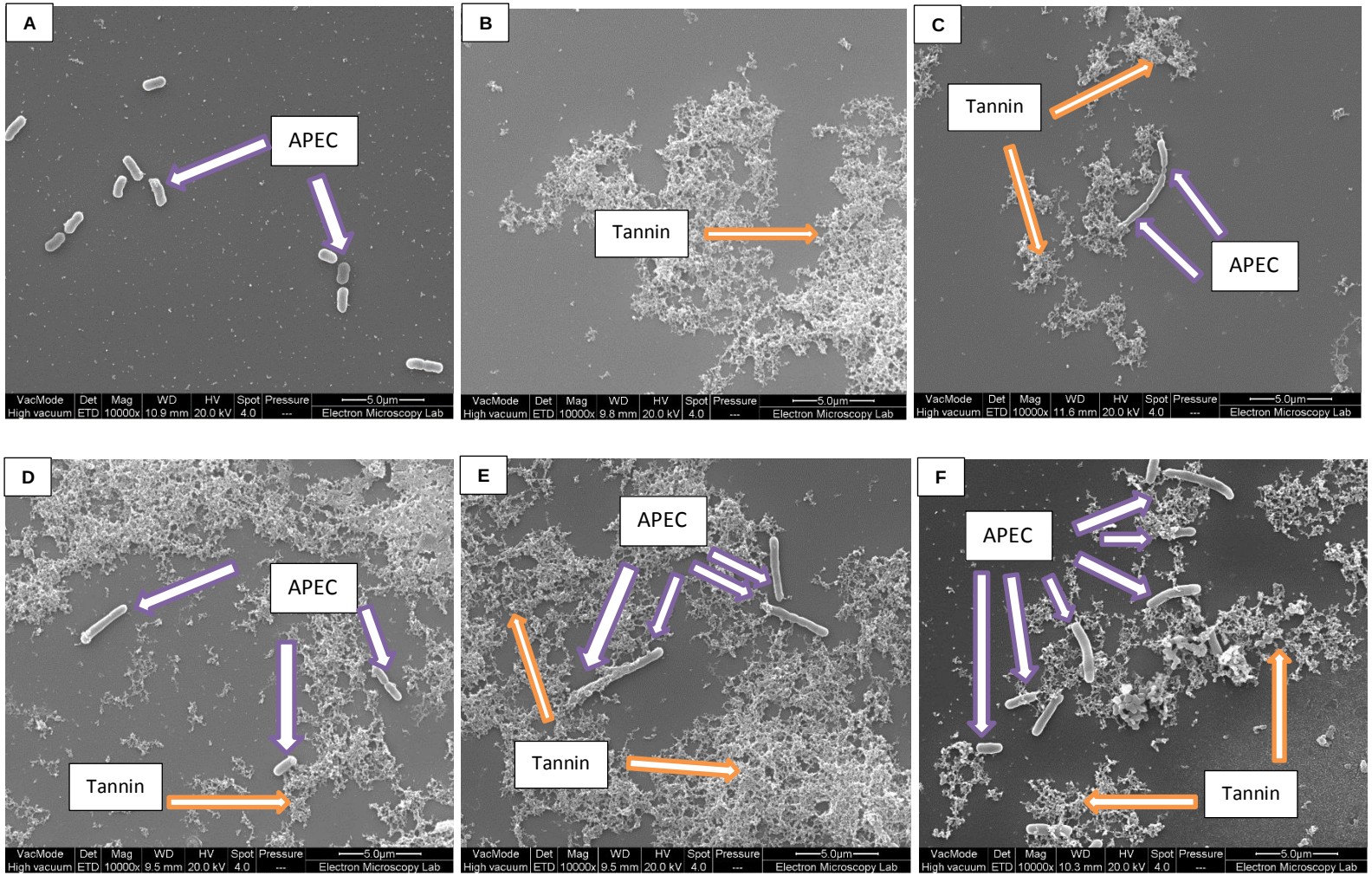



**Figure 3.7:** SEM images of APEC that treated with or without CTs from tilia flowers, PC type: **(A)** control; **(B)** 10 mg/ml; **(C)** 5 mg/ml; **(D)** 2.5 mg/ml; **(E)** 1.25 mg/ml; **(F)** 0.6 mg/ml**.**

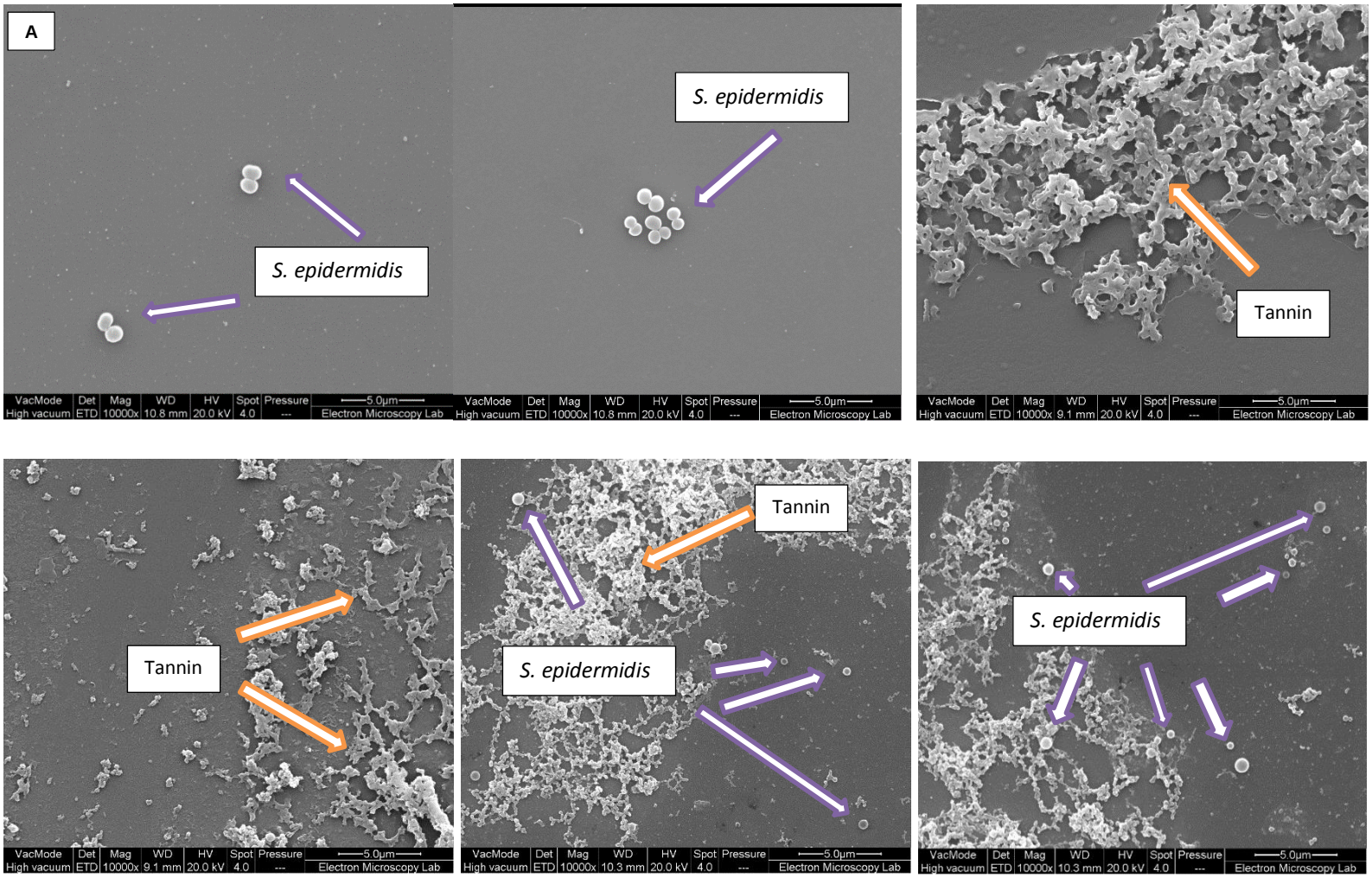



**Figure 3.8:** SEM images of *S. epidermidis* that treated with or without CTs from black locust, PD type: **(A)** control; **(B)** 10 mg/ml; **(C)** 5 mg/ml; **(D)** 2.5 mg/ml; **(E)** 1.25 mg/ml.

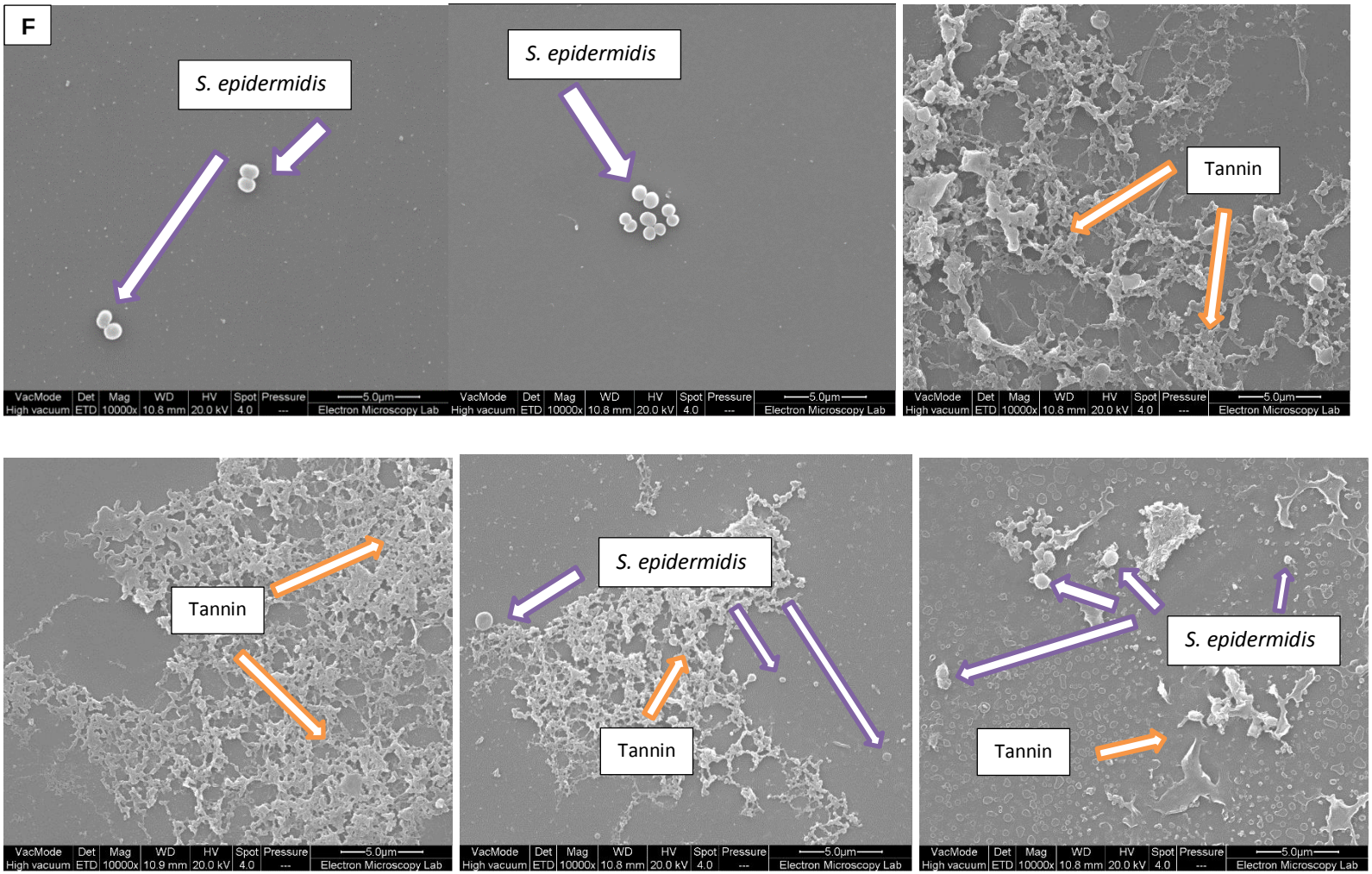



**Figure 3.9:** SEM images of *S. epidermidis* that treated with or without CTs from tilia flowers, PC type: **(A)** control; **(B)** 10 mg/ml; **(C)** 5 mg/ml; **(D)** 2.5 mg/ml; **(E)** 1.25 mg/ml.

### 3.5 Overall conclusion

This chapter investigated different concentrations and compositions of CT (PC/PD types). It can be concluded that there were significant antibacterial effects on a range of Gram-positive and Gram-negative bacteria. The study could provide support for the use of CT extracts in the management of some diseases, such as *colibacillosis* in chickens. The findings can form the basis for further studies to prepare and optimise CT preparation and to further evaluate them against a wide range of bacterial strains. Relatively high CT concentrations were used and showed several antimicrobial activities against APEC by affecting the growth curve, biofilm formation and motility. However, low concentrations of some CTs, particularly the PC type, had either a weak effect or sometimes no impact as antimicrobial agents, especially on the Gram-negative APEC.

SEM techniques revealed that CTs caused malformations of the bacterial cells. Further, SEM micrographs revealed that shrinkage of the bacterial cell was apparent in CT-treated cells compared to the untreated groups of both microbes. Further, CTs affected the binary fission of the bacterial cells, which is an important process in bacterial reproduction by division a single cell into two daughter cells. These results are likely to be related to CT effect on cells by compering to controls. However, further investigations will need to determine the effects of CT compositions by Transmission electron microscopy (TEM) investigations to investigate the dysfunction and apparent loss of turgidity and leakage of the cytoplasm from the bacterial cells. Nonetheless, bacteria can be directly inhibited by CTs apparently interacting with the cell walls or their extracellular proteins.

In total, the hypothesis was proved by testing different CT extracts, which showed inhibitory effects on both Gram-positive and Gram-negative bacteria. However, extractable CTs showed less anti-bacterial impact against APEC compared to *Staphylococcus epidermidis*. Furthermore, the exposure of CTs to sub-lethal concentrations (0.6 mg/ml) resulted in less effect against Gram-negative (APEC) specifically, with evidence of growth enhancement with high PC types. This finding needs further investigation in the future. The question as to the mechanism of action of these CT compositions on gut metabolites is to be addressed in the next chapter, and is based on an *in vitro* fermentation and metabolism study of the caecal contents or individual APEC culture.

### 3.6 References:


Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., and Iwatsuki, K. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *J Antimicrob Chemother.* 48, 487-491.

Baurhoo, B., Phillip, L., and Ruiz-Feria, C. A. (2007). Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. *Poult Sci* 86, 1070-8.

Bendaoud, M., Vinogradov, E., Balashova, N. V., Kadouri, D. E., Kachlany, S. C., and Kaplan, J. B. (2011). Broad-spectrum biofilm inhibition by *Kingella kingae* exopolysaccharide. *J Bacteriol* 193, 3879-86.

Borges, A., Saavedra, M. J., and Simoes, M. (2012). The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling* 28, 755-67.

Brown, R. H., Mueller-Harvey, I., Zeller, W. E., Reinhardt, L., Stringano, E., Gea, A., Drake, C., Ropiak, H. M., Fryganas, C., Ramsay, A., and Hardcastle, E. E. (2017). Facile Purification of Milligram to Gram Quantities of Condensed Tannins According to Mean Degree of Polymerization and Flavan-3-ol Subunit Composition. *J Agric Food Chem*.

Butler, L. G. (1992). Antinutritional effects of condensed and hydrolyzable tannins. *In* "Plant polyphenols", pp. 693-698. Springer.

Carson, C. F., Mee, B. J., and Riley, T. V. (2002). Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob Agents Chemother* 46, 1914-20.

Castanon, J. I. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poult Sci* 86, 2466-71.

Darah, I., Lim, S. H., and Nithianantham, K. (2013). Effects of Methanol Extract of *Wedelia chinensis* Osbeck (*Asteraceae*) Leaves against Pathogenic Bacteria with Emphasise on *Bacillus cereus*. *Indian J Pharm Sci* 75, 533-9.

Dibner, J. J., and Richards, J. D. (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* 84, 634-43.

Djurisic, A. B., Leung, Y. H., Ng, A. M. C., Xu, X. Y., Lee, P. K. H., Degger, N., and Wu, R. S. S. (2015). Toxicity of Metal Oxide Nanoparticles: Mechanisms, Characterization, and Avoiding Experimental Artefacts. *Small* 11, 26-44.

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15, 167-93.

Doss, A., Mubarack, H. M., and Dhanabalan, R. (2009). Antibacterial activity of tannins from the leaves of *Solanum trilobatum* Linn. *Indian J* Sci *Technol.* 2, 41-43.

Frutos, P., Hervas, G., Giráldez, F. J., and Mantecón, A. (2004). Review. Tannins and ruminant nutrition. *Spanish J. Agri. Res.* 2, 191-202.

Groschke, A., and Evans, R. (1950). Effect of antibiotics, synthetic vitamins, vitamin B12 and an APF supplement on chick growth. *Poult Sci* 29, 616-618.

Holloway, A., Mueller-Harvey, I., Gould, S., Fielder, M., Naughton, D., and Kelly, A. (2015). Heat treatment enhances the antimicrobial activity of (+) Catechin when combined with copper sulphate. *Lett Appl Microbiol* 61, 381-389.

Jones, G. A., McAllister, T. A., Muir, A. D., and Cheng, K. J. (1994). Effects of Sainfoin (*Onobrychis viciifolia* Scop.) Condensed Tannins on Growth and Proteolysis by Four Strains of Ruminal Bacteria. *Appl Environ Microbiol* 60, 1374-8.

Kaper, J. B., Nataro, J. P., and Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2, 123-40.

Kaya, I., Yigit, N., and Benli, M. (2008). Antimicrobial activity of various extracts of *Ocimum basilicum* L. and observation of the inhibition effect on bacterial cells by use of scanning electron microscopy. *AJTCAM* 5, 363-369.

Kearns, D. B. (2010). A field guide to bacterial swarming motility. *Nat Rev Microbiol* 8, 634-44.

Kroismayr, A., Schedle, K., Sehm, J., Pfaffl, M., Plitzner, C., Foissy, H., Ettle, T., Mayer, H., Schreiner, M., and Windisch, W. (2008). Effects of antimicrobial feed additives on gut microbiology and blood parameters of weaned piglets. *Bodenkultur* 59, 111-20.

Labrecque, J., Bodet, C., Chandad, F., and Grenier, D. (2006). Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and

adherence of Porphyromonas gingivalis. *J Antimicrob Chemother* 58, 439-43.

Lee, J. H., Park, J. H., Kim, J. A., Neupane, G. P., Cho, M. H., Lee, C. S., and Lee, J. (2011). Low concentrations of honey reduce biofilm formation, quorum sensing, and virulence in *Escherichia coli* O157:H7. *Biofouling* 27, 1095-104.

Lewu, F. B., Grierson, D. S., and Afolayan, A. J. (2006). Extracts from *Pelargonium sidoides* inhibit the growth of bacteria and fungi. *Pharm. Biol.* 44, 279-282.

Liu, H. W., Tong, J. M., and Zhou, D. W. (2011). Utilization of Chinese Herbal Feed Additives in Animal Production. *Agri Sci China* 10, 1262-1272.

Maisak, H., Jantrakajorn, S., Lukkana, M., and Wongtavatchai, J. (2013). Antibacterial Activity of Tannin from Sweet Chestnut Wood Against *Aeromonas* and *Streptococcal* Pathogens of Tilapia (*Oreochromis niloticus*). *Thai J Vet Med* 43, 105-111.

Medellin-Pena, M. J., Wang, H., Johnson, R., Anand, S., and Griffiths, M. W. (2007). Probiotics affect virulence-related gene expression in *Escherichia coli* O157:H7. *Appl Environ Microbiol* 73, 4259-67.

Niewold, T. A. (2007). The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poult Sci* 86, 605-9.

O'May, C., Ciobanu, A., Lam, H., and Tufenkji, N. (2012). Tannin derived materials can block swarming motility and enhance biofilm formation in *Pseudomonas aeruginosa*. *Biofouling* 28, 1063-1076.

O'May, C., and Tufenkji, N. (2011). The Swarming Motility of *Pseudomonas aeruginosa* Is Blocked by Cranberry Proanthocyanidins and Other Tannin-Containing Materials. *Appl. Environ. Microbiol.* 77, 3061-3067.

O'Toole, G., Kaplan, H. B., and Kolter, R. (2000). Biofilm formation as microbial development. *Annu Rev Microbiol.* 54, 49-79.

Pratt, L. A., and Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30, 285-93.

Sachs, J. L. (2008). Resolving the first steps to multicellularity. *Trends Ecol Evol* 23, 245-8.

Scalbert, A. (1991). Antimicrobial Properties of Tannins. *Phytochemistry* 30, 3875-3883.

Schluter, J., Nadell, C. D., Bassler, B. L., and Foster, K. R. (2015). Adhesion as a weapon in microbial competition. *Isme Journal* 9, 139-149.

Shao, D., Li, J., Li, J., Tang, R., Liu, L., Shi, J., Huang, Q., and Yang, H. (2015). Inhibition of Gallic Acid on the Growth and Biofilm Formation of *Escherichia coli* and *Streptococcus mutans*. *J Food Sci* 80, M1299-305.

Sheng, L., Olsen, S. A., Hu, J., Yue, W., Means, W. J., and Zhu, M. J. (2016). Inhibitory effects of grape seed extract on growth, quorum sensing, and virulence factors of CDC "top-six" non-O157 Shiga toxin producing *E. coli*. *Int J Food Microbiol* 229, 24-32.

Smith, J. L., Drum, D. J., Dai, Y., Kim, J. M., Sanchez, S., Maurer, J. J., Hofacre, C. L., and Lee, M. D. (2007). Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichia coli* strains colonizing broiler chickens. *Appl Environ Microbiol* 73, 1404-14.

Torres, A. G., Zhou, X., and Kaper, J. B. (2005). Adherence of diarrheagenic *Escherichia coli* strains to epithelial cells. *Infect Immun* 73, 18-29.

Van Houdt, R., and Michiels, C. W. (2005). Role of bacterial cell surface structures in *Escherichia coli* biofilm formation. *Res Microbiol* 156, 626-33.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., and Michiels, J. (2008). Living on a surface: swarming and biofilm formation. *Trends Microbiol* 16, 496-506.

Waghorn, G. (2008). Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-Progress and challenges. *Anim Feed SciTechnol.* 147, 116-139.

Wang, M. L., Suo, X., Gu, J. H., Zhang, W. W., Fang, Q., and Wang, X. (2008). Influence of grape seed proanthocyanidin extract in broiler chickens: effect on chicken *coccidiosis* and antioxidant status. *Poult Sci* 87, 2273-80.

Windisch, W., Schedle, K., Plitzner, C., and Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *J Anim Sci* 86, E140-8.

Zhang, J. M., Rui, X., Wang, L., Guan, Y., Sun, X. M., and Dong, M. S. (2014). Polyphenolic extract from Rosa rugosa tea inhibits bacterial quorum sensing and biofilm formation. *Food Control* 42, 125-131.

Zhu, M. J., Olsen, S. A., Sheng, L., Xue, Y., and Yue, W. (2015). Antimicrobial efficacy of grape seed extract against *Escherichia coli* O157:H7 growth, motility and Shiga toxin production. *Food Control* 51, 177-182.

# Chapter 4: The effects of condensed tannins on *in vitro* metabolic profile of caecal contents and APEC culture

## 4.1 Introduction:

Different dietary interventions in farm animals have been widely investigated to determine their effects on gut function and gut microbial profile and their subsequent effect on animal health (Jana et al., 2015). In addition, the increase in antibiotic resistance by microbes isolated from both humans and animals has led to a ban on the use of antibiotics as growth promoters in EU, and drives a reduction in the prophylactic use of antibiotics in animal production. This has led to the search for alternative remedies in order to support animal production (Stokes et al., 2008).

Various studies have reported that several plants or their extracts can be used as antimicrobial agents against certain pathogens. The most common natural antimicrobial compounds are polyphenols (Tzounis et al., 2008). The present study focussed on tannins which are well-known as antioxidants but there is also evidence that some possess antimicrobial and anti-inflammatory activities (Pozuelo et al., 2012). Moreover, there is some evidence that tannins can generate bioactive metabolites that may affect the composition of bacterial populations (Selma et al., 2009), which, if this could lead to the suppression of pathogenic and/or antimicrobial resistant bacteria, would be very advantageous.

Condensed tannins (CTs) have long been considered as anti-nutritional substances as their presence in animal feeds can have negative effects on animal performance (Lowry et al., 1996). However, several *in vivo* and *in vitro* studies over the last few years have also shown some beneficial effects of administering these compounds in mono-gastric feeding (Brenes et al., 2016). Evidence has emerged that some tannins are metabolised by the human gut flora, and there are suggestions that these polyphenolic metabolites may possess beneficial health effects (Calvani et al., 2014). CTs possess many different structures and this thesis hypothesises that it is the concentration and composition of CTs that determines their bioactivity and the yield and composition of metabolic end products.

Caecal bacterial communities and their metabolites are one example of biological activity that characterises the systemic interactions between the host animal diet and the environment (including the composition of the microbiome) in the gut. These interactions, and the resultant metabolite profile, may have both beneficial and adverse effects, and thus require comprehensive and systematic profiling (Beckonert et al., 2007).

$^{1}$H-NMR spectroscopy can provide an insight into the qualitative and quantitative changes of metabolites of host microbes (Zheng et al., 2011). These metabolites originate from microbial conversion of nutrients and host metabolites in the gut and, once absorbed, are transported throughout the body (Calvani et al., 2014). Therefore, NMR-based metabolic profiling of caecal digesta incubated with CT is a means of characterising the biological impact of condensed tannins in the caecum, and is a potentially valuable tool for investigating the mechanism by which different CT may exert their effects within, for example, the chicken caecum.

The aim of the experiments reported in this chapter was to explore how CT compositions affected the metabolite profile of caecal digesta (taken from chickens) or of individual pathogenic bacteria (avian pathogenic *E. coli*, APEC). These data could then be used to determine the potential for adding these compounds to chicken diets to improve aspects of chicken health and performance.

## 4.2 Materials and methods

Two experiments were conducted in which condensed tannins (CTs) were incubated *in vitro* with either avian caecal contents or a culture of avian pathogenic *E. coli* (APEC).

### 4.2.1 Preparation of samples from caecal contents (Experiment - 1)

Broiler chickens (Ross 308, n=8), which had been reared in floor pens on wood shavings and fed a diet based on maize and soyabean meal were used. They were sacrificed by cervical dislocation at 37 days of age and mean body weight of 1.700±0.200 kg.

Caecal fluid was taken from each caecum of the birds as quickly as possible after excision from the carcass by squeezing them into a pre-weighed sterile bottle that was then placed immediately inside an anaerobic jar. The caecal fluid from all the birds was pooled, and to ensure sample homogeneity, the contents were mixed by multiple inversions. The anaerobic jars with samples were transferred rapidly to the laboratory where the bottle was weighed, and Viade Levure nutrient broth (VL), which contained (g/l distilled water) tryptose; NaCl; yeast extract; glucose; beef extract; bacto agar; and cystine hydrochloride, was added to the caecal contents to give a 75:25 v/w dilution to produce the caecal slurry. This method, which has been used to prepare samples for analysis by $^{1}$H-NMR, was reported by Le Roy et al. (2016).

### 4.2.2 Incubation of condensed tannins with caecal slurry *in vitro*

Duran bottles (250 ml) were inoculated with caecal slurry (10 ml) prepared as described in 4.2.1 and VL nutrient medium (50 ml) and either nutrient medium only (Control, 20 ml) or nutrient medium with CTs of high PD% (0.6 mg/ml) or CTs with high PC% (0.6 mg/ml). Three replicate bottles were prepared for each treatment. The bottles were transferred to an anaerobic chamber (Whitley MG1000 anaerobic workstation, Don Widley, England, UK), which was adjusted to the pH value at the start of the experiments that was equivalent to the caecal environment (6.5) and the temperature was maintained at 41 °C in the chamber. The model was run as the closed fermentation method, meaning that the exchange of air was prohibited and no outside contamination with yeast or bacteria was allowed.

During the incubation, a sample (3ml) of incubation medium was taken from each bottle at 0, 10, 24 and 48 h. These samples were then snap frozen in liquid nitrogen before being stored (-80$^{0}$C) pending analysis by $^{1}$H-NMR.

### 4.2.3 Nuclear magnetic resonance spectroscopy (NMR) analysis of slurry

#### 4.2.3.1 Equipment

Samples were analysed by $^{1}$H-NMR (Bruker Avance III 700 MHz, Billerica, MA, USA); with flow-injection probe FI TXI 600 SB 5 mm with Z gradient; Tube NMR of 5 mm of 700 MHz (Sigma-Aldrich); and Gilson 215 flow-injection system with Icon-NMR; Autosampler BACS60 for tube NMR (Sigma-Aldrich); Gilson 215 sample

preparation robot with Sample-Track; Genevac EZ2 solvent evaporator (Genevac Ltd., Ipswich, UK); Eppendorf tubes 1.5 ml (Sigma-Aldrich).

### 4.2.4 Methods

#### 4.2.4.1 Preparation of phosphate buffer

To prepare the phosphate buffer solution (pH 7.0), $Na_2HPO_4$ (28.85 g), $NaH_2PO_4$ (28.85 g); 0.172 g of 2,2′,3,3′-tetradeuteropropionic acid 1 mM (TSP, 1 mM, used as an indicator in the NMR technique); sodium azide ($NaN_3$, 0.193 g) were weighed into a 1 l volumetric flask, dissolved and the solution made up to volume with deuterium oxide ($D_2O$).

#### 4.2.4.2 Sample preparation for NMR analysis

Samples (stored at - 80 °C), which had been taken from the incubation of the caecal slurry (see section 4.2.2), were thawed at room temperature and then vortexed before being centrifuged (10,000 g) for 10 minutes. Then 400 μl of the supernatant was transferred to a sterile microfuge tube to which 200 μl phosphate buffer solution was added.

Afterwards, these samples were vortexed and centrifuged at 10,000 g for 10 minutes, and then 550 μl of the supernatant was transferred to a 5 mm internal diameter NMR tube.

#### 4.2.4.3 Setup of NMR instrument

Samples were analysed by 1D Nuclear Overhauser Enhancement Spectroscopy (NOESY-1D), and the sources were acquired using a Bruker-FID pulse sequence to limit signal contribution. For each sample, the acquisition was 256 scans (as transients or scans) and recorded as 32768 data points over a spectral width of 9803.922 Hz as for NOESY-1D. The temperature was 299.98K at 700.19 MHz magnetic frequency at time domain 16384 points. This NOESY-1D was achieved for each experiment on one representative sample for metabolic identification purposes.

#### 4.2.4.4 Processing the data for analysis

All spectral peaks were measured using MestReNova software (Mnova, version: 11.0.3-18688; © 2017 Mestrelab Research S.L., Santiago de Compostela, Spain). By copying or dragging the raw data from a file 'fid' that was generated by the NMR software, these files were applied into MestReNova window/NMR software, which automatically transforms the raw data files into frequency domains.

After Measuring of metabolite concentrations, spectra were phased, processed in order to realign spectrum phasing calibration on TSP at 0.00 ppm and baseline correction using MestReNova software. Stacked spectra were imported into MATLAB (version R2017a, MathWorks, Inc., Natick, MA, USA) MathWork software where spectra were digitised between 0.5 - 9 ppm in order to delete useless information and avoid data bias; the region containing the water peak was deleted between 4.8 and 5.0 (Le Roy et al., 2016). Each peak was then related to a metabolic product by reference to the Human Metabolome Database (HMDB) websites (http://www.hmdb.ca/ or http://www.bmrb.wisc.edu). Once identified, these metabolic products were then analysed by Chenomx software (NMR suit; version 8.31; © 2001-2017 Chenomx Inc., Edmonton, AB, Canada) and ChemBioDraw software (version: 14.0.0117; © 1998-2014, PerkinElmer Inc., MA, USA) to record the concentrations of metabolites.

By using MatLab (version R2017a, MathWorks, Inc., Natick, MA, USA), multivariate statistical analysis was evaluated using principal component analysis (PCA) plots and analyses of the loading of the principal components were done to evaluate the metabolic variations existing between the treatments. Orthogonal projections to latent structure (OPLS) regressions were run in between each group for each time. OPLS $R^2Y$ values around 0.7 were indicative of a good model, with $Q^2$ values of around 0.5 indicating good predictive ability. PCA score plots and OPLS correlation plots were also produced to visualise differences in the metabolome between treatments. Loading and contribution plots were extracted to reveal the variables that bear class discriminating power. Moreover, to improve model visualisation and interpretation, S-line plots were extracted to detect the metabolites that influence variable selection as they display the overall importance

of each variable (X) on all responses (Y) cumulatively over all components. These data were analysed as previously reported by (Le Roy et al., 2016).

The effects of treatment and time, and the interaction between treatment and time, on the concentrations of individual metabolites was determined by analysis of variance (General Linear Model) using Minitab software (version 18.0; Minitab Inc., PA, USA). Least square means and the pooled standard error (SEM) were determined. Means were separated (when $P<0.05$) by the Tukey test.

### 4.2.5 Results and discussion

Representative $^{1}$H-NMR spectra of the treated and untreated CTs with different time points (at 0, 10, 24 and 48 hours) were determined and presented in this study (for further information of numerical key for annotation see figs. E, F, G and H – appendix 3) to produce a basic database of the metabolites found in the caecal contents (*in vitro*) after treatment with different CT compositions. This study showed that an endpoint in fermentation was reached by 24 h, as there were no significant differences in metabolite concentrations between the 24h and 48 h incubations. As anticipated the spectra at time-point 0h were very similar for all samples, although new peaks were recognised in the tannin treatment groups related to the CT composition with the aromatic area of the spectra (> 5.5 ppm) showing the signals of CT residues in the treated groups, which were absent in the control. The metabolites of treatments are shown in table 4.1 at 10 and 24 hours.

The raw experimental spectra contained a large number of data points. However, the whole spectra were adjusted to the TSP (0.00 ppm) peak as an indicator for the NMR standard (Le Roy et al., 2016). To reduce the computational burden, some regions were cut from the general spectra such as the beginning (from the start to 0.5 ppm), water areas (from 4.50 to 4.80 ppm) and the end region (from 9.5 ppm until the end of the spectrum) (Zheng et al., 2011). The signals at 5.90 to 7.50 ppm illustrated the presence of PC or PD units (dependent on the particular CT source) as well as other compounds that were present in the CT compositions. Moreover, the signals that appeared at 4.50 ppm are typical of the presence of the PC/PD subunits catechin/ epicatechin or gallocatechin/ epigallocatechin, respectively, and the region between 2.50 and 2.80 ppm is due to other signals of flavan-3-ol units. $^{1}$H-NMR spectra-based metabolic profiling allowed monitoring

and determination of the change in concentrations of some important metabolic end compounds that could play a role in energy metabolism of the microbes and the concentrations of these metabolites were affected by the presence or absence of CT, although not by the composition of the CT.

Table 4.1 summarises the effect of CT on the caecal metabolic profile. Addition of CT was characterised by low concentrations of amino acids, particularly glutamate, pyroglutamate, phenylalanine, lysine and leucine. This result also showed significant differences ($P \leq 0.05$) in decreasing of the concentrations of these amino acids in the caecum containing CTs at different times compared to controls. Anaerobic bacteria could utilise glutamate, which is an important amino acid that participates actively in TCA cycle, into fatty acids such as acetate, butyrate and other molecules e.g. ammonia. This amino acid can also contribute in transamination with pyruvate that fermented into fatty acids by gut microbes and reduce this amino acid (Meister, 1957). Further, phenylalanine, which is an aromatic amino acids that is produced from bacterial degradation of with microbial metabolism showed a significant decreasing ($P \leq 0.05$) at different time-points. Gut microbiota such as *Bacteroidetes* spp. and *Lactobacilli* spp. could get involved in this process (Fuchs et al., 2014). This observation confirmed that CT treatments enhanced the metabolic processes of amino acids and that lead to be considered as promotion to generate energy by decreasing the amino acids concentrations and improving the oxidation (Nutrition, 1971). As observed recently (Amorim Franco and Blanchard, 2017) branched chain amino acids can undergo oxidative transamination at an appreciable rate by enzyme that is called aminotransferase. This enzyme, thus, is responsible for the final step in the biosynthesis of these amino acids such as leucine, isoleucine and valine, in bacteria. This study showed that the decrease of these amino acids also presented evidence of amino acids transamination into pyruvate, which can be fermented by gut microbes into different fatty acids such as propionate, butyrate and acetate.

On the other hand, the functional outputs of some gut microbiota, including fatty acids and amino acids, are thought to be important to prevent of any disorders. Gut bacteria can alter the bioavailability of amino acids by utilisation of several amino acids originating from certain amino acids, in particular the aromatic and branched

chain amino acids (Neis et al., 2015). Thus, these amino acids that could release by gut microbes can stimulate by synthesis of some of fatty acids.

In contrast, the concentrations of some fatty acids such as acetate, butyrate, and propionate were significantly increased ($P \leq 0.05$) in fermentations containing CTs compared with the control at 10h and 24 h, perhaps suggesting that fermentation of carbohydrates had been increased. However, the fermentation of some short chain fatty acids was possibly interrupted in the presence of CTs by affecting succinate pathway, which is placed into one of the most important cyclic pathways in metabolism (Neis et al., 2015), whilst acetate is the end product of anaerobic fermentation (Macfarlane and Macfarlane, 2012). Lactate concentrations also decreased gradually in the CT treatments compared to the controls ($P \leq 0.05$). These findings can suggest that gut microbes respired aerobically to produce the anticipated end products of energy metabolism. This result seems to indicate that these CT compositions could encourage the proliferation of lactic acid producing microorganisms and interrupting the TCA as mentioned previously (Tjahjani, 2017). Further, lactic acid tends to reduce pH more than other fatty acids. Lactate produced by lactic acid bacteria is normally rapidly absorbed from the intestine or used as a substrate for lactate-utilising bacteria (Rinttila and Apajalahti, 2013). Further microbiological studies are needed to investigate the relationship between individual fatty acids and bacterial populations in the presence of different CT types.

Figure 4.1 and 4.2 illustrates more details for the key metabolites that demonstrate significant differences at S-line plot, which that contributed to this discrimination between the interaction of the treatments and different time-points (after 10h and 24h) in the presence or absence of CT compositions, high PC and PD. Further, a colour code indicating the weights of the discriminatory variables, as the peak colour gradually changes from blue to red, the absolute value of correlation coefficient increases, indicating the resonances were important for discriminating metabolite profiles of pairwise analyses. The result clearly differentiated the controls' samples and treated CTs by different component concentrations between them. In addition, carbohydrates such as mannitol, glucose, fructose, lactose and galactose demonstrated slight concentration changes between the treated and untreated groups. However, the statistical analysis of these carbohydrates did not

show any significant differences (P≥0.05) between the interaction of times and treatments, but some of them, e.g. mannitol, glucose, fructose, lactose and galactose, measured a significant difference (P≤0.05) at time-points only. Beneficial bacteria in the gut are susceptible to changes in dietary carbohydrates, which in turn have an effect on caecum fatty acid concentrations. Once carbohydrate sources are exhausted in the caecum, sources of protein material are fermented and metabolised to energy (Niba et al., 2009).

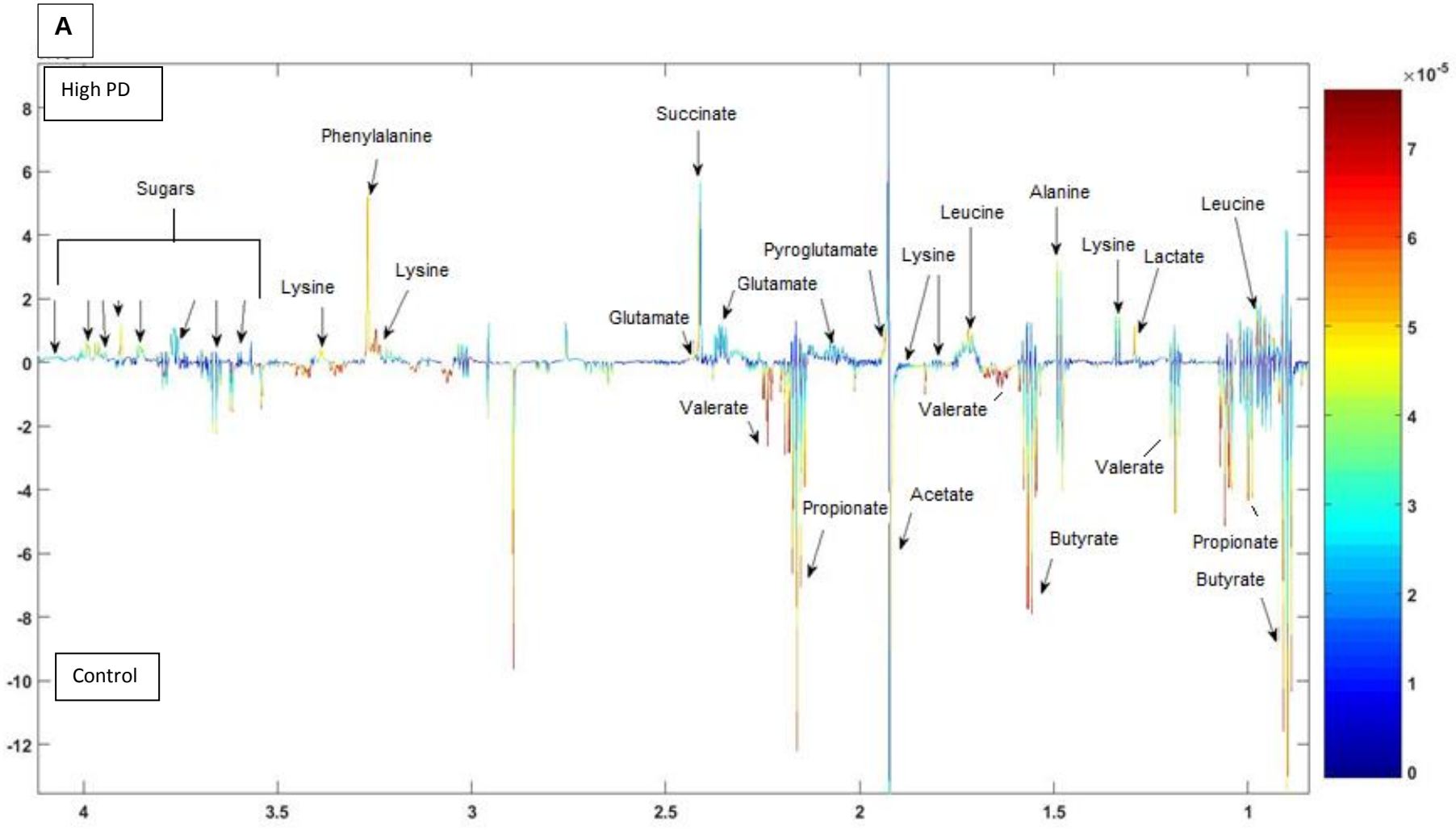
Finally, the current study shows that supplementation with CTs can have different effects on metabolic end products compared to controls and these impacts might be related to changes in gut microbiota. Further, this study indicated that PD and PC type of CTs have the same effects on the caecal contents. More specific studies are required to clarify how these CTs influence gut microbiota *in vitro* and *in vivo*.

**Table 4.1:** The concentrations (*mM) of metabolites of caecal contents in the presence or absence CTs that covered PD and PC, at 0, 10, 24 and 48 h incubation

| Metabolites | | High PD% | | | | High PC% | | | | Control | | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0h | 10h | 24h | 48h | 0h | 10h | 24h | 48h | 0h | 10h | 24h | 48h | Time | Treat. | T*T |
| Fatty acids | Acetate | 35.0 | 46.0 | 70.0 | 71.0 | 35.0 | 50.0 | 77.0 | 78.0 | 35.0 | 68.0 | 97.0 | 95.0 | 0.01 | 0.02 | 0.03 |
| | Butyrate | 9.0 | 15.0 | 28.0 | 27.0 | 9.0 | 14.0 | 30.0 | 28.5 | 9.0 | 21.0 | 36.0 | 35.0 | 0.01 | 0.05 | 0.05 |
| | Lactate | 3.0 | 2.0 | 0.9 | 0.8 | 3.0 | 1.9 | 0.6 | 0.55 | 3.0 | 1.1 | 0.3 | 0.2 | 0.01 | 0.03 | 0.02 |
| | Succinate | 2.4 | 4.2 | 3.5 | 3.5 | 2.4 | 4.2 | 3.3 | 3.4 | 2.3 | 4.0 | 3.1 | 3.0 | 0.01 | 0.07 | 0.08 |
| | Propionate | 2.3 | 4.0 | 6.8 | 6.9 | 2.3 | 3.1 | 7.3 | 7.5 | 2.3 | 4.9 | 15.0 | 14.0 | 0.02 | 0.06 | 0.05 |
| | Isobutyrate | 1.8 | 1.5 | 1.0 | 0.9 | 1.8 | 1.5 | 1.2 | 1.2 | 1.8 | 1.7 | 2.0 | 1.9 | 0.06 | 0.08 | 0.12 |
| | Isovalerate | 1.6 | 1.2 | 1.1 | 1.1 | 1.6 | 1.0 | 0.9 | 0.8 | 1.6 | 1.3 | 1.4 | 1.3 | 0.07 | 0.06 | 0.11 |
| | Valerate | 1.6 | 1.5 | 3.0 | 2.9 | 1.6 | 1.5 | 4.0 | 3.9 | 1.6 | 2.0 | 6.5 | 6.0 | 0.06 | 0.06 | 0.09 |
| Amino acids | Glutamate | 7.5 | 7.0 | 2.0 | 2.0 | 7.5 | 6.9 | 1.9 | 1.9 | 7.5 | 3.1 | 1.2 | 1.2 | 0.01 | 0.17 | 0.07 |
| | Leucine | 7.0 | 7.5 | 5.9 | 5.9 | 7.0 | 7.2 | 6.0 | 6.0 | 7.0 | 6.4 | 3.5 | 3.5 | 0.02 | 0.04 | 0.03 |
| | Valine | 5.5 | 4.3 | 5.8 | 5.8 | 5.5 | 4.2 | 5.7 | 5.7 | 5.6 | 4.2 | 3.1 | 3.1 | 0.14 | 0.12 | 0.13 |
| | Alanine | 4.8 | 4.9 | 4.7 | 4.7 | 4.8 | 5.0 | 4.8 | 4.8 | 4.8 | 4.5 | 2.9 | 2.9 | 0.11 | 0.07 | 0.12 |
| | Threonine | 4.0 | 2.5 | 0.3 | 0.3 | 4.0 | 2.4 | 0.4 | 0.4 | 4.0 | 2.2 | 0.4 | 0.4 | 0.11 | 0.17 | 0.15 |
| | Isoleucine | 3.8 | 4.0 | 3.6 | 3.7 | 3.8 | 3.9 | 3.8 | 3.8 | 3.7 | 3.0 | 2.3 | 2.3 | 0.17 | 0.02 | 0.13 |
| | Lysine | 3.1 | 2.2 | 0.5 | 0.5 | 3.1 | 2.3 | 0.8 | 0.8 | 3.1 | 2.5 | 1.5 | 1.2 | 0.01 | 0.03 | 0.03 |
| | Pyroglutamate | 2.4 | 3.8 | 1.8 | 1.8 | 2.4 | 3.7 | 1.7 | 1.7 | 2.5 | 2.0 | 0.7 | 0.7 | 0.05 | 0.03 | 0.08 |
| | Phenylalanine | 2.2 | 1.9 | 1.0 | 1.0 | 2.2 | 1.8 | 1.0 | 1.1 | 2.2 | 1.1 | 0.2 | 0.2 | 0.01 | 0.02 | 0.07 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Proline | 2.0 | 1.1 | 0.3 | 0.3 | 2.0 | 1.0 | 0.5 | 0.5 | 2.0 | 0.9 | 0.1 | 0.1 | 0.03 | 0.08 | 0.16 |
| | Sarcosine | 0.1 | 0.4 | 0.1 | 0.1 | 0.1 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.02 | 0.28 | 0.14 |
| Carb. | Mannitol | 1.7 | 2.1 | 1.4 | 1.4 | 1.7 | 2.3 | 0.7 | 0.6 | 1.7 | 2.6 | 0.7 | 0.6 | 0.01 | 0.35 | 0.17 |
| | Glucose | 1.6 | 2.1 | 0.1 | 0.1 | 1.6 | 2.0 | 0.08 | 0.08 | 1.6 | 1.4 | 0.06 | 0.05 | 0.04 | 0.42 | 0.22 |
| | Fructose | 1.3 | 1.6 | 1.2 | 1.2 | 1.3 | 1.5 | 0.7 | 0.7 | 1.3 | 1.5 | 0.6 | 0.6 | 0.05 | 0.09 | 0.12 |
| | Lactose | 0.4 | 0.76 | 0.3 | 0.3 | 0.4 | 0.77 | 0.2 | 0.2 | 0.4 | 0.8 | 0.2 | 0.2 | 0.01 | 0.32 | 0.17 |
| | Galactose | 0.1 | 0.4 | 0.3 | 0.3 | 0.1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.3 | 0.1 | 0.1 | 0.01 | 0.03 | 0.11 |
| | Fucose | 0.1 | 0.4 | 0.2 | 0.2 | 0.1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.3 | 0.1 | 0.1 | 0.06 | 0.08 | 0.12 |
| | Mannose | 0.1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.3 | 0.06 | 0.06 | 0.1 | 0.2 | 0.04 | 0.04 | 0.08 | 0.09 | 0.32 |
| | Maltose | 0.01 | 0.07 | 0.04 | 0.04 | 0.01 | 0.03 | 0.01 | 0.01 | 0.01 | 0.03 | 0.01 | 0.01 | 0.06 | 0.32 | 0.19 |
| Others | Pyruvate | 1.1 | 0.5 | 0.1 | 0.1 | 1.1 | 0.4 | 0.1 | 0.1 | 1.1 | 0.4 | 0.2 | 0.2 | 0.05 | 0.42 | 0.51 |
| | Trimeth-N-oxid | 0.5 | 0.5 | 0.06 | 0.06 | 0.5 | 0.45 | 0.05 | 0.05 | 0.5 | 0.2 | 0.05 | 0.05 | 0.06 | 0.22 | 0.13 |
| | Uracil | 0.4 | 0.6 | 0.3 | 0.3 | 0.4 | 0.5 | 0.3 | 0.3 | 0.4 | 0.4 | 0.04 | 0.03 | 0.07 | 0.27 | 0.16 |
| CTs | Prodelphinidin | 1.0 | 0.6 | 0.05 | 0.05 | 1.0 | 0.2 | 0.03 | 0.03 | 0.01 | 0.00 | 0.00 | 0.00 | 0.05 | 0.16 | 0.02 |
| | Procyanidin | 1.0 | 0.1 | 0.01 | 0.01 | 1.0 | 0.8 | 0.08 | 0.08 | 0.01 | 0.00 | 0.00 | 0.00 | 0.02 | 0.18 | 0.03 |

*(mM) = the molar concentration of each compound; All values represent the mean of three replicates; Significant differences ($P \leq 0.05$) between the treatments; Time-*P-value* indicates significant values in time-points; Treat.-*P-value* indicates significant values in treatments; T*T-*P-value* indicates significant values in interactions between the times and treatments;


A
High PD
Control
Sugars
Lysine
Phenylalanine
Lysine
Succinate
Glutamate
Glutamate
Pyroglutamate
Valerate
Propionate
Lysine
Leucine
Valerate
Acetate
Alanine
Butyrate
Lysine
Lactate
Valerate
Leucine
Propionate
Butyrate
×10-5
8
6
4
2
0
-2
-4
-6
-8
-10
-12
4
3.5
3
2.5
2
1.5
1
7
6
5
4
3
2
1
0

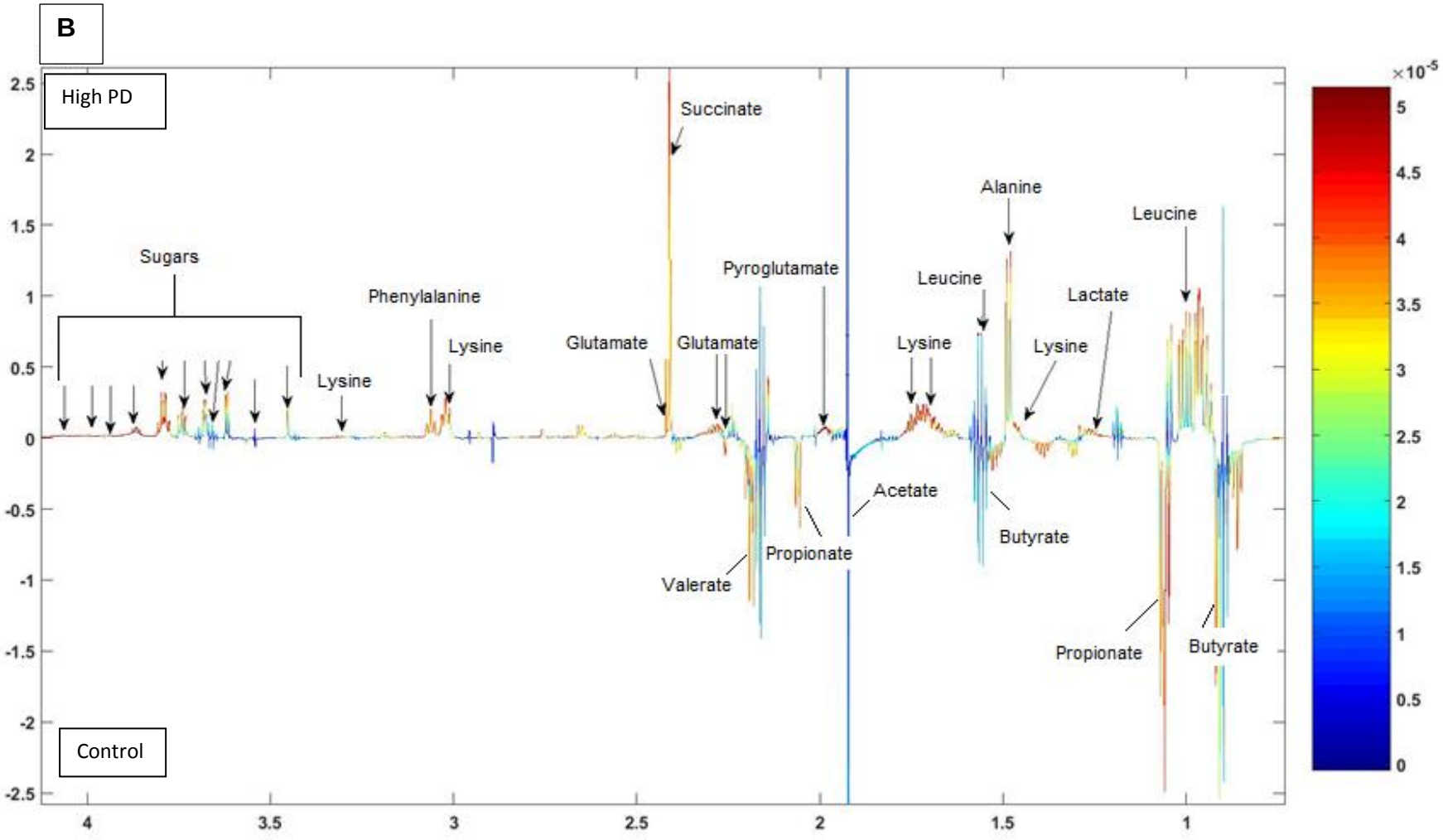
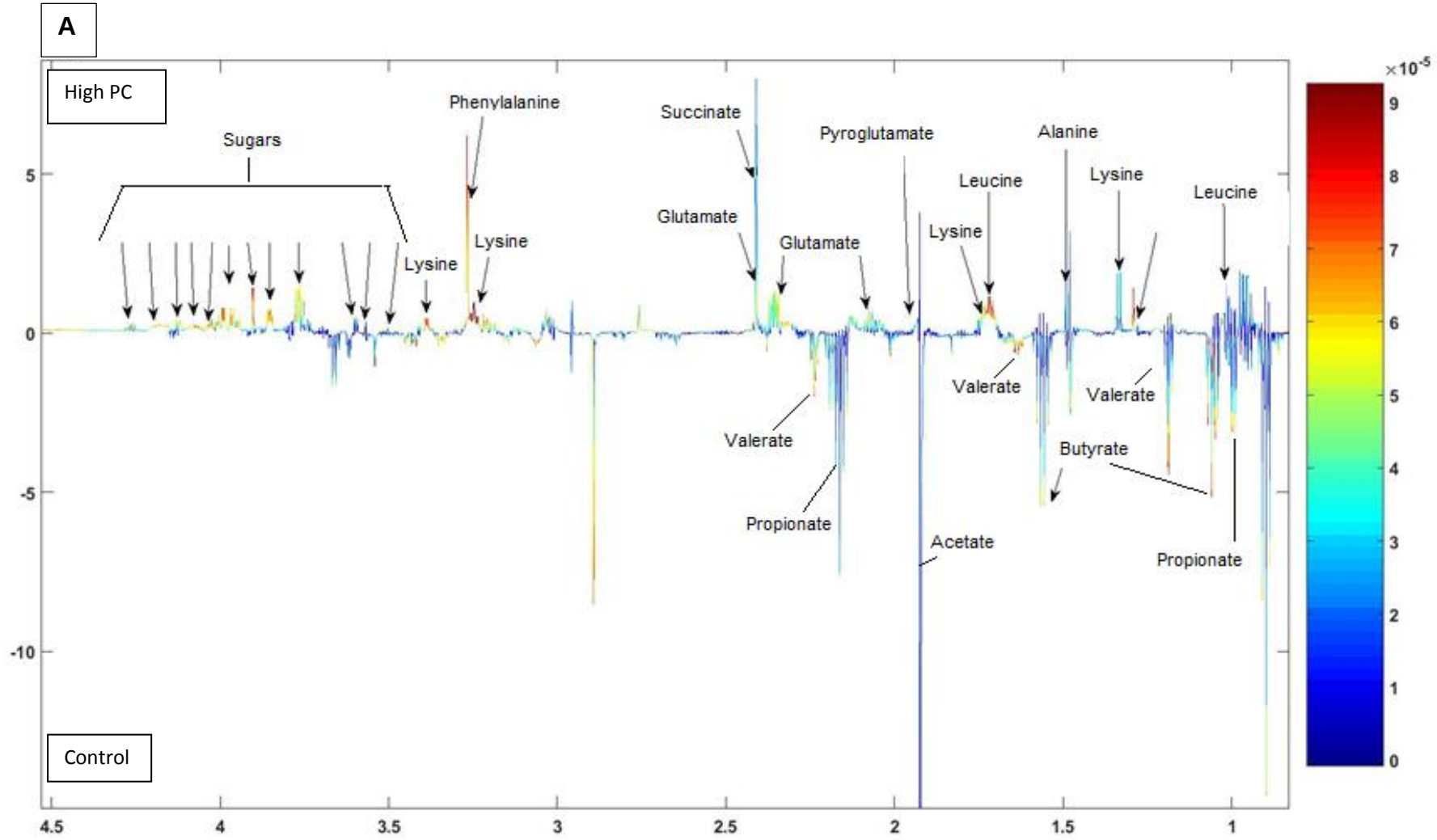



**Figure 4.1:** The colour plot of $^{1}$H-NMR spectra highlights the most important metabolites of caecal contents for the differentiation between controls (bottom) and treated with or without CT - black locust (high PD at 0.6 mg/ml) (top) after: **(A)** 10h and **(B)** 24h

*The peak colours generated from blue to red, which indicates the value of correlation coefficient increases at $P \leq 0.05$.


A
High PC
Control
Sugars
Phenylalanine
Succinate
Pyroglutamate
Alanine
Lysine
Leucine
Glutamate
Lysine
Glutamate
Lysine
Leucine
Lysine
Valerate
Valerate
Valerate
Butyrate
Propionate
Acetate
Propionate
×10-5
9
8
7
6
5
4
3
2
1
0
5
0
-5
-10
4.5
4
3.5
3
2.5
2
1.5
1

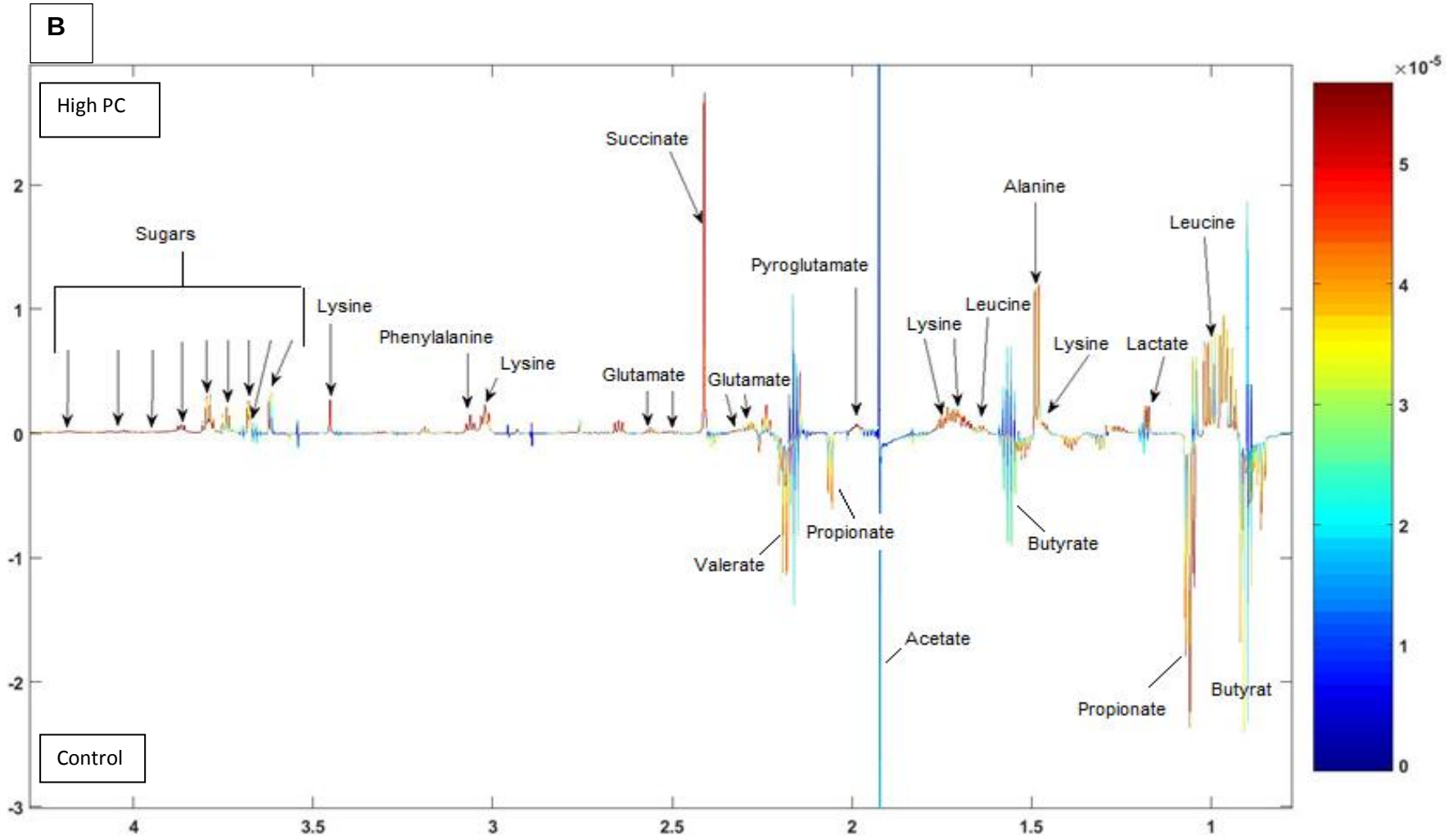



**Figure 4.2:** The colour plot of $^{1}$H-NMR spectra highlights the most important metabolites of caecal contents for the differentiation between controls (bottom) and treated with or without CT- tilia flowers (high PC at 0.6 mg/ml) (top) after: **(A)** 10h and **(B)** 24h

*The peak colours generated from blue to red, which indicates the value of correlation coefficient increases at $P \leq 0.05$.

Unsurprisingly, the addition of CT to the incubation medium resulted in NMR peaks associated with different CT residues. This indicated that indicating the PC and PD were metabolised to a limited extent and considering undigested CTs were fermented in caecal content in this *in vitro* experiment. To identify metabolic variation based on correlation coefficient values between the samples assignment of spectral lines over time between the different treatments for all four-times points collected was applied to metabolic profiles. Figures 4.3 and 4.4 provide an overview of the principal component analysis (PCA) that is based on orthogonal partial least squares (OPLS) to statistically draw the graphs from a class model that examined the homogeneity among the samples. The metabolic profile of the data resulted in $R^2$ (0.77) and $Q^2$ (0.54) values of 63 % for the first two components, which are illustrated in colour plot to describe the metabolic variability between all groups at different time points.

Furthermore, an OPLS plot discriminated between the control samples and the CT samples along the first principal component at time point 10h and 24h. These plots showed that there are good model and predictive ability in the concentrations of metabolites between treated and untreated groups, and these differences have been plotted in different colours at different time points. In these models, a clear separation was evident based on the different CT types and controls within the time recorded. The result clearly also differentiated the controls from both treated CTs by metabolic peaks and the different component concentrations between them at different time-points. Moreover, the metabolic variation at time 24h and 48h showed very close results in metabolites.

Thus, the results could suggest that different metabolites were observed. However, one of the major differences between the treatments is the impact of CTs, both compositions, on metabolites and could contribute to the differences seen. These are shown in a colour plot and describe the metabolic variability of the treatments. These plots were also produced to visualise differences in the metabolome between treatments.

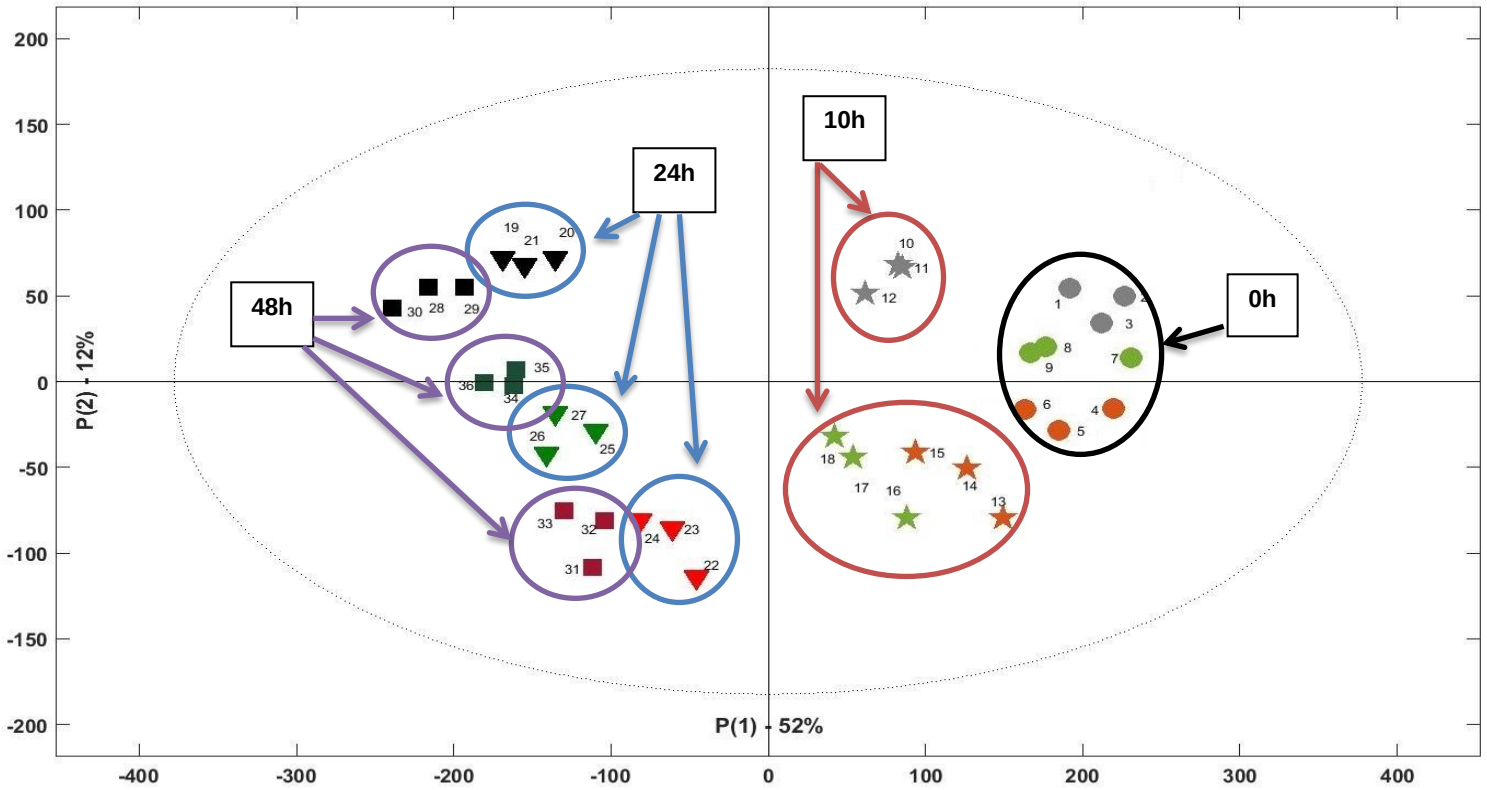



**Figure 4.3:** Principal component analysis (PCA) - score plot obtained from the $^{1}$H-NMR spectra of caecal contents that were treated with CTs of either high PD% or high PC% for different time-points; $R^2 = 0.77$, $Q^2 = 0.54$. Circles indicate the treatments at 0h, stars indicate the treatments at 10h, triangles indicate the treatments at 24h and squares indicate the treatments at 48h; (Red = high PD% group; Green = high PC% group; Black = controls)

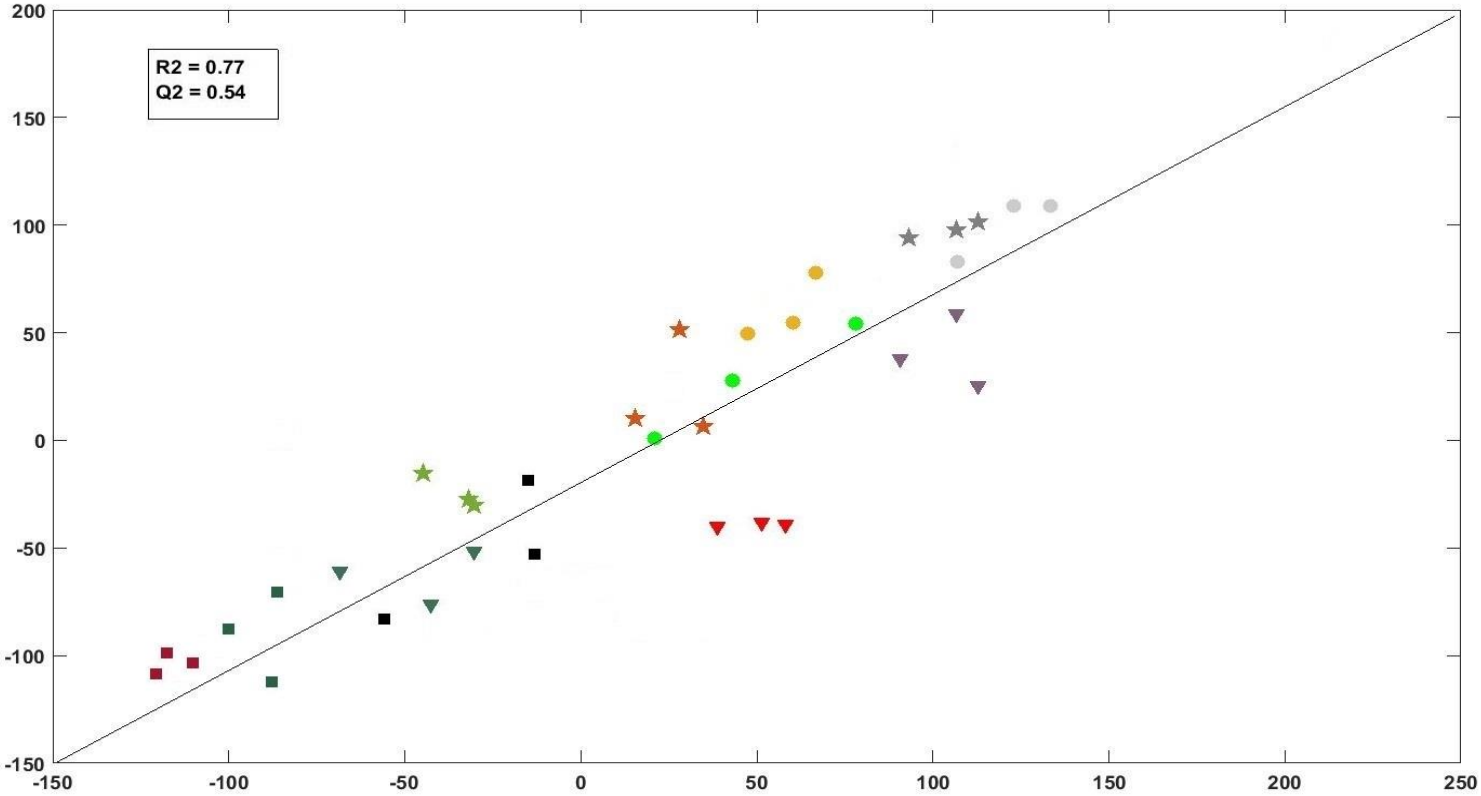



**Figure 4.4:** Orthogonal partial least squares (OPLS) - score plot for metabolites of controls and treated CT either high PD% or PC% of CT types. Circles indicate the treatments at 0h, stars indicate the treatments at 10h, triangles indicate the treatments at 24h and squares indicate the treatments at 48h; (Red = high PD% group; Green = high PC% group; Black = controls)

## 4.3 Materials and Methods for APEC (Experiment 2)

### 4.3.1 Preparation of samples with APEC culture

For the NMR metabolic analysis, 2-3 APEC colonies were incubated overnight in 10 ml of Luria-Bertani medium (LB) at 37 °C with shaking at 100 rpm. Subsequently the culture was incubated at 37 °C with shaking was obtained, and this culture was then incubated with medium containing either medium or condensed tannins in medium.

Separately, three vessels for the treatments were prepared that included 6 replicates in each treatment. Each vessel contained 50 ml of LB medium that contained CTs with either high PD% or high PC% (concentrations were adjusted to 0.6 mg CT/ml) plus 10 ml APEC culture, whereby control vessels contained APEC and LB broth only. This experiment was performed at pH 6.5 and 37 °C as reported previously (Hoerr et al., 2016).

Afterwards, Bottles and their contents were incubated for 24h under aerobic conditions. A sample of incubation medium, 6 ml was withdrawn from each vessel at 0h, 10h and 24h. These samples were snap-frozen in liquid nitrogen and stored directly in a freezer at - 80 °C pending analysis by NMR.

### 4.3.2 Nuclear magnetic resonance spectroscopy (NMR) for APEC

The reagents and the equipment for this experiment were similar to Experiment 1 (Section 4.2.3).

### 4.3.3 Methods

#### 4.3.3.1 Preparation of phosphate buffer

For this assay, the same PBS used in Experiment 1 (section 4.2.4.1) was used.

#### 4.3.3.2 Preparation samples of APEC for NMR analysis

Tubes containing samples of incubation medium were defrosted in a water bath at 25 C°. The same procedure used in Experiment 1 was then used. 400 μl of each sample was transferred to a sterile microfuge tube to mix with 200 μl phosphate

buffer was added. Then these samples were vortexed and centrifuged (10,000 g) for 10 minutes, and 550 μl of each supernatant was transferred into a NMR tube.

#### 4.3.3.3 Setup of NMR instrument

The procedure adopted for NMR analysis was as described in Section 4.2.4.

#### 4.3.3.4 Processing the data for analysis

All data for this part were analysed in the same way as described in section 4.2.4 before. Furthermore, the same computer software was used.

### 4.3.4 Results and discussion

The analysis of the $^{1}$H-NMR spectra from this experiment are presented in the tables and figures below. These data may serve as a new database of APEC end products after CT treatments, because this is the first study that investigated by affecting of CTs on metabolites of APEC culture. This study also has been achieved at three different time-points (0, 10, 24 hours) with controls and CT treatments. This research hypothesised that in the presence of CTs some differences in metabolites will be detectable especially those metabolites that have the highest concentrations such as amino acids and fatty acids.

Captured data were analysed using whole metabolic analysis tools that assign the metabolite description and relative concentration (location and height of peaks, respectively). To analyse these complex data sets Principle Component Analysis (PCA) was used (Figures below). The direction and distance covered by the samples can be considered as respective indicators of the differences between the metabolic profiles of APEC under the two test conditions, with and without CTs.

The metabolic profile of APEC grown in LB medium was tightly clustered indicating minimal sample to sample variation between treated and untreated groups. However, the metabolic end products of CT treatments were more dispersed but significantly discrete from APEC, particularly the PD treatment. Thus, it is obvious that the metabolites of APEC differed in the CTs. However, each CT treatment had a different effect on APEC culture. This suggests that the different CTs impacted not only on bacteria but also on their metabolites.

Table 4.2 shows the effect of CTs on APEC metabolism. There were significant differences (P≤0.05) of the interaction between treatment and time on the concentration of metabolites in presence or absence of CTs. The most important results to emerge from the data are that the concentrations of amino acids (such as lysine, glutamate, pyroglutamate and phenylalanine) were significantly increased (P≤0.05) after 10 and 24 h incubation, and this increase was greater when APEC was incubated with high PD% CT but there was little difference between the control and high PC% CT. These results are probably due to the fact that CT can make a complex with other compounds, particularly with proteins (Terrill et al., 1992; McSweeney et al., 2001). Consistent with these findings, some of the amino acids, e.g. aromatic and branched amino acids, showed also significant differences (P≤0.05) between these treatments but not at time-points (P>0.05). The PC group gave results that were close to control when compared to the PD group. Therefore, this study recommends that it would be worthwhile to investigate further the effects of different CT types on fermentation and putrefaction of these amino acid families in the chicken gut in term of chicken health.

In contrast, some short chain fatty acids, particularly in the high PD treatment, such as butyrate, iso-butyrate, valerate and iso-valerate showed a slight increase in their concentrations compared to the high PC treatment and controls; but these increases were not statistically significant (P>0.05) between the integration of times and treatments. However, they showed significant differences (P≤0.05) at the times. In the intestine, butyrate is a product of fermentation depending on the intestinal microflora. Butyrate plays a major role not only as an important energy source for the host but also as maintain factor of the tissues (Liu et al., 2012). Further, acetate, lactate and succinate had the lowest concentrations in the PD group compared to the PC and control groups (P≤0.05/ treatment).

The short chain fatty acids were also increased in concentration over time as would be expected in controls as they are the products of bacterial fermentation. The apparent inhibition of fermentation with PD but not PC confirmed the hypothesis that the effect of CTs on bacterial fermentation is affected by CT composition and not by CT concentration alone. The changes in particularly fatty acid but also amino acid concentrations could indicate these changes in

metabolism of energy pathways as a result of the addition of PD to the APEC medium although Krebs cycle intermediates such as succinate were affected (Kotze et al., 1969; Liu et al., 2012). This finding also indicated that APEC bacteria might survive in both the PC and control groups, and could utilise these acids by mixed acid pathways, which converts glucose into pyruvate and then produces more fatty acids. This finding, thus, confirmed that CTs, particularly PD, could influence the biological process of mixed acid fermentation by effecting lactate fermentation pathway. This contrasts with *E. coli*, where last normally increased by anaerobic respiration (Forster and Gescher, 2014).

Further, Figure 4.5 illustrates the interaction between treatment and time on these metabolites. The peak colours generated from blue to red, which indicates of the value of correlation coefficient increases. The result showed the different component concentrations between controls and treated CTs. However, this figure shows several peaks that correlate with end products of energy metabolism such as lactate, acetate and succinate, which were significantly different in treated CTs, especially PD, and APEC. Succinate is the intermediary synthetic product of TCA cycle, whilst acetate is the end products of the TCA cycle (Liu et al., 2012). These findings suggest that APEC respired aerobically to produce the anticipated end products of energy metabolism, but with treated CTs these fatty acid pathways were interrupted. Additionally, there were few significant effects of either treatment or time on the concentrations of carbohydrates, but both mannitol and fructose increased in concentration with time, and the concentration of both these metabolites was greater when APEC was incubated with high PD% CT. There was, however, no significant interaction between treatment and time. The addition of PD to an incubation of APEC, therefore, resulted in the production of mannitol, fructose, lysine, glutamate, pyroglutamate and phenylalanine but an inhibition of the production of acetate and lactate.

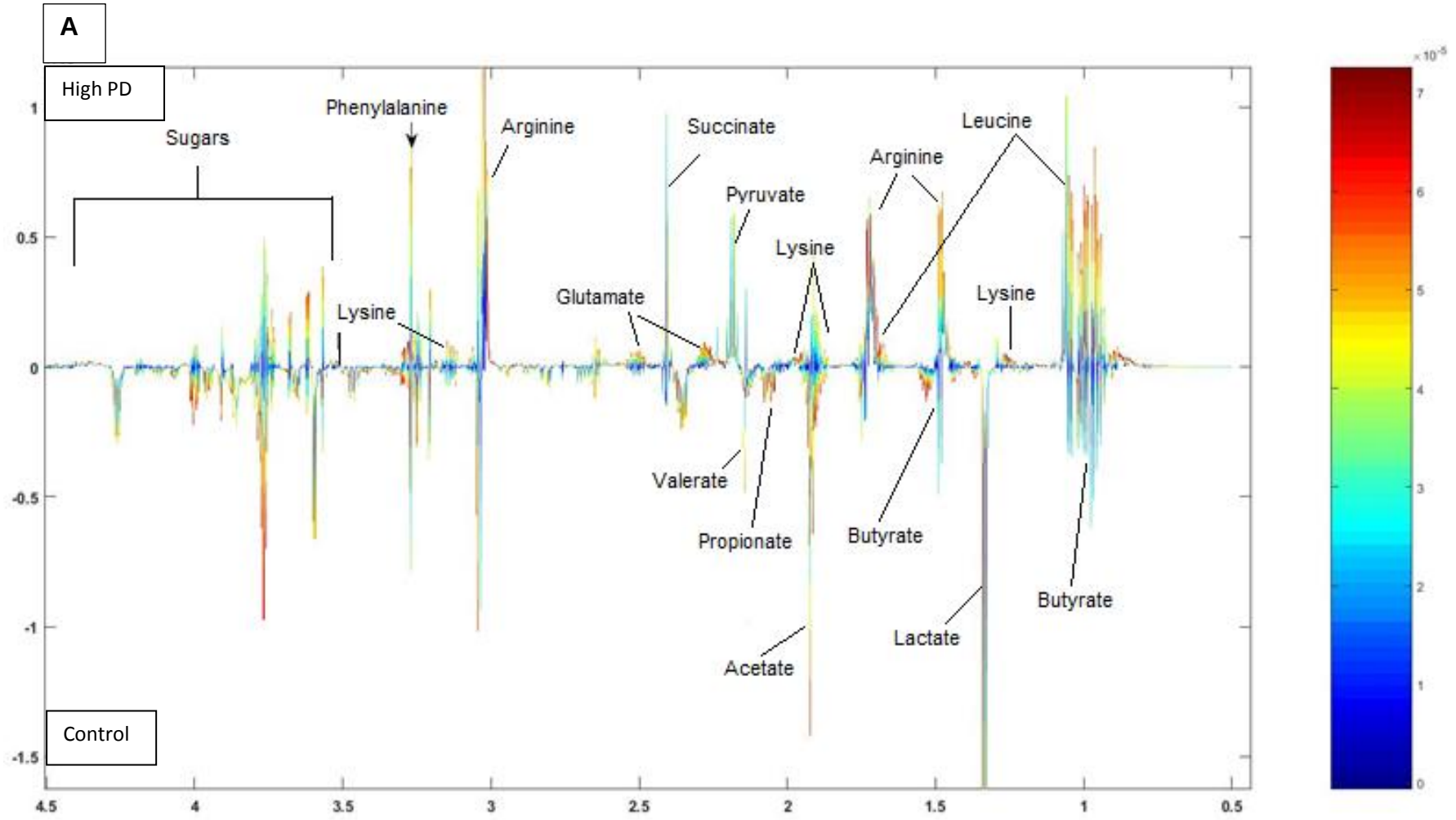
The CT peaks, which presented in the aromatic area of the spectra, showed signals > 5.5 ppm that demonstrated the presence of CTs in the treated groups. However, some of these peaks were absent after 24h, suggesting (as in Experiment 1) that some of the CT compositions had been, probably, metabolised by bacteria as source of nutrients. As expected, there was no evidence for any CT peaks in the control group (Fig 4.6).

**Table 4.2:** The concentrations (*mM) of metabolites from APEC culture incubated with condensed tannins that had either high molar percentages of PD or PC after 10 and 24h incubation

| Metabolites | | High PD% | | | High PC% | | | Control | | | *P-value* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0h | 10h | 24h | 0h | 10h | 24h | 0h | 10h | 24h | Time | Treat. | T*T |
| Fatty acids | Acetate | 0.6 | 2.2 | 2.1 | 0.6 | 2.3 | 2.4 | 0.6 | 2.9 | 3.0 | 0.01 | 0.05 | 0.03 |
| | Lactate | 0.3 | 0.3 | 0.4 | 0.2 | 0.5 | 0.6 | 0.2 | 0.5 | 0.9 | 0.10 | 0.08 | 0.05 |
| | Propionate | 0.2 | 1.5 | 1.9 | 0.2 | 1.6 | 2.0 | 0.2 | 1.8 | 2.3 | 0.01 | 0.13 | 0.11 |
| | Isobutyrate | 0.2 | 0.6 | 0.7 | 0.2 | 0.6 | 1.0 | 0.2 | 0.6 | 1.1 | 0.04 | 0.44 | 0.14 |
| | Valerate | 0.2 | 0.4 | 0.4 | 0.2 | 0.3 | 0.5 | 0.2 | 0.3 | 0.8 | 0.07 | 0.62 | 0.13 |
| | Succinate | 0.1 | 0.5 | 0.6 | 0.1 | 1.1 | 1.2 | 0.2 | 1.2 | 2.6 | 0.04 | 0.14 | 0.06 |
| | Butyrate | 0.1 | 0.3 | 0.4 | 0.1 | 0.4 | 0.6 | 0.1 | 0.4 | 0.7 | 0.05 | 0.21 | 0.07 |
| | Isovalerate | 0.1 | 0.3 | 0.2 | 0.1 | 0.3 | 0.3 | 0.1 | 0.3 | 0.4 | 0.02 | 0.43 | 0.18 |
| Amino acids | Lysine | 3.2 | 7.3 | 8.3 | 3.2 | 5.9 | 6.3 | 3.2 | 5.5 | 5.5 | 0.12 | 0.01 | 0.05 |
| | Leucine | 2.0 | 4.7 | 5.1 | 2.0 | 4.4 | 4.1 | 2.0 | 4.5 | 4.0 | 0.11 | 0.02 | 0.05 |
| | Glutamate | 1.4 | 3.2 | 3.7 | 1.4 | 2.4 | 2.8 | 1.4 | 2.3 | 2.7 | 0.11 | 0.01 | 0.05 |
| | Alanine | 1.1 | 2.5 | 2.6 | 1.1 | 2.6 | 2.4 | 1.1 | 2.4 | 2.4 | 0.10 | 0.07 | 0.06 |
| | Phenylalanine | 0.5 | 2.3 | 2.2 | 0.5 | 1.8 | 1.6 | 0.5 | 1.7 | 1.5 | 0.14 | 0.03 | 0.05 |
| | Valine | 0.9 | 2.3 | 2.1 | 0.9 | 2.1 | 1.9 | 0.9 | 2.1 | 2.0 | 0.15 | 0.07 | 0.25 |
| | Pyroglutamate | 0.8 | 2.1 | 2.2 | 0.8 | 1.9 | 1.7 | 0.8 | 1.7 | 1.5 | 0.12 | 0.02 | 0.05 |
| | Isoleucine | 0.8 | 2.0 | 2.1 | 0.8 | 1.6 | 1.8 | 0.8 | 1.7 | 1.7 | 0.13 | 0.06 | 0.09 |
| | Arginine | 0.5 | 1.0 | 1.1 | 0.5 | 0.8 | 0.8 | 0.5 | 0.8 | 0.8 | 0.10 | 0.07 | 0.06 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Carb. | Mannitol | 0.3 | 0.8 | 0.7 | 0.3 D | 0.55 | 0.5 | 0.3 | 0.5 | 0.4 | 0.09 | 0.06 | 0.10 |
| | Fructose | 0.2 | 0.5 | 0.6 | 0.2 | 0.3 | 0.4 | 0.2 | 0.4 | 0.3 | 0.09 | 0.06 | 0.11 |
| | Glucose | 0.1 | 0.3 | 0.5 | 0.1 | 1.0 | 0.02 | 0.1 | 0.02 | 0.02 | 0.11 | 0.10 | 0.10 |
| | Lactose | 0.08 | 0.3 | 0.4 | 0.08 | 1.0 | 0.01 | 0.08 | 0.02 | 0.01 | 0.10 | 0.08 | 0.10 |
| | Mannose | 0.08 | 0.2 | 0.5 | 0.08 | 0.04 | 0.02 | 0.08 | 0.03 | 0.01 | 0.15 | 0.15 | 0.12 |
| | Galactose | 0.01 | 0.1 | 0.5 | 0.01 | 0.07 | 0.03 | 0.01 | 0.04 | 0.02 | 0.14 | 0.12 | 0.11 |
| | Maltose | 0.01 | 0.05 | 0.3 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.16 | 0.15 | 0.13 |
| Others | Pyruvate | 0.1 | 0.3 | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.10 | 0.09 | 0.10 |
| CTs | Prodelphinidins | 1.0 | 0.5 | 0.05 | 1.0 | 0.2 | 0.04 | 0.01 | 0.001 | 0.00 | 0.16 | 0.06 | 0.15 |
| | Procyanidins | 1.0 | 0.2 | 0.01 | 1.0 | 0.8 | 0.09 | 0.01 | 0.001 | 0.00 | 0.24 | 0.05 | 0.14 |

(*mM) = the molar concentration of each compound; All values represent the mean of three replicates; Significant differences ($P \leq 0.05$) between the treatments; Time-*P-value* indicates significant values in time-points; Treat.-*P-value* indicates significant values in treatments; T*T-*P-value* indicates significant values in interactions between the times and treatments.


A
High PD
Control
Sugars
Phenylalanine
Arginine
Succinate
Pyruvate
Lysine
Glutamate
Lysine
Arginine
Leucine
Lysine
Valerate
Propionate
Butyrate
Acetate
Lactate
Butyrate
1
0.5
0
-0.5
-1
-1.5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
×10⁻⁵
7
6
5
4
3
2
1
0

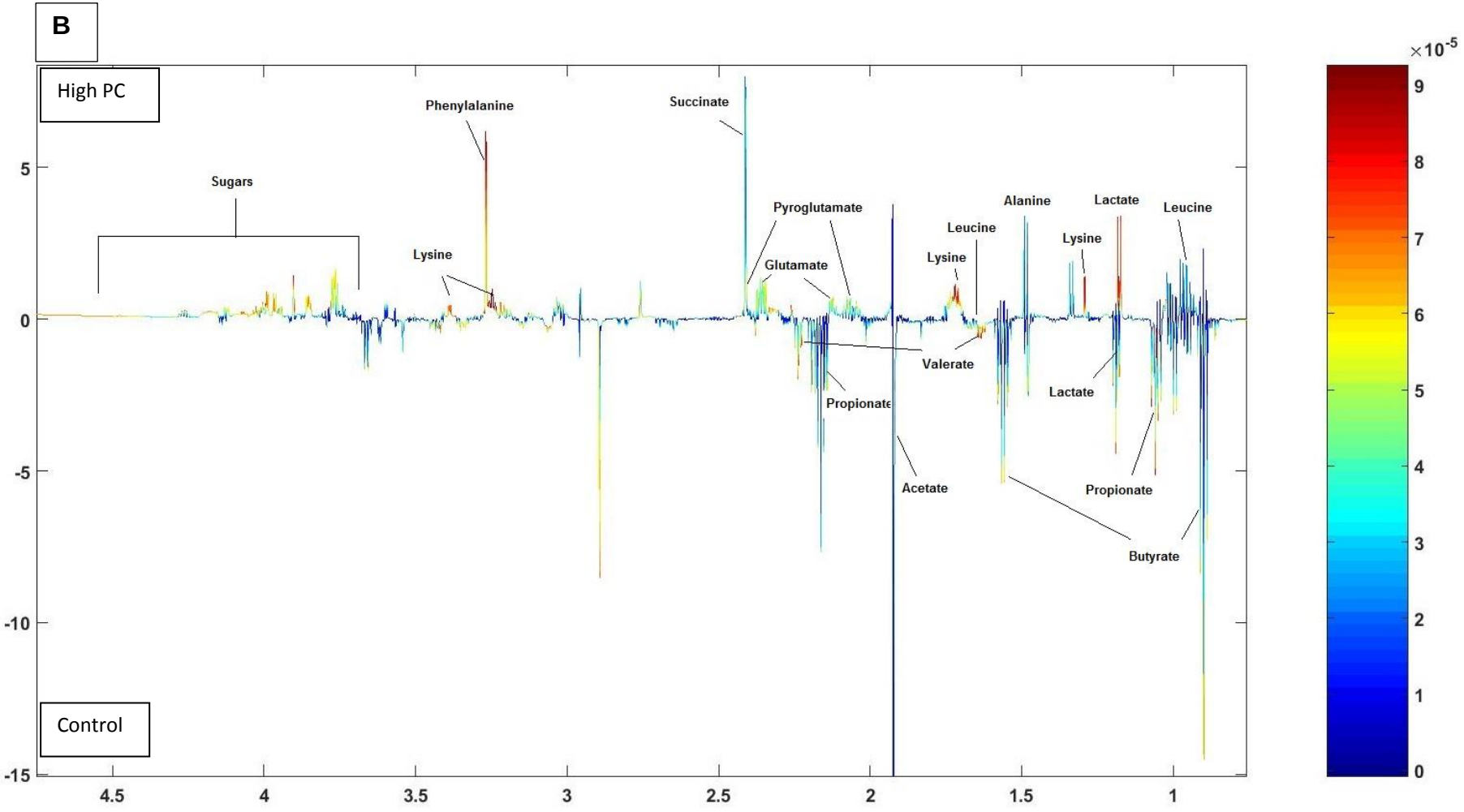



**Figure 4.5:** The colour plot of $^{1}$H-NMR spectra highlights the most important metabolites of APEC after 24h between controls (bottom) and treated CTs (top) either **(A)** black locust (high PC at 0.6 mg/ml) or **(B)** tilia flowers (high PC at 0.6 mg/ml)

*The peak colours generated from blue to red, which indicates the value of correlation coefficient increases at $P \leq 0.05$.

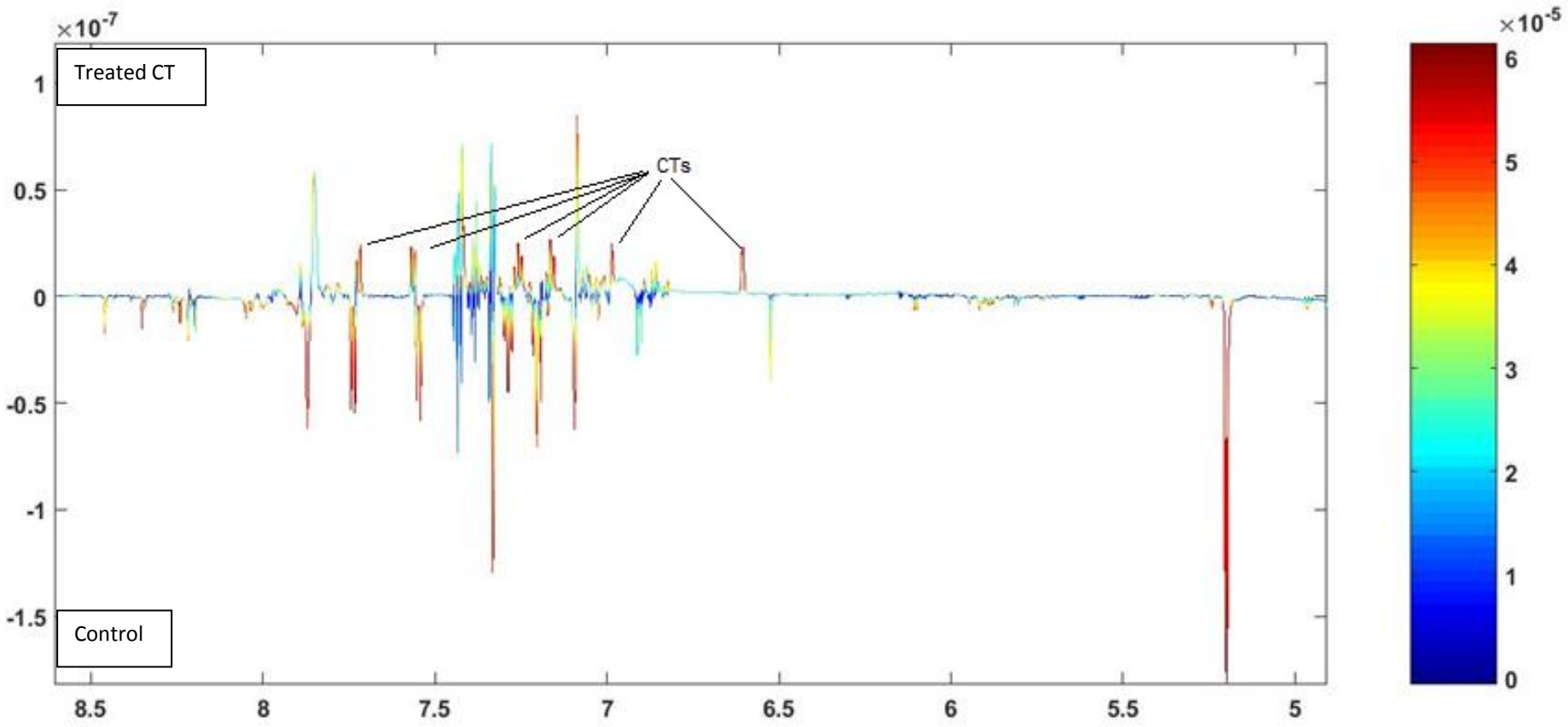



**Figure 4.6:** The colour plot of $^{1}$H-NMR spectra shows the aromatic region (> 5.5 ppm) in APEC culture after 24 h with or without CT compositions.

Figures 4.7 and 4.8 present the principal component analysis (PCA) linked to orthogonal partial least squares (OPLS); this data generated these graphs from a class model that examined the homogeneity among the samples. The metabolites of these data resulted in $R^2$ and $Q^2$ values of 65 % for the first two components and are illustrated in a colour plot that describes the metabolic variability of the treatments at the different time points (0, 10 and 24 hours). The OPLS plot differentiated between the controls and CT treatments along the first principal component at time points 10 h and 24h. These plots illustrated that there are obviously significant differences ($P \leq 0.05$) in the amount of metabolites between treated and untreated groups. Further, these differences have been plotted in different colours at different time-points. Therefore, these metabolites not only changed during times, 0h, 10h and 24h, but also the metabolites were affected significantly by the different CT compositions (PC vs PD types).

As result of these experiments (1 and 2), the final hypothesis of this study was proven that CT compositions may not have potential effects on the metabolites of caecal contents, but may have affecting the important metabolic end products of APEC. However, unlike the mixed culture used in Experiment 1, the monoculture APEC responded differently to CT depending on their compositions. Therefore, these findings highlight the need for more research in this field.

In general, extractable CTs that contained high molar percentages of PC generated a slightly improving composition of metabolic end products along different time-points compared to CTs with high PD, and these results were close to the controls. A possible explanation for these results may be either a lack of antimicrobial effects of the PC compared to PD; or PD has a greater effect on amino acids particularly because they are more effective at complexing with proteins because of their larger number of OH groups. However, in previous research of Holloway et al. (2015) concluded that monomeric CTs, e.g. catechin and epicatechin, that combined with inorganic substance such as copper sulphate, which could generate an antimicrobial agent such as $H_2O_2$ and influence pathogens.

Finally, this raises the question about how chicken diets can be formulated to influence the microbes in caecum that are important for the health and nutrition of chicken (Jana et al., 2015). Is it possible that manipulation of the microbiota may

influence metabolism? Indeed, this *in vitro* study demonstrated that caeca contents treated with a sort of CT produced different levels of metabolites. Thus, adding these CTs may be beneficial by having a positive nutritional impact if such changes are associated with enhancement of certain metabolites. However, this study also suggests that choosing the right CT compositions will be important, especially because PD had a significant effect on APEC. Thus, *in vivo* studies will be required to assess their efficacy in practice.

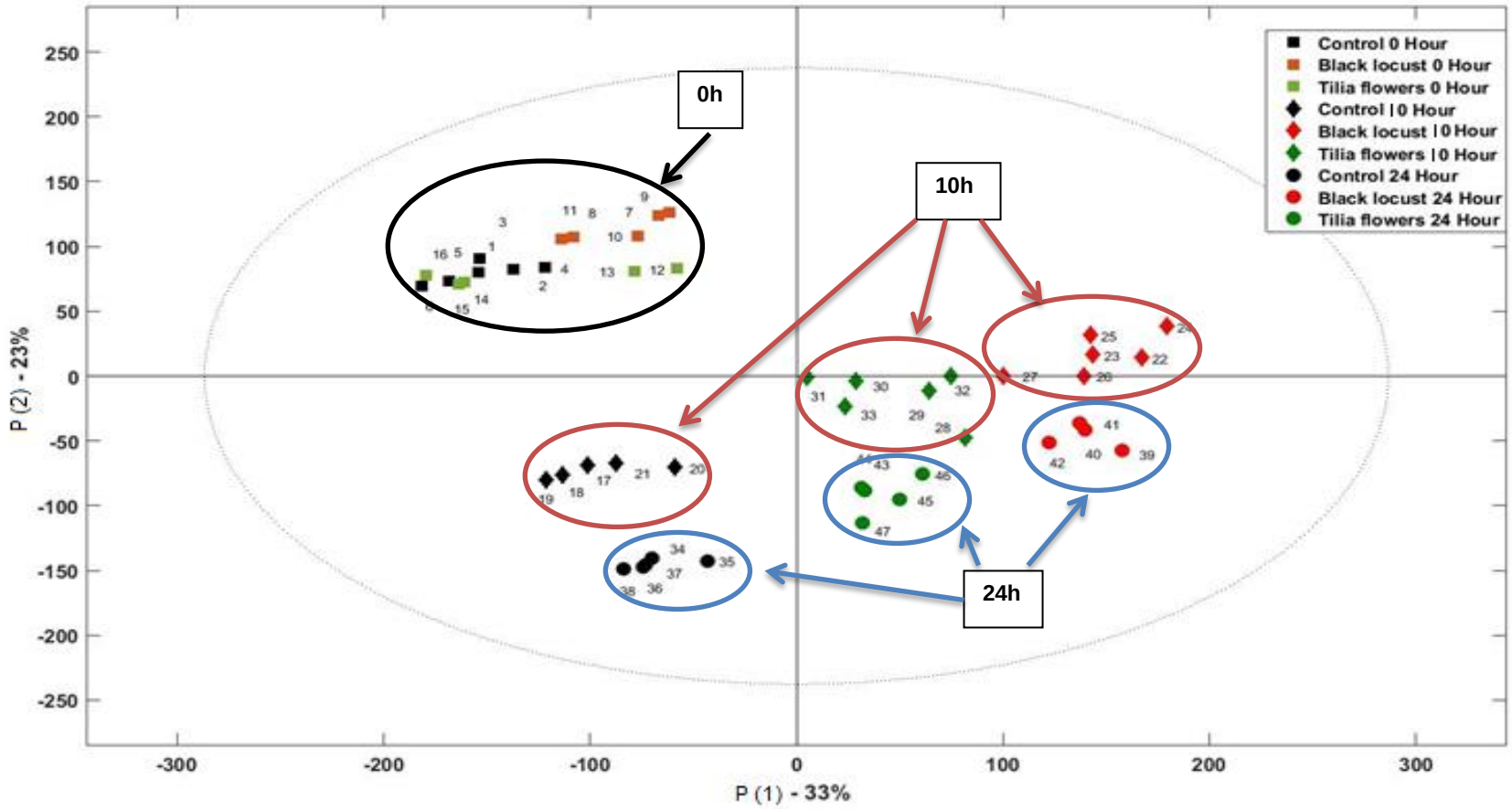



**Figure 4.7:** Principal component analysis (PCA) - score plot obtained from the $^{1}$H-NMR spectra of APEC culture that treated with high PD% or high PC% for different time-points; $R^2$ = 0.52; $Q^2$ = 0.42; (Red = high PD% group; Green = high PC% group; Black = controls).

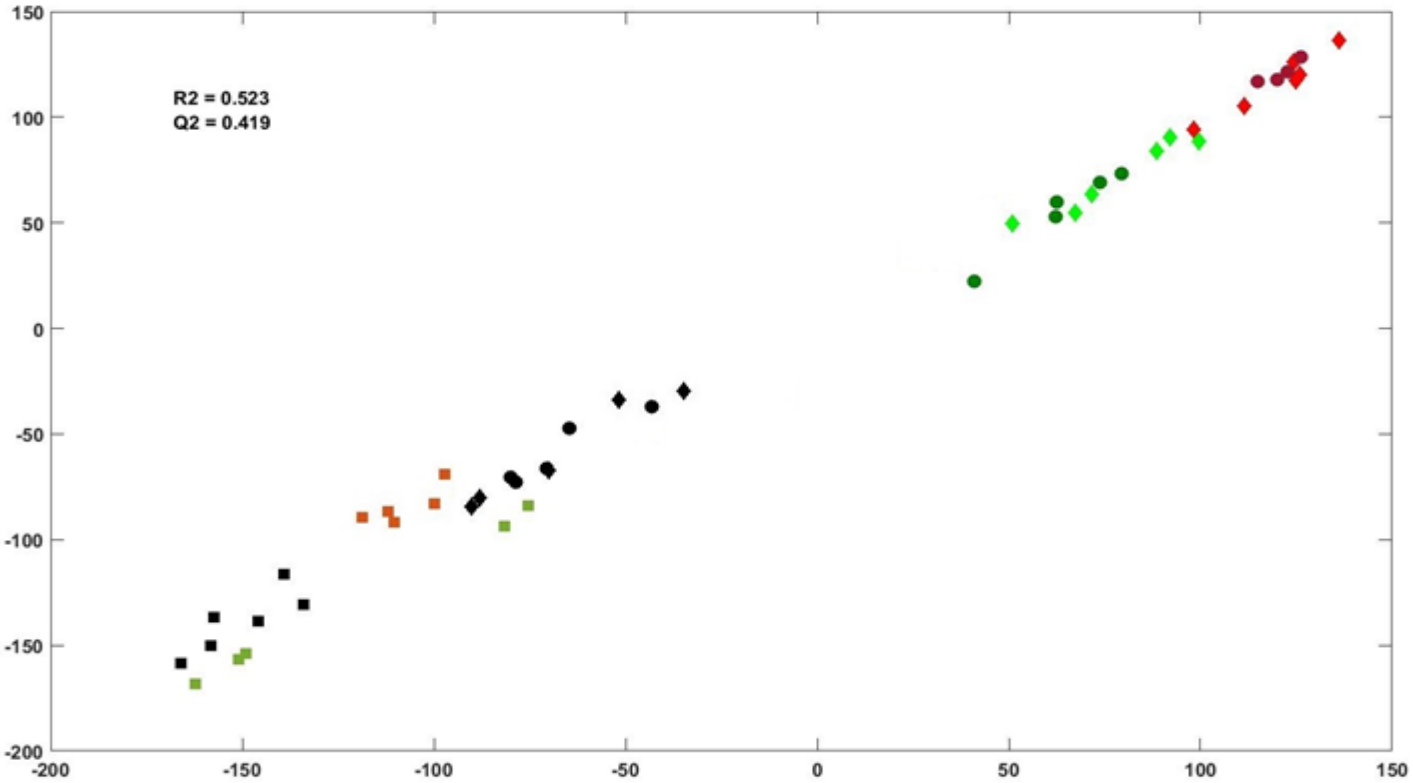



**Figure 4.8:** Orthogonal partial least squares (OPLS) - score plot for metabolites of APEC culture for untreated and treated groups with a two sub-MIC concentration of CTs (0.6 mg/ml), either high PD% or high PC%, at different time points; N = 6 for each sample; $R^2$ = estimates goodness of fit; $Q^2$ = estimates goodness of samples (Red = high PD% group; Green = high PC% group; Black = controls)

## 4.5 Overall conclusion

The aim of the present research was to examine the effect of different compositions of CT, PD vs PC type, on various fermentation models such as caecal contents or APEC culture and to determine the metabolites that generated in the presence of these extracts. The use of CTs in poultry nutrition has not been investigated to any great extent, but this study indicated that the composition of CT has an effect on fermentation and metabolism *in vitro* of single cultures (such as APEC, investigated in Experiment 2), although this was not apparent with the mixed culture (Experiment 1), in which CTs certainly had an effect on the fermentation but there was no evidence of an effect of their composition.

The results from this study indicated significant ($P \leq 0.05$) changes of some important metabolites, such as amino acids, occurred at different time-points. These amino acids can play an important role by oxidations of amino acid synthesis that give rise to it are being used as sources of energy. It has been suggested that by increasing the concentrations of some amino acids may influence the diversity of microbiota and this could impact the oxidative reactions along in the gut (Lemme et al., 2004; Amorim Franco and Blanchard, 2017).

The most obvious finding to emerge from this study is that the stimulation of fermentation with both CT types did not increase the fatty acids compared to the control. However, CTs produced significant ($P \leq 0.05$) higher succinate and acetic acid concentrations than the controls in the caecal experiment. As a result, these findings indicate that CTs could assist to induce a low pH environment by promotion of these acids. This may lead to the inhibition of the growth rate for gut pathogens such as APEC. In addition, a significant response in PD treatment ($P \leq 0.05$) was observed on increasing and decreasing the levels of metabolites on APEC experiment, particularly fatty acids and amino acids respectively. More importantly, CT with high molar proportions of PD showed significant interruption of APEC fermentation that disturbed the growth of this pathogen in an *in vitro* experiment. This study also found that PD, in general, significantly affected this pathogen in contrast to PC.

In conclusion, these effects of differing CT compositions may depend on the individual variation in gut microbial ecology, which may also affect the extent of CT

absorption from the gut, before the CT arrives at the caecum. Further research will be needed to better understand, even *in vitro*, the interaction between microbial communities and CTs. Moreover, *in vivo* studies will be necessary to probe the effects of CT composition and/or concentration on the different parts of the gastrointestinal tract. It is hoped that CT extracts may find use for improving aspects of chicken health.

More specific studies are required to understand the fate of CTs and clarify how they affect intestinal microbiota *in vitro* and *in vivo*. However, it is essential that caution be advised in extrapolating results from static gut models (Duenas et al., 2015). Conversely, several effects were observed in the first 10 h of incubation and that may more readily reflect the impact in the gut. Changes later between 10h and 24h may be purely artefacts of extending incubation in an inappropriate system for too long. That said, what has been learnt here is that there are bacteria that thrive in the model in the presence of CTs.

## 4.6 References:


Amorim Franco, T. M., and Blanchard, J. S. (2017). Bacterial Branched-Chain Amino Acid Biosynthesis: Structures, Mechanisms, and Drugability. *Biochemistry* 56, 5849-5865.

Beckonert, O., Keun, H. C., Ebbels, T. M., Bundy, J., Holmes, E., Lindon, J. C., and Nicholson, J. K. (2007). Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2, 2692-703.

Brenes, A., Viveros, A., Chamorro, S., and Arija, I. (2016). Use of polyphenol-rich grape by-products in monogastric nutrition. A review. *Anim Feed Sci Technol* 211, 1-17.

Calvani, R., Brasili, E., Pratico, G., Sciubba, F., Roselli, M., Finamore, A., Marini, F., Marzetti, E., and Miccheli, A. (2014). Application of NMR-based metabolomics to the study of gut microbiota in obesity. *J Clin Gastroenterol* 48 Suppl 1, S5-7.

Forster, A. H., and Gescher, J. (2014). Metabolic Engineering of *Escherichia coli* for Production of Mixed-Acid Fermentation End Products. *Front Bioeng Biotechnol* 2, 16.

Fuchs, S., Behrends, V., Bundy, J. G., Crisanti, A., and Nolan, T. (2014). Phenylalanine metabolism regulates reproduction and parasite melanisation in the malaria mosquito. *PLOS ONE* 9, e84865.

Hoerr, V., Duggan, G. E., Zbytnuik, L., Poon, K. K., Grosse, C., Neugebauer, U., Methling, K., Loffler, B., and Vogel, H. J. (2016). Characterization and prediction of the mechanism of action of antibiotics through NMR metabolomics. *BMC Microbiol* 16, 82.

Holloway, A., Mueller-Harvey, I., Gould, S., Fielder, M., Naughton, D., and Kelly, A. (2015). Heat treatment enhances the antimicrobial activity of (+) Catechin when combined with copper sulphate. *Lett Appl Microbiol* 61, 381-389.

Jana, A., Adak, A., Halder, S. K., Das, A., Paul, T., Mondal, K. C., and Mohapatra, P. K. D. (2015). A new strategy for improvement of tamarind seed based chicken diet after microbial detannification and assessment of its safety aspects. *Acta Biol. Szeged* 59, 1-9.

Kotze, J., Thiel, P., and Hattingh, W. (1969). Anaerobic digestion II. The characterization and control of anaerobic digestion. *Water Research* 3, 459-494.

Lemme, A., Ravindran, V., and Bryden, W. (2004). Ileal digestibility of amino acids in feed ingredients for broilers. *World's Poult Sci J.* 60, 423-438.

Le Roy, C. I., Mappley, L. J., La Ragione, R. M., Woodward, M. J., and Claus, S. P. (2016). NMR-based metabolic characterization of chicken tissues and biofluids: a model for avian research. *Metabolomics* 12, 157.

Liu, W., Le, A., Hancock, C., Lane, A. N., Dang, C. V., Fan, T. W., and Phang, J. M. (2012). Reprogramming of proline and glutamine metabolism contributes to the proliferative and metabolic responses regulated by oncogenic transcription factor c-MYC. *Proc Natl Acad Sci USA* 109, 8983-8.

Lowry, J. B., McSweeney, C. S., and Palmer, B. (1996). Changing perceptions of the effect of plant phenolics on nutrient supply in the ruminant. *Australian J. Agri. Res.* 47, 829-842.

Macfarlane, G. T., and Macfarlane, S. (2012). Bacteria, colonic fermentation, and gastrointestinal health. *JAOAC Int* 95, 50-60.

McSweeney, C. S., Palmer, B., McNeill, D. M., and Krause, D. O. (2001). Microbial interactions with tannins: nutritional consequences for ruminants. *Anim Feed Sci Technol* 91, 83-93.

Meister, A. (1957). "Biochemistry of the amino acids." *New York: Academic Press Inc.*, xiii + 485 pp.

Neis, E. P., Dejong, C. H., and Rensen, S. S. (2015). The role of microbial amino acid metabolism in host metabolism. *Nutrients* 7, 2930-46.

Niba, A. T., Beal, J. D., Kudi, A. C., and Brooks, P. H. (2009). Bacterial fermentation in the gastrointestinal tract of non-ruminants: Influence of fermented feeds and fermentable carbohydrates. *Trop Anim Health Prod* 41, 1393-1407.

Nutrition, N. R. C. C. o. A. (1971). "Nutrient requirements of poultry," Sixth revised edition, *National Academy of Sciences*, Washington, D.C

Pozuelo, M. J., Agis-Torres, A., Hervert-Hernandez, D., Elvira Lopez-Oliva, M., Munoz-Martinez, E., Rotger, R., and Goni, I. (2012). Grape antioxidant dietary fiber stimulates Lactobacillus growth in rat cecum. *J Food Sci* 77, H59-62.

Rinttila, T., and Apajalahti, J. (2013). Intestinal microbiota and metabolites--Implications for broiler chicken health and performance1. *J Appl Poult Res* 22, 647-658.

Selma, M. V., Espin, J. C., and Tomas-Barberan, F. A. (2009). Interaction between Phenolics and Gut Microbiota: Role in Human Health. *J. Agric. Food Chem.* 57, 6485-6501.

Stokes, D. J., Kelly, A. F., Gould, S. W. J., Cassar, C. A., and Fielder, M. D. (2008). The withdrawal of antimicrobial treatment as a mechanism for defeating resistant microorganisms. *Fems Immunol Medical Microbiol* 53, 300-305.

Terrill, T. H., Rowan, A. M., Douglas, G. B., and Barry, T. N. (1992). Determination of Extractable and Bound Condensed Tannin Concentrations in Forage Plants, Protein-Concentrate Meals and Cereal-Grains. *J. Sci. Food Agric.* 58, 321-329.

Tjahjani, S. (2017). Antimalarial activity of *Garcinia mangostana* L rind and its synergistic effect with artemisinin *in vitro*. *BMC Complement Altern Med* 17, 131.

Tzounis, X., Vulevic, J., Kuhnle, G. G., George, T., Leonczak, J., Gibson, G. R., Kwik-Uribe, C., and Spencer, J. P. (2008). Flavanol monomer-induced changes to the human faecal microflora. *Br J Nutr* 99, 782-92.

Zheng, C., Zhang, S., Ragg, S., Raftery, D., and Vitek, O. (2011). Identification and quantification of metabolites in (1)H NMR spectra by Bayesian model selection. *Bioinformatics* 27, 1637-44.

# Chapter 5: General discussion and conclusions

## 5.1 Characterisation of CTs

This thesis focused on exploring the antimicrobial effects of different CT-types in order to assess their potential use in chicken health. Initially (Chapter 2), a large number of plant materials were screened with the HCl-butanol acetone assay (ABA) in order to rank the plants according to their CT content (Grabber et al., 2013). This study screened a wide range of plant materials to detect CTs. Sixteen of the selected samples with the highest CT contents, which ranged from 3.2 to 14.0, were then analysed for their CT compositions by thiolysis and HPLC/MS analysis. An important constraint in the quest for valid CT data is the requirement for high purity standards for quantitation, which means that the CT concentration and the purity of the standards needs to be assessed by CT-specific methods such as thiolyic reaction (Gea et al., 2011; Grabber et al., 2013). This approach enabled characterisation of the CTs in terms of the mean degree of polymerisation (mDP), molar percentages of procyanidins (PC%) and prodelphinidins (PD%), *trans*- and *cis*- flavan-3-ols and CT concentrations (Schofield et al., 2001). Table 2.3 showed the correlation between these compositions by using a statistical nonparametric correlation. The following ranges were found for mDP: 4.1 to 33.6; for PC: 5.0 to 100.0, and for *trans*-flavan-3-ols: 2.3 to 76.4. There were positive correlations between CT concentration and molar percentages of PD (P≤0.05=0.61), between mDP and CT concentration (P≤0.05=0.54), and between molar percentages of PD and mDP (P≤0.05=0.5) (Karonen et al., 2006).

The results from the thiolysis revealed that samples from the following plant materials could serve as the appropriate sources for different CT-types, e.g. PD vs PC of CT-types, which will be examined in microbial and fermentation experiments in the next chapters. Those showed different values with high and/or low mDP and PC/PD ratios such as climbing hydrangea (mDP=4.1; PC/PD=5.0:95.0), yew (mDP=4.3; PC/PD=50.9:49.1), chuckle berry (mDP=4.4; PC/PD=100.0:0.0), strawberry tree (mDP=4.5; PC/PD=59.5:40.5), norway spruce (mDP=4.5; PC/PD=85.6:14.4), bracken (mDP=5.8; PC/PD=68.4:31.6), St John's wort (mDP=7.2; PC/PD=89.3:10.7), winged spindle (mDP=7.4; PC/PD=72.8:27.2), bistort (mDP=7.7; PC/PD=100.0:0.0), blackcurrant (mDP=8.2; PC/PD=9.3:90.7),

hazel (mDP=8.8; PC/PD=25.6:74.4), london plane (mDP=12.0; PC/PD=31.0:68.7), allspice (mDP=12.9; PC/PD=37.0:63.0), laurel-leaf cistus (mDP=13.9; PC/PD=57.5:42.5), persian ironwood (mDP=26.8; PC/PD=12.0:88.0), black locust (mDP=27.6; PC/PD=7.0:93.0). However, a wide variation of CT compositions and contents was found as well as that the CT concentrations were lower than expected in these plant materials. Therefore, the next step was to increase CT concentrations by Sephadex LH-20 column as suggested by (Brown et al., 2017).

Generally, the stereochemistry of CTs at (heterocyclic ring) gives a chemical structure of either 2–3 *cis*- or 2–3 *trans*-flavan-3-ols and the average polymer length of CT mixtures is described in terms of the mean degree of polymerisation (mDP) (Mueller-Harvey, 2006; Sivakumaran et al., 2006). It is important to note that most condensed tannin-containing plants tend to contain complex mixtures of PC and PD types (homo- and hetero-) that are difficult to separate (Porter et al., 1985; Ropiak et al., 2016). However, a few plants specialise in the synthesis of either PCs or PDs and can thus serve as valuable sources for the different tannin types and as research tools for probing structure–activity relationships (Klongsiriwet et al., 2015).

This study has observed the correlations between the compositions of CTs, which are illustrated in table 2.4. However, this reseearch focused on CT compounds that contain differnet PC/PD ratios of flavan-3-ol, it did not include samples with PC/PD galloylated flavan-3-ol subunits (Holloway et al., 2015), and since these galloylated CTs could interfere with the calculation of CT concentrations. Thus, these extracts are of interest as they have been implicated in numerous health effects (Ropiak et al., 2016).

The CTs were then extracted from chosen plant materials and purified on a Sephadex LH-20 column and eluted with acetone/water (3:7 and 8:2, v/v, for low and high molecular weight respectively) (Williams et al., 2014). Larger scale purifications of crude extracts from a variety of plant materials were also performed using this method and it yielded several fractions with 1 g of CTs (Brown et al., 2017). These investigations resulted in a set of CT fractions, which had CT concentrations ranging from 10 to 50 g CT/100 g fraction, mDP values of 1.6 to 9.8, and PD/PC ratios of 0:100 to 75:25. However, this method was repeated twice

to obtain a sufficient quantity of purified CT fractions. Therefore, the final concentrations of these fractions were increased to reach up to 95 g CT /100 g CT fraction for subsequent use. The identification of these extracts was confirmed by thiolytic degradation and HPLC.

### 5.2 Antimicrobial analyses

The present study has investigated the *in vitro* inhibitory activity of different compositions of CTs against two bacteria, APEC and *S. epidermidis*. This study has also provided some observations for what happened to these microbes that were treated with CT extracts. Scanning electron microscopy showed that CTs had a direct effect on bacteria as they disrupted the bacterial cells; a similar finding was reported previously by (Jones et al., 1994), who examined CTs on ruminal bacteria. The reaction of CTs on proteins and other compounds depended on the spatial configuration of the molecules and the availability of reactive phenolic groups. The size and structure of the CT extracts was important because the larger molecular weight allows for more stable cross-linking of different protein molecules (Scalbert, 1991). Conformational mobility and flexibility also influences the effectiveness of complexation, and it has been observed that CTs have the possibly to make a tannin-protein bond (Terrill, 1992). Thus, strong complexes with proteins are formed by free phenolic hydroxyl groups in the aromatic ring structure of CTs and protein groups of other compounds, particularly proteins. There is evidence for selective protein interaction as CTs have a higher chance of affinity for proteins with larger molecular weight, possibly due to the more open and flexible conformation of these compounds (Smith et al., 2005). Another point of interest is the similarity of responses of different pathogens to tannins extracted from chestnut wood (Maisak et al., 2013) which suggests that all Gram negative bacteria may be equally susceptible to tannins but this assumes that the complexation described above is similar for these two and other Gram-negative genera. This assumption needs testing as there may be different surface architectures dependent upon genera and also on their physiological state (e.g. stress response).

A complexation of metal ions by CTs also results in the inhibition by making these nutrients, many of which are essential, unavailable for bacteria by affecting the

electron transporter chains such as iron-sulphur proteins (Holloway et al., 2015). Akiyama et al. (2001) illustrated this in an *in vitro* antibacterial study that examined the antibacterial effects of several types of tannins such as tannic acid on *Staphylococcus aureus* and these authors also suggested continuing research into different tannin compositions for further investigation as a possible adjutant agent against different pathogens, especially infections that are currently treated with antibiotics.

The present study explored two CT-types (PC vs PD) in order to determine their MIC values against APEC and *S. epidermidis*. These CTs have shown their inhibition activity on these bacteria. Depending on the low or high molecular weight of the CT fractions, MBCs were recorded between 5 - 10 mg/ml ($P \geq 0.05$) for APEC, and between 1.25 - 5 mg/ml ($P \leq 0.05$) for *S. epidermidis*. This MBC difference between Gram negative and positive bacteria may indicate that the outer lipopolysaccharide membrane of Gram negative bacteria (e.g. APEC) may be capable of repelling the CT effects compared to Gram positive (e.g. *S. epidermidis*). The findings reported here matched those of Taguri et al. (2004) who evaluated the antibacterial activities of 10 different plant polyphenols, including CT extracts, by comparing their antibacterial effects against several food-borne pathogens such as various strains of *Staphylococcus aureus*, *Salmonella* and *E. coli*. Thus, Gram negative bacteria were less susceptible to CTs and this has been reported previously (Smith et al., 2007). The major finding to emerge from the present research was the ability of the lowest concentration, 0.06 mg/ml to promote the growth of APEC. However, this was only observed with CTs containing high molar PC percentages which enhanced the growth of APEC significantly ($P \leq 0.05$) in contrast to high and medium molar PC/PD percentages (Fig 3.3 - chapter 3). Sheng et al. (2016) reported a similar effect of a low concentration of grape seeds that contain tannins on different strains of *E. coli*. It remains to be seen whether this effect stems from a competition between the growth promoting effect of nutrients that are present as contaminants in plant extracts or CT fractions, and the growth inhibiting effect of the CTs or CT-bound by-products that are generated by the pathogens to prevent CT-adherence to the pathogens themselves. However, it could be shown that all high CT concentrations had a significant impact ($P \leq 0.05$) by inhibiting APEC growth. Further, CT

concentrations also played an important role on the outcome of these antibacterial tests. These results were consistent with the previous research, in which the difference in the source, these compositions of CTs may play a significant role in the antibacterial action (Tang et al., 2017).

The SEM study has also provided an observation into what happened to these microbes, APEC and *S. epidermidis,* which were treated with CT extracts by using the SEM technique (Jones et al., 1994). This technique can contribute to a further understanding of these described changes and the antimicrobial impacts (Carson et al., 2002). CT extracts showed the disruption on the cell walls of the bacteria compared to the controls of both, APEC and *S. epidermidis*, which showed a smooth surface on typical bacterial cells. As Darah et al. (2013) reported previously that antimicrobial agents such as flavonoids and tannins could attack the cell membrane and make morphological changes. However, low CT concentrations were unable to inhibit the growth of these bacteria compared to high concentrations of the same CT compositions. Nonetheless, low CT showed impacts on fission cells by preventing the cell division into new daughter cells. Therefore, this SEM study provided some insight into what happened to the bacterial cells when they were treated with different CT concentrations and how these treatments caused some distinct morphological and cytological alterations. In contrast, CTs could cause the apparent malformation of *S. epidermidis* (Gram-positive) to a greater extent than of APEC (Gram-negative). These differences can play an important part, as the cell wall can act as a barrier that prevents the penetration of foreign substances into the cells (Lewu et al., 2006).

This variation of antimicrobial activity may be attributed to their linking methods and their CT-type differences, reflected by PC/PD ratios. Therefore, high PC, med PC/PD and high PD of CTs have been chosen for further investigation as antimicrobial agents. In addition, the stimulation effect on the growth of APEC had also been reported and the tested concentration range for a given PC/PD contributed to this totally different action.

The results showed that high PD of CT exhibited a similar concentration effect on APEC. However, high PC of CT inhibited the growth of this pathogen at concentrations higher than 5 mg/ml, but enhanced their growth at concentrations

lower than 0.6 mg/ml. This antimicrobial activity of high PC with dose-manner on APEC may more accurately explain why PC increased the growth of APEC (Tang et al., 2017). Our study suggested that high doses of PC or PD could have a bactericidal effect on APEC. However, the lowest doses could not impact bacterial growth, as similar dose-dependent PC did before against some types of *E. coli*, which was also reported by (Freestone et al., 2007). Thus, the reported data suggested that the PC of CTs may stimulate the growth of bacteria by providing extra carbon sources (Mena et al., 2014), or being metabolised to the phenolic acids metabolites that increased the growth of *E. coli* (Tzounis et al., 2008).

Biofilms are microbial communities that adhere to surfaces and are an important development during the initiation of bacterial infections. Donlan and Costerton (2002) suggested that attachment and biofilm formation serve as a kind of defence mechanism for microbes to survive in harsh environments. As CTs are known to interact strongly with several different compounds (proteins, carbohydrates and lipids (Brown et al., 2017); i.e. CTs are very 'sticky' compounds, this thesis also explored whether CTs could prevent the attachment of APEC to surfaces. However, the findings showed that CTs significantly reduced APEC cell attachment especially at high concentrations (2.5 – 10 mg/ml). Nonetheless, the lowest concentrations of CT were less able to prevent biofilm formation, especially when low molecular weights CTs were used. There is, therefore, a possibility that these bacteria might become resistant to low molecular weight CTs in preference to high molecular weights CTs when exposed to low concentrations of low molecular weight CTs. However, future experiments will be needed to test whether this is the case and perhaps search for genes that might mutate and lead to resistance against low molecular weight CTs.

Apart from molecular weight, the molar proportion of PDs was also an important factor. Both low and high molecular weight CTs from black locust that contains a high PD percentage, showed a large effect on anti-biofilm formation. However, the same CTs exhibited particularly weak activity at low CT concentrations when compared to other CT extracts. This may indicate that low concentrations of CTs that are not inhibitory to APEC growth could actually contribute physically to increased binding and biofilm formation (Donlan and Costerton, 2002). This is, therefore, a new finding that has not been mentioned by previous studies.

Although, CT compositions are the main factors that influenced pathogens, other factors such as genetic diversity and environmental conditions could exhibit differences in their efficacy against pathogens (Theivendran et al., 2006). Consequently, these anti-biofilm formation effects of various CT compositions and concentrations showed a wide variety of bacterial sensitivity (APEC). This can explain why bacteria having extreme lack of nutrition or other external impacts that could influence their growth, may develop a biofilm formation as a survival strategy (Borges et al., 2012).

On the other hand, this study represents also the first research that investigated the inhibitory effects of CTs on APEC motility, swimming and swarming activities. These experiments demonstrated that APEC was highly resistant to PC, and PD had a much better effect as an antibacterial agent. Consistent with these findings, there could be a link between the motility results and anti-biofilm findings as CTs could act on the motile structures, such as flagella and pili, which in turn could influence the movement function by physically adhering to a surface (Pratt and Kolter, 1998). Interestingly, both CT types, i.e. PCs and PDs, showed a significant impact ($P \leq 0.05$) on swimming and on swarming properties and this is probably due to the fact that CTs are 'sticky', i.e. can bind to and precipitate several different compounds (O'May and Tufenkji, 2011). Therefore, these results could suggest that the anti-biofilm and anti-motility effects of these CT compositions can be caused by the CT binding to bacterial surfaces, especially pili and flagella.

In general, the hypothesis of this study was proved by showing several antimicrobial activities of these CT extracts on both bacteria. However, extractable CTs showed less antibacterial effect on APEC than *Staphylococcus epidermidis*. Further, the exposure to CTs in sub-lethal concentrations at 0.6 mg/ml resulted in less effect against Gram-negative (APEC), specifically with evidence of growth enhancement that showed when treated by high PC. Therefore, prodelphinidins of CT is the most important descriptor of CT characteristics in terms of antimicrobial agents.

### 5.3 *In vitro* analyses of fermentation and metabolism

A fermentation and metabolism study was conducted with two *in vitro* experiments. They investigated the possible effects of CT compositions on the metabolic end

products of either a caecal chicken content or an avian pathogenic *E. coli* (APEC) culture in an *in vitro* fermentation system. A simple batch culture experiment with fermented caecal contents was conducted. This model served as a versatile tool to generate the most important metabolic products *in vitro* in the presence of CT under anaerobic (caecal contents) and aerobic (APEC culture) conditions. The $^{1}$H-NMR technique was used to investigate the metabolism and degradation process of some important metabolites such as amino acids, fatty acids and carbohydrates.

The results of these experiments were illustrated in various tables in chapter 4 and represent different time-points. The metabolic variability of the principal component analysis (PCA) and orthogonal partial least squares (OPLS) for treatments and control were also demonstrated.

The findings of these experiments can serve as a useful database of metabolic products in the chicken caecum in the presence of different CT compositions. The identification of these products can help to provide an understanding of the mechanistic basis of CT effects on the chicken gut.

### 5.3.1 NMR analysis of caecal contents

The concentrations of most amino acids, especially glutamate, leucine, lysine, pyroglutamate, phenylalanine, proline and sarcosine decreased during incubation suggesting that they were being utilised. Glutamate can be metabolised to pyruvate by glutamic pyruvate transaminase (GPT), and then to some short chain fatty acids such as acetate, propionate and butyrate as well as other molecules e.g. ammonia. This amino acid may also present evidence for transamination of some amino acids into pyruvate which will be fermented by gut microbes into glucose by gluconeogenesis (Meister, 1957). Phenylalanine was produced during the incubation, possibly from the bacterial degradation of aromatic amino acids microbial metabolism of phenylalanine producing derivatives (Windey et al., 2012). Gut bacteria such as *Bacteroides* spp. and *Lactobacilli* spp. could get involved in this processing (Fuchs et al., 2014). This observation suggested that the addition of CT changed the metabolic processes of amino acids, leading to the production of metabolic energy (ATP) from the oxidation in the citric acid cycle (Nutrition, 1971). Further, branched chain amino acids, such as leucine, isoleucine and valine can undergo oxidative transamination at an appreciable rate by aminotransferase,

which is responsible for the biosynthesis of these amino acids (Amorim Franco and Blanchard, 2017). While, fatty acids such as acetate, butyrate and propionate showed significant increase (P≤0.05) in CT treatments compared to control at the interaction between the time-point and treatments. Nonetheless, these fatty acids possibly broke down and were fermented by the active caecal microbes (Neis et al., 2015). Further, propionate was found significantly more in control cultures at the time 24 h rather than CT treatments, although by the time 48h the amount of propionate was increased slightly but not significantly in the CT treatments. To produce propionate and butyrate, there are various pathways; it is mostly formed through the succinate and/or lactate and acetate pathways (Salonen et al., 2014; Louis et al., 2014). Therefore, the relative abundance of propionate was probably associated with some microbes among normal gut microbes. This result indicates that CTs could impact the lactic acid producing by microflora and interrupting the tricarboxylic acid cycle (TCA) as mentioned previously (Tzonis, 2008). Lactic acid has an impact on pH as it is the strongest fatty acid produced by gut microbes. This acid could be utilised as a substrate for lactate-utilising bacteria (Rinttila and Apajalahti, 2013).

Additionally, CTs had a slight effect on carbohydrates such as mannitol, glucose, fructose, lactose and galactose during the timed periods by fluctuations on their concentrations at 10h and 24h. Carbohydrate concentration initially increased, but then decreased as they were utilised. The microflora in the caecum content could digest enzymatically these kinds of sugars and provide a source of energy by glycolysis (Niba et al., 2009).

### 5.3.2 NMR analysis of APEC culture

This is the first study, to our knowledge, that investigated the effect of CT compositions on APEC culture using $^{1}$H-NMR spectroscopy at different time-points. Therefore, this experiment provided new database information, which probably will be used for more investigation on the effect of these CT on other gut pathogens, probably.

In table 4.2, the $^{1}$H-NMR results of the interaction between CT treatment and time on the concentration of different metabolites were presented. Some of these findings demonstrated significant differences (P≤0.05) of the interaction between

treatments and time-points on their metabolites in the presence or absence of CTs. The most important results were recorded by amino acid concentrations such as lysine, leucine, glutamate, phenylalanine and pyroglutamate, which showed significant differences ($P \leq 0.05$) between the treatments. However, lysine, glutamate, phenylalanine and pyroglutamate interacted significantly on time-point and these treatments when APEC was incubated with CTs. These results argued that CT compositions have the ability to make a complex with other compounds, particularly with proteins (Terrill et al., 1992; McSweeney et al., 2001). Nonetheless, PC measured a close result to controls compared to PD. Therefore, further investigation needs to determine the effects of CTs on the fermentation of these amino acids.

In contrast, fatty acids, particularly butyrate, iso-butyrate, valerate and iso-valerate were increased in high PD treatment compared to high PC and controls. These results did not show a statistical difference ($P > 0.05$) between the interaction of times and treatments, but they showed a significant difference ($P \leq 0.05$) at different time-points. This could explain how these CT compositions, particularly high PD%, could interrupt the fermentation processing of the TCA cycle and lead to the resultant accumulation of fatty acids (Kotze et al., 1969). This finding also demonstrated that a high PD treatment can influence the biological changing on mixed acid fermentation by decreasing lactate, which should normally be increased as showed by treated high PC and controls. High PD treatment showed a significant difference in acetate concentration compared with the high PC and controls, while the concentration of lactate was lower with PD compared with PC and control. This finding indicated that surviving APEC in both PC and controls could ferment the fatty acids by normal mixed acid pathways. CTs could influence the mixed acid fermentation by changing concentrations of acetate and lactate. All these variations of concentrations in fatty acids and amino acids could be associated with changed energy production in APEC culture in the presence of CTs (Tzonis, 2008).

In addition, other metabolites such as carbohydrates and pyruvate did not demonstrate significant differences ($P > 0.05$) between the treatments. Collectively the findings from this thesis, reveal that the response of bacteria exposed to high PD of CTs is a slowing of growth that may be associated with a shift from

respiration to fermentation as indicated by the accumulation of short chain fatty acids. Further, Lactate was already present in all the samples tested but it showed a high level of both PC and controls after 24h. This is an evidence of a switch from respiration to fermentation as part of the strategy of APEC to survive (Forster and Gescher, 2014).

The most important finding of this research is that demonstrated by the similarity in the metabolites of controls (APEC) and treated by high PC, whereas there was a significant impact on APEC metabolism by high PD of CT. This may indicate that either PC could be rapidly metabolised by surviving APEC, as Chapter 3 showed that a high PC group had a less inhibitory effect on APEC compared with one with a high PD content, or the number of surviving bacteria was more when in PC treated compared to those PD treated.

In conclusion, these results confirmed that using different CT compositions could affect the metabolic end products of APEC at different time-points. Further, $^{1}$H-NMR spectroscopy provided good monitoring to determine these metabolic end products during fermentation by APEC (Jacobs et al., 2008). Consequently, high PD can reduce the growth of APEC. These findings could provide insights for host responses to the pathogens that may lead to improvement in the gut health of chickens. More investigation is needed to determine how these CTs will interact with other gut pathogens.

As a result of these experiments (1 and 2), the hypothesis of this research was proved that CT compositions, PC vs PD, have no potential impact on the metabolites of caecal contents, i.e. both CT compositions showed a similar effect compared to controls. However, these compositions have different effects on the important metabolic end products of APEC culture. Therefore, these observations supported the hypothesis that CT compositions may or may not have impacts on the metabolism from the chicken gut by affecting the important end products and the biochemical pathways.

### 5.4 Future perspectives

Future research could extend these studies to investigate the effects of condensed tannins (CTs) from different plants and herbs that can be planted in Middle East

regions, especially Iraq. In *in vivo* experiments will be needed to assess their effects in terms of antioxidant status, immune response and gastrointestinal parasites (e.g. coccidian parasites) in chicken health and performance or other mono-gastric animals such as rabbits.

The present study has indicated that CT extracts may have potential for the development of alternatives to synthetic antibiotics; these alternatives could be used as growth promoter agents. Feeding trials will be needed to ensure that such additives do not impact negatively on animal productivity. Future studies will also need to probe the mechanisms of action of these alternatives, which could induce changes in gut microflora. Furthermore, these studies will need to assess also the potential for resistance development amongst gut pathogens to CTs and how these alternative treatments differ from antibiotics.

Another aspect, several tannin-containing plants, which are considered to possess biological activities, has been used alone as bioactive compounds. However, our future work will focus to synergistically boost this bioactivity of some tannin compositions.

Therefore, the purpose of these future studies will be to determine the benefits of CT extracts with specific concentrations as beneficial feed additives on chicken diets, in terms of feed intake, growth performance, immunology, biochemical parameters, and carcass yields.

### 5.5 Conclusions

In conclusion, these studies elucidated some of the relationships between the chemical compositions of CTs and their *in vitro* effects on the fermentation and metabolism of selected bacteria. Use of different CT-types clearly indicated that PD was more effective than PC at reducing bacterial activities, such as growth, biofilm formations and motility. Further these CT compositions, PC/PD, showed significant effects on the minimum inhibitory concentration of two contrasting bacterial species, APEC and *S. epidermidis*. Scanning electron microscopy also illustrated the effect that CT, particularly high PD of CT, had on the Gram-positive *S. epidermidis* and how this was greater than with the Gram-negative APEC. This

study concluded that CTs can inhibit both bacterial strains but with different concentrations (1.25 mg/ml for *S. epidermidis*, but 10 mg/ml for APEC).

Conversely, these CTs also had significant effects on the production and utilisation of metabolites in an *in vitro* fermentation using caecal contents extracted from chickens. NMR spectroscopy revealed that CTs affected amino acid and carbohydrate concentrations in this caecal model, and that CT composition had little effect on these changes. In contrast, fatty acids were less affected by CTs. On the other hand, high PC of CTs had little effect on fermentation by APEC culture, whereas high PC of CT maintained its inhibitory effects.

Although CTs have a marked *in vitro* impact on microbial growth and fermentation, it is necessary to confirm these findings in an *in vivo* trial. These results will further our understanding as to how CTs may be useful additives for poultry diets in terms of antimicrobial activity and enhanced nutritional quality of poultry diets.

**5.6 References:**


Akiyama, H., Fujii, K., Yamasaki, O., Oono, T., and Iwatsuki, K. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *J Antimicrob Chemother* 48, 487-491.

Amorim Franco, T. M., and Blanchard, J. S. (2017). Bacterial Branched-Chain Amino Acid Biosynthesis: Structures, Mechanisms, and Drugability. *Biochemistry* 56, 5849-5865.

Borges, A., Saavedra, M. J., and Simoes, M. (2012). The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling* 28, 755-67.

Brown, R. H., Mueller-Harvey, I., Zeller, W. E., Reinhardt, L., Stringano, E., Gea, A., Drake, C., Ropiak, H. M., Fryganas, C., Ramsay, A., and Hardcastle, E. E. (2017). Facile Purification of Milligram to Gram Quantities of Condensed Tannins According to Mean Degree of Polymerisation and Flavan-3-ol Subunit Composition. *J Agric Food Chem*.

Carson, C. F., Mee, B. J., and Riley, T. V. (2002). Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by

time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob Agents Chemother* 46, 1914-20.

Darah, I., Lim, S. H., and Nithianantham, K. (2013). Effects of Methanol Extract of *Wedelia chinensis* Osbeck (*Asteraceae*) Leaves against Pathogenic Bacteria with Emphasise on *Bacillus cereus*. *Indian J Pharm Sci* 75, 533-9.

Donlan, R. M., and Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15, 167-93.

Forster, A. H., and Gescher, J. (2014). Metabolic Engineering of *Escherichia coli* for Production of Mixed-Acid Fermentation End Products. *Front Bioeng Biotechnol* 2, 16.

Freestone, P. P., Walton, N. J., Haigh, R. D., and Lyte, M. (2007). Influence of dietary catechols on the growth of enteropathogenic bacteria. *Int J Food Microbiol* 119, 159-69.

Fuchs, S., Behrends, V., Bundy, J. G., Crisanti, A., and Nolan, T. (2014). Phenylalanine metabolism regulates reproduction and parasite melanisation in the malaria mosquito. *PLOS ONE* 9, e84865.

Gea, A., Stringano, E., Brown, R. H., and Mueller-Harvey, I. (2011). *In situ* analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high-throughput germplasm screening. *J Agric Food Chem* 59, 495-503.

Grabber, J. H., Zeller, W. E., and Mueller-Harvey, I. (2013). Acetone Enhances the Direct Analysis of Procyanidin- and Prodelphinidin-Based Condensed Tannins in Lotus Species by the Butanol-HCl-Iron Assay. *J. Agric. Food Chem.* 61, 2669-2678.

Holloway, A., Mueller-Harvey, I., Gould, S., Fielder, M., Naughton, D., and Kelly, A. (2015). Heat treatment enhances the antimicrobial activity of (+) Catechin when combined with copper sulphate. *Lett Appl Microbiol* 61, 381-389.

Jacobs, D. M., Deltimple, N., van Velzen, E., van Dorsten, F. A., Bingham, M., Vaughan, E. E., and van Duynhoven, J. (2008). $^{1}$H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR in Biomedicine* 21, 615-626.

Jones, G. A., McAllister, T. A., Muir, A. D., and Cheng, K. J. (1994). Effects of Sainfoin (*Onobrychis viciifolia* Scop.) Condensed Tannins on Growth and Proteolysis by Four Strains of Ruminal Bacteria. *Appl Environ Microbiol* 60, 1374-8.

Karonen, M., Ossipov, V., Sinkkonen, J., Loponen, J., Haukioja, E., and Pihlaja, K. (2006). Quantitative analysis of polymeric proanthocyanidins in birch leaves with normal-phase HPLC. *Phytochem Anal* 17, 149-56.

Klongsiriwet, C., Quijada, J., Williams, A. R., Mueller-Harvey, I., Williamson, E. M., and Hoste, H. (2015). Synergistic inhibition of *Haemonchus contortus* exsheathment by flavonoid monomers and condensed tannins. *Int J Parasitol Drugs Drug Resist* 5, 127-34.

Kotze, J., Thiel, P., and Hattingh, W. (1969). Anaerobic digestion II. The characterization and control of anaerobic digestion. *Water Research* 3, 459-494.

Lewu, F. B., Grierson, D. S., and Afolayan, A. J. (2006). Extracts from *Pelargonium sidoides* inhibit the growth of bacteria and fungi. *Pharm. biol.* 44, 279-282.

Louis, P., Hold, G. L., and Flint, H. J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. *Nat Rev Microbiol* 12, 661-72.

Maisak, H., Jantrakajorn, S., Lukkana, M., and Wongtavatchai, J. (2013). Antibacterial Activity of Tannin from Sweet Chestnut Wood Against *Aeromonas* and *Streptococcal* Pathogens of Tilapia (*Oreochromis niloticus*). *Thai J Vet Med* 43, 105-111.

McSweeney, C. S., Palmer, B., McNeill, D. M., and Krause, D. O. (2001). Microbial interactions with tannins: nutritional consequences for ruminants. *Anim Feed Sci Technol* 91, 83-93.

Meister, A. (1957). "Biochemistry of the amino acids." *New York: Academic Press Inc.*, xiii + 485 pp.

Mena, P., Calani, L., Bruni, R., and Del Rio, D. (2014). Bioactivation of high-molecular-weight polyphenols by the gut microbiome. *In* "Diet-Microbe Interactions in the Gut", pp. 73-101. *Elsevier*.

Mueller-Harvey, I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.* 86, 2010-2037.

Neis, E. P., Dejong, C. H., and Rensen, S. S. (2015). The role of microbial amino acid metabolism in host metabolism. *Nutrients* 7, 2930-46.

Niba, A. T., Beal, J. D., Kudi, A. C., and Brooks, P. H. (2009). Bacterial fermentation in the gastrointestinal tract of non-ruminants: Influence of fermented feeds and fermentable carbohydrates. *Trop Anim Health Prod* 41, 1393-1407.

Nutrition, N. R. C. C. o. A. (1971). "Nutrient requirements of poultry," Sixth revised edition, *National Academy of Sciences*, Washington, D.C.

O'May, C., and Tufenkji, N. (2011). The Swarming Motility of *Pseudomonas aeruginosa* Is Blocked by Cranberry Proanthocyanidins and Other Tannin-Containing Materials. *Appl. Environ. Microbiol.* 77, 3061-3067.

Porter, L. J., Hrstich, L. N., and Chan, B. G. (1985). The Conversion of Procyanidins and Prodelphinidins to Cyanidin and Delphinidin. *Phytochemistry* 25, 223-230.

Pratt, L. A., and Kolter, R. (1998). Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30, 285-93.

Rinttila, T., and Apajalahti, J. (2013). Intestinal microbiota and metabolites--Implications for broiler chicken health and performance1. *J Appl Poult Res* 22, 647-658.

Ropiak, H. M., Ramsay, A., and Mueller-Harvey, I. (2016). Condensed tannins in extracts from European medicinal plants and herbal products. *J Pharm Biomed Anal* 121, 225-231.

Salonen, A., Lahti, L., Salojarvi, J., Holtrop, G., Korpela, K., Duncan, S. H., Date, P., Farquharson, F., Johnstone, A. M., Lobley, G. E., Louis, P., Flint, H. J., and de Vos, W. M. (2014). Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *Isme Journal* 8, 2218-30.

Scalbert, A. (1991). Antimicrobial Properties of Tannins. *Phytochemistry* 30, 3875-3883.

Schofield, P., Mbugua, D. M., and Pell, A. N. (2001). Analysis of condensed tannins: a review. *Anim Feed Sci Technol* 91, 21-40.

Sheng, L., Olsen, S. A., Hu, J., Yue, W., Means, W. J., and Zhu, M. J. (2016). Inhibitory effects of grape seed extract on growth, quorum sensing, and virulence factors of CDC "top-six" non-O157 Shiga toxin producing *E. coli. Int J Food Microbiol* 229, 24-32.

Sivakumaran, S., Rumball, W., Lane, G. A., Fraser, K., Foo, L. Y., Yu, M., and Meagher, L. P. (2006). Variation of proanthocyanidins in *Lotus species*. *J Chem Ecol* 32, 1797-816.

Smith, A. H., Zoetendal, E., and Mackie, R. I. (2005). Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microb Ecol* 50, 197-205.

Smith, J. L., Drum, D. J., Dai, Y., Kim, J. M., Sanchez, S., Maurer, J. J., Hofacre, C. L., and Lee, M. D. (2007). Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichia coli* strains colonising broiler chickens. *Appl Environ Microbiol* 73, 1404-14.

Taguri, T., Tanaka, T., and Kouno, I. (2004). Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biol Pharm Bull* 27, 1965-9.

Tang, C., Xie, B. J., and Sun, Z. D. (2017). Antibacterial activity and mechanism of B-type oligomeric procyanidins from lotus seedpod on enterotoxigenic *Escherichia coli*. *J Funct Foods* 38, 454-463.

Terrill, T. H., Rowan, A. M., Douglas, G. B., and Barry, T. N. (1992). Determination of Extractable and Bound Condensed Tannin Concentrations in Forage Plants, Protein-Concentrate Meals and Cereal-Grains. *J. Agric. Food Chem.* 58, 321-329.

Theivendran, S., Hettiarachchy, N. S., and Johnson, M. G. (2006). Inhibition of *Listeria monocytogenes* by nisin combined with grape seed extract or green tea extract in soy protein film coated on turkey frankfurters. *J Food Sci.* 71, M39-M44.

Tjahjani, S. (2017). Antimalarial activity of *Garcinia mangostana* L rind and its synergistic effect with artemisinin *in vitro*. *BMC Complement Altern Med* 17, 131.

Tzounis, X., Vulevic, J., Kuhnle, G. G., George, T., Leonczak, J., Gibson, G. R., Kwik-Uribe, C., and Spencer, J. P. (2008). Flavanol monomer-induced changes to the human faecal microflora. *Br J Nutr* 99, 782-92.

Williams, A. R., Ropiak, H. M., Fryganas, C., Desrues, O., Mueller-Harvey, I., and Thamsborg, S. M. (2014). Assessment of the anthelmintic activity of medicinal plant extracts and purified condensed tannins against free-living and parasitic stages of *Oesophagostomum dentatum*. *Parasit Vectors* 7, 518.

Windey, K., De Preter, V., and Verbeke, K. (2012). Relevance of protein fermentation to gut health. *Mol Nutr Food Res* 56, 184-96.

# Appendix 1: Further information for Chapter 2

**Table A:** Botanical, family and common names of plant materials that were screened by ABA methods

| No | Botanical name | Family name | Common name |
|---|---|---|---|
| 1 | *Abies nordmannia* | *Pinaceae* | Nordmann fir or Caucasian fir |
| 2 | *Acanthus spinosus* | *Acanthaceae* | Spiny bear's breeches |
| 3 | *Acer platnoides* | *Sapindaceae* | Norway maple |
| 4 | *Achillea millefolium* | *Asteraceae* | Yarrow |
| 5 | *Actinidia deliciosa* | *Actinidiaceae* | Kiwi fruit |
| 6 | *Aesculus hippocastanum* | *Sapindaceae* | Horse-chestnut or conker tree |
| 7 | *Aesculus hippocastanum fr.* | *Sapindaceae* | Horse-chestnut or conker tree |
| 9 | *Agastache foeniculum* | *Lamiaceae* | Anise hyssop, blue giant hyssop, fragrant giant hyssop |
| 10 | *Agropyron repens* | *Poaceae* | Elymus repens or couch grass |
| 11 | *Ajuga reptans* | *Lamiaceae* | Bugle, blue bugle, bugle herb, bugleweed and carpetweed |
| 12 | *Alchemilla alpina* | *Rosaceae* | Alpine lady's-mantle |
| 13 | *Alchemilla vulgaris L.* | *Rosaceae* | Lady's mantle |
| 14 | *Alliaria petiolate* | *Brassicaceae* | Garlic mustard, garlic root, hedge garlic, sauce-alone, jack-in-the-bush, penny hedge and poor man's mustard |
| 15 | *Allium Cepa* | *Amaryllidaceae* | Onion or bulb onion |
| 16 | *Allium porum* | *Amaryllidaceae* | Leek |
| 17 | *Alnus glutinosa* | *Betulaceae* | Alder, black alder and European alder |
| 18 | *Amelanchier canadensis* | *Rosaceae* | Canadian serviceberry, chuckle berry, currant-tree, June berry, shadblow |

| | | | |
|---|---|---|---|
| | | | serviceberry, shadblow, sugarplum or thicket serviceberry |
| 20 | *Amoracia rusticana* | *Brassicaceae* | Horseradish |
| 21 | *Anemone hybrida* | *Ranunculaceae* | Chinese anemone, Japanese anemone, thimbleweed and windflower |
| 22 | *Anthemis cotula* | *Asteraceae* | Stinking chamomile |
| 23 | *Anthemis nobilis Or Chamaemelum nobile* | *Asteraceae* | Chamomile or camomile, Roman chamomile, English chamomile, garden chamomile, ground apple, low chamomile and whig plant |
| 24 | *Aquilegia vulgaris* | *Ranunculaceae* | European columbine, Common columbine, Granny's nightcap, Granny's bonnet |
| 25 | *Arbutus unedo* | *Ericaceae* | Strawberry tree |
| 26 | *Arctium minus* | *Asteraceae* | Lesser burdock, burweed, louse-bur, common burdock, button-bur, cuckoo-button, and wild rhubarb |
| 27 | *Arctostaphylos uva-ursi* | *Ericaceae* | Bearberry |
| 28 | *Aristolochia durior* | *Aristolochiaceae* | Dutchman's pipe or pipevine |
| 29 | *Artemisia abrotanum* | *Asteraceae* | Old man, boy's love and wormwood |
| 30 | *Artemisia absinthium* | *Asteraceae* | Absinthium, absinthe wormwood, wormwood, green ginger |
| 31 | *Arum maculatum* | *Araceae* | Snakeshead, adder's root, arum, wild arum, arum lily, lords-and-ladies, devils and angels or cows and bulls |
| 32 | *Asparagus officinalis* | *Asparagaceae* | Asparagus or garden asparagus |
| 33 | *Asparagus* | *Asparagaceae* | Asparagus fern, Lace fern and |

| | | | |
|---|---|---|---|
| | *plumosus* | | Climbing asparagus |
| 34 | *Aster frikartii* | *Hybrid* | Aster |
| 35 | *Astragali radix* | *Fabaceae* | Astragalus, milkvetch, locoweed and goat's-thorn |
| 36 | *Astrantia major L.* | *Apiaceae* | Great masterwort |
| 37 | *Athyrium filix-femina* | *Athyriaceae* | Lady fern and female fern |
| 38 | *Atriplex species* | *Amaranthaceae* | Saltbush and orache |
| 39 | *Ballota nigra L.* | *Lamiaceae* | Black horehound |
| 40 | *Berberis valdiviana* | *Berberidaceae* | clen or espina en cruz |
| 41 | *Bergenia crassifolia* | *Saxifragaceae* | Badan, Siberian tea, Mongolian tea, leather bergenia, winter-blooming bergenia, and elephant-ears |
| 42 | *Beta vulgaris altissima* | *Amaranthaceae* | Sugar beet |
| 43 | *Beta vulgaris crassa* | *Amaranthaceae* | Beet |
| 44 | *Betonica (=Stachys) officinalis* | *Lamiaceae* | Betony, purple betony, wood betony, bishopwort or bishop's wort |
| 45 | *Betula pendula* Roth | *Betulaceae* | Silver birch or warty birch |
| 46 | *Borago officinalis* | *Boraginaceae* | Borage or starflower |
| 47 | *Buxus sempervirens* | *Buxaceae* | Common box, European box and boxwood |
| 48 | *Calamintha clinopodium* | *Lamiaceae* | Calamints |
| 49 | *Calendula arvensis L.* | *Asteraceae* | Field marigold |
| 50 | *Calendula officinalis L.* | *Asteraceae* | Pot marigold, ruddles or garden marigold |
| 51 | *Caltha palustris* | *Ranunculaceae* | Kingcup or Marsh marigold |

| | | | |
|---|---|---|---|
| 52 | *Camellia sinensis* | *Theaceae* | Tea plant |
| 53 | *Capsicum sp.* | *Solanaceae* | Pepper or bell pepper |
| 54 | *Carthamus tinctorius* L. | *Asteraceae* | Safflower |
| 55 | *Carum carvi* | *Apiaceae* | Caraway, meridian fennel and Persian cumin |
| 56 | *Cassia tora* | | Sickle senna, sickle pod, tora, coffee pod, tovara, chakvad or foetid cassia |
| 57 | *Castanea sativa* fr. | *Fagaceae* | Sweet chestnut |
| 58 | *Catalpa speciosa* (Warder ex.) | *Bignoniaceae* | Catalpa, cigar tree and catawba-tree |
| 59 | *Chelidonium majus L.* | *Papaveraceae* | Greater celandine or tetterwort |
| 61 | *Chenopodium bonus henricus* **Or** *Blitum bonus-henricus* | *Amaranthaceae* | Good-King-Henry, poor-man's asparagus and perennial goosefoot |
| 62 | *Cichorium intybus* | *Asteraceae* | Chicory |
| 63 | *Cimicifuga racemosa* **Or** *Actaea racemosa* | *Ranunculaceae* | Black cohosh, black bugbane, black snakeroot and fairy candle |
| 64 | *Cinnamonum cassia* | *Lauraceae* | Chinese cassia or cinnamon |
| 65 | *Cirsium arvense* | *Asteraceae* | Creeping thistle |
| 66 | *Cistus laurifolius* | *Cistaceae* | Laurel-leaf cistus or laurel-leaved rock rose |
| 67 | *Citrus reticulate* | *Rutaceae* | Mandarin orange |
| 68 | *Clematis vitalba* L. | *Ranunculaceae* | Old man's beard and traveller's joy |
| 69 | *Convolvulus* | *Convolvulaceae* | Field bindweed |

| | | | |
|---|---|---|---|
| | *arvensis* L. | | |
| 70 | *Coptidis rhizome* | --- | Goldthread |
| 71 | *Corataegus cuneata* | *Rosaceae* | May, mayblossom, maythorn, quickthorn, whitethorn, motherdie or haw |
| 72 | *Coriandrum sativum* | *Apiaceae* | Cilantro, Chinese parsley and dhania |
| 74 | *Cornus sanguinea* | *Cornaceae* | Dogwood |
| 75 | *Corylus avellana* | *Betulaceae or Corylaceae* | hazelnut |
| 76 | *Crataegus monogyna* | *Rosaceae* | Hawthorn, thornapple, May-tree, whitethorn and hawberry |
| 77 | *Cucumis melo* | *Cucurbitaceae* | Muskmelon |
| 78 | *Cucurbita pepo* | *Cucurbitaceae* | Pumpkin |
| 79 | *Cuminum cyminum* | *Apiaceae* | Cumin or zeera |
| 80 | *Cynara scolymus* | *Asteraceae* | Globe artichoke |
| 81 | *Datisca cannabina L.* | *Datiscaceae* | Acalbir or Akalbir |
| 84 | *Dianthus caryophyllus L.* | *Caryophyllaceae* | Carnation or clove pink |
| 86 | *Dryopteris filix-mas* | *Dryopteridaceae* | Male fern |
| 87 | *Echinacea purpurea (MOENCH)* | *Asteraceae* | Eastern purple coneflower, Hedgehog coneflower or Purple coneflower |
| 88 | *Echium vulgare* L. | *Boraginaceae* | Viper's Bugloss or Blueweed |
| 89 | *Epilobium hirsutum* | *Onagraceae* | Codlins-and-cream, apple-pie and cherry-pie |
| 90 | *Epimedium brevicornum* | *Berberidaceae* | Barrenwort, bishop's hat, fairy wings, horny goat weed, rowdy lamb herb or |

| | | | |
|---|---|---|---|
| | | | randy beef grass |
| 91 | *Equisetum pratense* | *Equisetophyta* | Meadow horsetail, shade horsetail or shady horsetail |
| 92 | *Eucommia ulmoides* | *Eucommiaceae* | Rubber bark tree |
| 93 | *Eugenia caryophyllata* | *Myrtaceae* | Cloves |
| 94 | *Euonymus alatus* | *Celastraceae* | winged spindle, winged euonymus and burning bush |
| 95 | *Euonymus europaeus* | *Celastraceae* | Spindle, European spindle and common spindle |
| 96 | *Eupatorium cannabinum* | *Asteraceae* | Hemp-agrimony |
| 97 | *Eupatorium cannabinum* L. | *Asteraceae* | Hemp-agrimony |
| 98 | *Euphorbia cyparissias* | *Euphorbiaceae* | Cypress spurge |
| 99 | *Euphorbia lathyris* | *Euphorbiaceae* | Caper spurge, paper spurge or gopher spurge |
| 100 | *Fagopyrum esculentum* | *Polygonaceae* | Japanese buckwheat and silverhull buckwheat |
| 101 | *Fagus purpurea* | *Fagaceae* | Copper Beech |
| 102 | *Fagus sylvatica* | *Fagaceae* | European beech |
| 103 | *Filipendula ulmaria* | *Rosaceae* | Meadowsweet or mead wort |
| 104 | *Filipendula vulgaris* | *Rosaceae* | Dropwort or Fern-leaf Dropwort |
| 105 | *Foenum graecum* | *Fabaceae* | Fenugreek |
| 106 | *Forsythia intermedia* | *Forsythia (Genus)* | Border Forsythia |
| 107 | *Fraxinus excelsior* | *Oleaceae* | Ash or European ash |
| 108 | *Fructus forsythiae* | | Chinese herb |

| | | | |
|---|---|---|---|
| 109 | *Fructus forsythiae* | *Oleaceae* | Forsythia |
| 110 | *Gaultheria procumbens* | *Ericaceae* | Eastern teaberry, checkerberry, boxberry, or american wintergreen |
| 111 | *Genista germanica* L. | *Fabaceae* | German greenweed |
| 112 | *Gentiana asclepiadea* | *Gentianaceae* | Willow gentian |
| 113 | *Geum urbanum* **Or** *herba benedicta* | *Rosaceae* | Wood avens, herb bennet, colewort and St. Benedict's herb |
| 114 | *Ginkgo biloba* | *Ginkgoaceae* | Maidenhair tree |
| 115 | *Gleditsia japonica* | *Fabaceae* | Gleditsia or locust |
| 116 | *Glyceria maxima* | *Poaceae* | Great manna grass, Reed mannagrass and Reed sweet-grass |
| 117 | *Glycyrrhizae radix uralensis* | *Fabaceae* | Chinese liquorice |
| 118 | *Hedera helix* | *Araliaceae* | Ivy and English ivy or European ivy |
| 119 | *Helianthus annuus* | *Asteraceae* | Sunflower |
| 120 | *Helianthus tuberosus* L. | *Asteraceae* | The Jerusalem artichoke, sunroot, sunchoke, earth apple or topinambour |
| 121 | *Helichrysum Triplinervis* | *Asteraceae* | N/A |
| 122 | *Helleborus orientalis* **Or** *Helleborus viridis* | *Ranunculaceae* | Green hellebore |
| 123 | *Heracleum sphondylium* | *Apiaceae* | Hogweed or common hogweed |
| 124 | *Houttuynia cordata* | *Saururaceae* | Lizard tail, chameleon plant, heartleaf, fishwort and bishop's weed |
| 125 | *Hydrangea* | *Hydrangeacae* | Climbing hydrangea |

| | | | |
|---|---|---|---|
| | *petiolaris*<br>**Or**<br>*Hydrangea anomala subsp. Petiolaris* | | |
| 126 | *Hydrastis Canadensis* | *Ranunculaceae* | Goldenseal, orangeroot or yellow puccoon |
| 127 | *Hypericum perforatum* L. | *Hypericaceae* | Common St John's wort |
| 128 | *Hyssopus officinalis* | *Lamiaceae* | Hyssop |
| 129 | *Impatiens parviflora Dc.* | *Balsaminaceae* | Small balsam or small-flowered touch-me-not |
| 130 | *Indigofera tinctoria L.* | *Fabaceae* | True indigo |
| 131 | *Inula magnifica* | *Asteraceae* | Inula |
| 132 | *Iris germanica* L. | *Iridaceae* | German Iris |
| 133 | *Jasminum grandiflorum* | *Oleaceae* | Spanish jasmine, royal jasmine and Catalonian jasmin. |
| 134 | *Juglans nigra* | *Juglandaceae* | Eastern black walnut |
| 135 | *Juglans regia* L. | *Juglandaceae* | Walnut or Persian walnut, English walnut |
| 136 | *Juniperus communis* | *Cupressaceae* | Juniper |
| 137 | *Lactuca serriola* | *Asteraceae* | Prickly lettuce, milk thistle, compass plant or scarole |
| 138 | *Lactuca virosa* | *Asteraceae* | Wild lettuce, bitter lettuce, laitue vireuse, opium lettuce and tall lettuce |
| 139 | *Lavandula angustifolia* (MILL.) | *Lamiaceae* | Lavender, true lavender and narrow-leaved lavender |
| 140 | *Lavatera* | *Malvaceae* | Lavatera or malva |

| | | | |
|---|---|---|---|
| | *clementii* | | |
| 141 | *Levisticum officinale* | *Apiaceae* | Lovage |
| 142 | *Lewisia rediviva* | *Portulacaceae* | Bitterroot |
| 143 | *Ligustrum vulgare* L. | *Oleaceae* | Wild privet |
| 144 | *Linaria vulgaris MILL.* | *Scrophulariaceae* | Toadflax, Yellow Toadflax, or Butter-and-eggs |
| 145 | *Liriodendron tulipifera* L. | *Magnoliaceae* | Tulip tree, American tulip tree, tulip poplar, whitewood, fiddle-tree or yellow poplar |
| 146 | *Lonicera japonica* | | Japanese honeysuckle |
| 147 | *Lonicera periclymenum* | *Caprifoliaceae* | Honeysuckle European honeysuckle or woodbine |
| 148 | *Lotus corniculatus L.* | *Fabaceae* | Bird's foot trefoil |
| 149 | *Lycopersicum esculentum* | *Solanaceae* | Tomato |
| 150 | *Lycopus europaeus L.* | *Lamiaceae* | Gypsywort, gipsywort, bugleweed and water horehound |
| 151 | *Lythrum salicria L.* | *Lythraceae* | Purple loosestrife or purple lythrum |
| 152 | *Macleaya cordata* | *Papaveraceae* | *Bocconia cordata* willd |
| 153 | *Magnolia officinalis* | *Magnoliaceae* | Houpu Magnolia or Magnolia-bark |
| 154 | *Mahonia aquifolium* | *Berberidaceae* | Oregon grape |
| 155 | *Malus sylvestris* | *Rosaceae* | Forest apple, apple fruit and European crab apple |
| 156 | *Malva molucco* | *Malvaceae* | N/A |
| 157 | *Massa fermentata* | *(mixture of many Chinese herbs)* | Shen Qu, Chinese name, or medicated leaven |
| 158 | *Medicago* | *Fabaceae* | Black medick or nonesuch |

| | | | |
|---|---|---|---|
| | *lupulina* | | |
| 159 | *Melissa officinalis* | *Lamiaceae* | Lemon balm, balm or balm mint |
| 160 | *Mentha haplocalyx* | *Lamiaceae (mint family* | Greek mint or mentha |
| 161 | *Mentha piperta L.* | *Lamiaceae* | Peppermint |
| 162 | *Metasequoia glyptostroboides* | *Cupressaceae* | Dawn redwood |
| 163 | *Monarda didyma* | *Lamiaceae* | Crimson or scarlet beebalm, scarlet monarda, Oswego tea and bergamot |
| 164 | *Myrrhis odorata* | *Apiaceae* | Cicely |
| 165 | *Narissus sp.* | *Amaryllidaceae* | Daffodil, daffadowndilly, narcissus or jonquil |
| 167 | *Nepeta cataria* | *Lamiaceae* | Catnip, catswort and catmint |
| 168 | *Nigella sativa L.* | *Ranunculaceae* | Black cumin |
| 169 | *Ocium basilicum* | *Lamiaceae* | Basil, Thai basil, or sweet basil |
| 170 | *Oenothera biennis* | *Onagraceae* | Evening-primrose, evening star and sun drop |
| 171 | *Olea europaea* | *Oleaceae* | Olive |
| 172 | *Origanum majorana* | *Lamiaceae* | Marjoram or pot marjoram |
| 173 | *Origanum vulgare* | *Lamiaceae* | Oregano |
| 174 | *Paeoniae radix alba* **Or** *Paeonia lactiflora* | *Paeoniaceae* | Chinese peony or common garden peony |
| 175 | *Panax quinquefolius* | *Araliaceae* | Man root or man-like plant |
| 176 | *Parrotia persica* | *Hamamelidaceae* | Persian ironwood |
| 177 | *Peltiphyllum peltatum* **Or** *Darmera peltata* | *Saxifragaceae* | Indian rhubarb and umbrella plant |
| 178 | *Pericarpium citri* | *Rutaceae* | Tangerine Peel |

| | | | |
|---|---|---|---|
| | *reticulatae* | | |
| 179 | *Petasites hybridus* | *Asteraceae* | Butterbur |
| 180 | *Peucedanum graveolens* | *Apiaceae* | Dill |
| 181 | *Phacelia tanacetifolia* Benth. | *Boraginaceae* | Lacy phacelia or purple tansy |
| 182 | *Phyllostachys nigra* | *Poaceae* | Black bamboo |
| 183 | *Physalis peruviana L.* | *Solanaceae* | Cape gooseberry or sometimes simply Physalis |
| 184 | *Phytolacca mericana* L. | *Phytolaccaceae* | American Pokeweed, Virginia poke, American nightshade, cancer jalap, coakum, garget, inkberry, pigeon berry, pocan, pokeroot, pokeweed, pokeberry, redweed, scoke or red ink plant |
| 185 | *Picea abies* (acerocona) | *Pinaceae* | Norway spruce |
| 186 | *Pimenta officinalis* | *Myrtaceae* | Allspice or Jamaica pepper, myrtle pepper, English pepper and newspice |
| 187 | *Pimpinella anisum* | *Apiaceae* | Anise or aniseed |
| 188 | *Pimpinella major (l.) HUDS.* | *Apiaceae* | Greater burnet-saxifrage or hollowstem burnet saxifrage |
| 189 | *Pinus tabulaeformis* | *Pinaceae* | Chinese red pine |
| 190 | *Plantago lanceolata* | *Plantaginaceae* | English plantain, ribwort plantain, ribleaf, buckhorn plantain, buckhorn and lamb's tongue |
| 191 | *Platanus* X *hybrid* | *Platanaceae* | London plane |
| 192 | *Podophyllum* | *Berberidaceae* | Sinopodophyllum or Sinopodophyllum |

| | | | |
|---|---|---|---|
| | *hexandrum* | | hexandrum |
| 193 | *Polygonatum hybridum* | *Asparagaceae* | A hybrid plant between Common Solomon's-seal (*Polygonatum multiflorum*) and Angular Solomon's-seal (*Polygonatum odoratum*). |
| 194 | *Polygonum bistorta* L. | *Polygonaceae* | bistort, common bistort |
| 195 | *Polygonum japonicum* **Or** *Fallopia japonica* | *Polygonaceae* | Japanese knotweed |
| 196 | *Polygonus aviculture* | *Polygonaceae* | Knotweed, knotgrass, bistort, tearthumb and ars-smerte. |
| 197 | *Polyporus phyllostachydis* | *Polyporaceae* | Sotome, T. hatt. or kakish |
| 198 | *Potentilla anserine* | *Rosaceae* | Silverweed |
| 199 | *Potentilla anserine* | *Rosaceae* | Silverweed |
| 200 | *Potentilla aurea* TORN. | *Rosaceae* | Dwarf yellow cinquefoil |
| 201 | *Primula florindae* | *Primulaceae* | Tibetan cowslip and giant cowslip |
| 202 | *Prunus avium* | *Rosaceae* | Wild cherry or cherry |
| 203 | *Pteridium aquilinum* | *Dennstaedtiaceae* | Bracken or brake |
| 204 | *Pterocarya fraxinifolia* (LAM) | *Juglandaceae* | Caucasian wingnut or Caucasian walnut |
| 205 | *Pulmonaria officinalis* | *Boraginaceae* | Lungwort, common lungwort and our lady's milk drops |
| 206 | *Punica granatum* | *Lythraceae* | Pomegranate |
| 207 | *Quercus robur* | *Fagaceae* | English oak, pedunculate oak and French oak |

| | | | |
|---|---|---|---|
| 208 | *Raphanus sativus* | *Brassicaceae* | Radish |
| 209 | *Reseda luteola* L. | *Resedaceae* | Dyer's rocket, dyer's weed, weld, woold and yellow weed |
| 210 | *Rheum nobile* | *Polygonaceae* | Noble rhubarb or Sikkim rhubarb |
| 211 | *Rhizome atractylodis macrocephalae* | *Asteraceae* | Largehead Atractylodes *rhizome* |
| 212 | *Rhododendron ponticum* | *Ericaceae* | Rhododendron or pontic rhododendron |
| 213 | *Rhus typhina* | *Anacardiaceae* | staghorn sumac or stag's horn sumach |
| 214 | *Ribes nigrum* | *Grossulariaceae* | Blackcurrant |
| 215 | *Ricinus communis* | *Euphorbiaceae* | Castor oil plant |
| 216 | *Robinia pseudoacacia* | *Fabaceae* | Black locust and false acacia |
| 217 | *Rosa gallica* | *Rosaceae* | Gallic rose, French rose and rose of provins |
| 218 | *Rosa rubiginosa* | *Rosaceae* | Sweet briar or eglantine |
| 219 | *Rosmarinus officinalis* | *Lamiaceae* | Rosemary |
| 220 | *Rosmarinus officinalis* L. | *Lamiaceae* | Rosemary |
| 221 | *Rubia tinctoria* L. | *Rubiaceae* | Madder or dyer's madder |
| 222 | *Rubus idaeus* | *Rosaceae* | Red raspberry or European raspberry |
| 223 | *Ruta graveolen* L. | *Rutaceae* | Rue, common rue or herb-of-grace |
| 224 | *Salix caprea* | *Salicaceae* | Goat willow or pussy willow |
| 225 | *Salix fragilis* | *Salicaceae* | crack willow or brittle willow |
| 226 | *Salvia apiana Jepson* | *Lamiaceae* | White sage, bee sage and sacred sage. |
| 227 | *Salvia officinalis* | *Lamiaceae* | Sage |
| 228 | *Salvia pratensis* | *Lamiaceae* | Meadow clary or meadow sage |
| 229 | *Salvia Sclarea* | *Lamiaceae* | Salvia sclarea, clary and clary sage. |

| | | | |
|---|---|---|---|
| | *Sambucus nigra* | *Adoxaceae* | Elder, elderberry, black elder, European elder, European elderberry and European black elderberry |
| 230 | *Sambucus racemosa* | *Adoxaceae* | Red elderberry and Red-berried elderberry |
| 231 | *Saponaria officinalis* L. | *Caryophyllaceae* | Soapwort, bouncing-bet, crow soap, wild sweet William and soapweed |
| 232 | *Scorphularia auriculata* | *Scrophulariaceae* | Water figwort |
| 233 | *Scutellaria radix laterflora* | *Lamiaceae* | Skullcaps |
| 234 | *Sedum acre* | *Crassulaceae* | Goldmoss stonecrop, mossy stonecrop, goldmoss sedum, biting stonecrop and wallpepper |
| 235 | *Senecio erucifolius* | *Asteraceae* | Jacobaea erucifolia or hoary ragwort |
| 236 | *Senecio vulgaris* | *Asteraceae* | Groundsel |
| 237 | *Sequoiadendron giganteum* | *Cupressaceae* | Giant sequoia, giant redwood, Sierra redwood, Sierran redwood and Wellingtonia |
| 238 | *Silaum silaus* L. | *Apiaceae (Umbelliferae)* | Pepper-saxifrage |
| 239 | *Silene dioica* **Or** *Melandrium rubrum* | *Caryophyllaceae* | Red campion |
| 240 | *Sisymbrium officinale* | *Brassicaceae* | Hedge mustard |
| 241 | *Solanum dulcamara* | *Solanaceae* | Bittersweet, Bittersweet nightshade, Blue bindweed, Amara dulcis. |
| 242 | *Solanum* | *Solanacea* | Potato |

| | | | |
|---|---|---|---|
| | *tuberosum* | | |
| 243 | *Solidago canadensis* | *Asteraceae* | Canada golden-rod |
| 244 | *Solidago virgaurea* L. | *Asteraceae* | European goldenrod or woundwort |
| 245 | *Sophorae flavescentis radix* | *Fabaceae* | Shrubby sophora |
| 246 | *Sorbus aucuparia* | *Rosaceae* | Rowan and mountain-ash |
| 247 | *Stellaria media* | *Caryophyllaceae* | Chickweed, chickenwort, craches, maruns and winterweed |
| 248 | *Sucissa pratensis* | *Caprifoliaceae* | Devil's-bit or Devil's-bit Scabious |
| 249 | *Symphytum officinale* L. | *Boraginaceae* | Comfrey, quaker comfrey, cultivated comfrey, boneset and slippery-root |
| 250 | *Syringa vulgaris* | *Oleaceae* | Lilac |
| 251 | *Tanacetum balsamita* L. | *Asteraceae* | Costmary, alecost, balsam herb, bible leaf or mint geranium |
| 252 | *Tanacetum vulgare* L. | *Asteraceae* | Tansy, bitter buttons, cow bitter and golden buttons |
| 253 | *Taraxacum officinale* | *Asteraceae* | Blowballs or clocks |
| 254 | *Taxus baccata* L. | *Taxaceae* | Yew, English yew or European yew |
| 255 | *Teucrium scorodonia* | *Lamiaceae* | Wood sage or woodland germander |
| 256 | *Thuja plicata* (DONN ex D.Don) | *Cupressaceae* | Western or Pacific redcedar, giant or western arborvitae, giant cedar or shinglewood |
| 257 | *Thymus serphyllum var. citrodorus* | *Lamiaceae* | Breckland thyme, wild thyme and creeping thyme |
| 258 | *Thymus vulgaris* | *Lamiaceae* | Thyme or garden thyme |
| 259 | *Tilia cordata* | *Tiliaceae* | Small-leaved lime, small-leaved linden or little-leaf linden |
| 260 | *Tilia europea* | *Malvaceae* | Linden or lime |

| | | | |
|---|---|---|---|
| 261 | *Trichosanthes kirilowii maxim* | *Cucurbitaceae* | Chinese cucumber or Chinese snake gourd |
| 262 | *Tropaeolum majus (freeze dried)* | *Tropaeolaceae* | Garden nasturtium, Indian cress and monk cress |
| 263 | *Tulip asp.* | *Liliaceae* | It is about 75 wild species. |
| 264 | *Tussilago farfara* L. | *Asteraceae* | Coltsfoot |
| 265 | *Ulex europaeus* | *Fabaceae* | Gorse, furze or whin |
| 266 | *Urtica dioica* | *Urticaceae* | Nettle or stinging nettle |
| 267 | *Vaccinium myrtillus* | *Ericaceae* | Bilberry or blue whortleberry |
| 268 | *Vaccinium vitis-idaea* | *Ericaceae* | lingonberry or cowberry |
| 269 | *Valeriana officinalis* L. | *Caprifoliaceae* | Valerian |
| 270 | *Verbascum thapsus* | *Scrophulariaceae* | Great mullein or common mullein |
| 271 | *Verbena officinalis* L. | | Vervain or common verbena |
| 272 | *Viburnum opulus* | *Adoxaceae* | Guelder-rose |
| 273 | *Vicia sepium* | *Fabaceae* | bush vetch |
| 274 | *Vinca minor* | *Apocynaceae* | Lesser periwinkle or Dwarf periwinkle |
| 275 | *Viola odorata* | *Violaceae* | Wood violet, sweet violet, common violet and garden violet |
| 276 | *Vitellaria paradoxa* | *Sapotaceae* | shea tree, shi tree and vitellaria (shea meal) |
| 277 | *Vitex agnus-castus L.* | *Verbenaceae* | Vitex, chaste tree, chasteberry, Abraham's balm or Monk's pepper |
| 278 | *Zingiber officinale* | *Zingiberaceae* | Ginger or ginger root |

* N/A = the information are not available.

**Table B:** Subset of plants with high tannin contents selected for thiolysis and HPLC-MS analysis

| No | Botanical name | Family name | Native place (origin) | Common name |
|---|---|---|---|---|
| 1 | *Arbutus unedo* | *Ericaceae* | Mediterranean and western EU | Strawberry tree |
| 2 | *Cistus laurifolius* | *Cistaceae* | Mediterranean Countries | Laurel-leaf cistus or laurel-leaved rock rose |
| 3 | *Corylus avellana* | *Betulaceae* | Different parts of EU and Asia | Hazel |
| 4 | *Euonymus alatus* | *Celastraceae* | Northern china, Japan, and Korea | Winged spindle, winged euonymus and burning bush |
| 5 | *Hydrangea petiolaris* | *Hydrangeacae* | South east of Asia and north Russia | Climbing hydrangea |
| 6 | *Hypericum perforatum* | *Hypericaceae* | Europe and Asia | St John's wort |
| 7 | *Parrotia persica* | *Hamamelidaceae* | Northern Iran and southern Azerbaijan | Persian ironwood |
| 8 | *Picea abies* | *Pinaceae* | It comes from various regions in Europe | Norway spruce |
| 9 | *Pimenta officinalis* | *Myrtaceae* | The origin is from Jamaica, but it is also planted in European and Mediterranean | Allspice or Jamaica pepper, myrtle pepper, English pepper and new-spice |
| 10 | *Pinus L.* | *Pinaceae* | This plant is throughout in the world, except in Antarctica and desert areas | Pine buds |

| | | | | |
|---|---|---|---|---|
| 11 | *Platanus* X *hybrid* | *Platanaceae* | This kind is native in northern hemisphere | London plane |
| 12 | *Polygonum bistorta* **Or** *Persicaria bistorta* | *Polygonaceae* | Europe and north-western Asia | Bistort |
| 13 | *Ribes nigrum* | *Grossulariacea* | Europe and northern Asia | Blackcurrant |
| 14 | *Robinia pseudoacacia* | *Fabaceae* | It is throughout in the world | Black locust |
| 15 | *Taxus baccata* L. | *Taxaceae* | South-western and central of Europe, northwest Africa, and west and southwest Asia | Yew, English yew or European yew |
| 16 | *Tilia L.* | *Malvaceae* | It originates from UK. Trees or bushes, throughout most of the Northern Hemisphere | Tilia or lime tree |

**Table C:** Sites of plant collection or websites for purchased samples

| | **Latin names** | **Plant sources** | **Site of collection** | **Supplier (online website)** |
|---|---|---|---|---|
| 1 | *Arbutus unedo* | Strawberry tree | Emmer Green, UK | ---------- |
| 2 | *Corylus avellana* | Hazel | University of Reading, UK | ---------- |
| 3 | *Pinus* spp. | Pine buds | ---------- | https://www.kozlek.pl/ |
| 4 | *Platanus* X *hybrid* | London plane | Campus of University of Reading, UK | ---------- |
| 5 | *Polygonum bistorta* | Bistort | University of Reading, UK | ---------- |
| 6 | *Robinia pseudoacacia* | Black locust | University of Reading, UK | ---------- |
| 7 | *Taxus baccata* L. | Yew | Letchworth Garden City, UK | ---------- |
| 8 | *Tiliae* spp. | Tilia flowers | ---------- | https://www.kozlek.pl/ |
| 9 | *Theobroma cacao* | Cocoa beans | ---------- | https://www.buywholefoodsonline.co.uk/cacao-cocoa-beans |

Plants were purchased or collected between July and September 2015.

## Appendix 2: Publication in scientific conference proceedings

Dakheel M.M., Drake C., Mueller-Harvey I., Drake C., Rymer C. Can tannin-containing plants help maintain gut health in poltry (**2015**); In: The 22nd Annual Review Meeting of SCI Conference; (**Scientific conference/ poster presentation**)

Hoste, H., Torres-Acosta, J., Quijada, J., Chan-Perez, I., Dakheel, M., Kommuru, D., Mueller-Harvey, I., and Terrill, T. (**2016**). Chapter Seven-Interactions Between Nutrition and Infections With Haemonchus contortus and Related Gastrointestinal Nematodes in Small Ruminants. In "Advances in parasitology", Vol. 93, pp. 239-351. (**Chapter in scientific book**).

Dakheel M.M., Alkandari F.A.H., Woodward M.J., Mueller-Harvey I., Drake C., Rymer C. Effect of tannin structures as antimicrobial agents against pathogenic bacteria (**2016**); In: Book of Abstracts of the 67th Annual Meeting of the European Federation of Animal Science; ISBN: 978-90-8686-284-9; (**Scientific conference/ poster presentation**)

Dakheel M.M., Alkandari F.A.H., Woodward M.J., Mueller-Harvey I., Drake C., Rymer C. Effect of condensed tannins as antibcterials against pathogenic bacteria in poultry (**2017**); In: The Annual Meeting of the 71st AVTRW Conference; (**Scientific onference/ poster presentation**)

## Appendix 3: Achieved sessions and courses of this study

This table shows the identification training needs, as part of learning needs analysis and assessment during this PhD study.

| Course details | Course title | Course dates |
|---|---|---|
| RRPD (Reading Researcher Development Programme)<br><br>AEP (Academic English Programme).<br><br>Some library courses at Reading University<br><br>Health and safety courses<br><br>Scientific conferences and workshops<br><br>Public activities related to academic training | • Sourcing information for a literature review – information retrieval<br>• MATLAB<br>• An essential guide to critical academic writing<br>• How to avoid plagiarism<br>• Academic writing skills for non-native speakers<br>• Preparing poster - Theory (Part 1) & Practical (Part 2) / RRDP<br>• Presentation skills/<br>• Doctoral Research Conference<br>• Basic statistics refresher<br>• Academic Teaching Mentoring Scheme<br>• Listening and Speaking - AEP/ autumn term.<br>• Written Language Skills –AEP/Spring term.<br>• Research writing skills – AEP/ Summer term 2015<br>• EndNote Web: beginners' workshop – Library course<br>• Health and safety course/ HCl training– Chemical lab in TOB section<br>• Health and safety course – Reading University | 28/10/2014<br><br>19/11/2014<br>26/11/2014<br><br>09/12/2014<br>09/02/2015<br><br>27/04/2015<br><br>05/05/2015<br>Jan (2015-16-17)<br>18/06/2015<br>19/11/2015<br><br>Oct- Dec/ 2014<br><br>Jan-Feb 2015<br>Apr-May 2015<br><br>19/11/2014<br><br>01/10/2014<br><br>15/10/2014<br><br>23-24 /06/2015 |

| | | |
|---|---|---|
| | • Feed conference 2015 at Nottingham University.<br>• SCI Agri-sciences Young Researchers 2015.<br>• TEDx event at Reading University.<br>• ISO 17025- workshop | 08/07/2015<br>26/02/2015<br>29/01/2016<br>25/02/2016 |
| Learn a presentation skill and how to deal with it.<br>How to gain the academic writing skills, especially for non-native speakers. | • Presentation skills<br>• Studying in other cultures<br>• Academic writing skills for non-native speakers<br>• Writing a thesis as a collection of papers<br>• How to write a scientific paper | 05/05/2015<br>24/01/2018<br>09/02/2015<br>23/05/2017<br>08/03/2017 |
| English institute (AEP) at Reading University | • Listening and Speaking/ Autumn term<br>• Written Language Skills/ Spring term<br>• Research writing skills/ Summer term 2015 | Oct- Dec/2014<br>Jan-Feb/ 2015<br>Apr-May/ 2015 |
| How to manage and reference your academic sources. | • EndNote Web: (Library course)<br>• Self-management: Managing academic pressure<br>• Self-management: increasing concentration<br>• Getting your first post-doc position<br>• Open access for research publications<br>• How to get published | 19/11/2014<br>08/11/2016<br>17/05/2016<br>28/02/2017<br>10/02/2017<br>23/02/2016 |
| Encourage for subscription in the scientific conferences. | • Doctoral Research Conference 2015/ RRDP.<br>• Doctoral Research Conference 2016.<br>• Doctoral Research Conference 2017. | 18/06/2015<br>23/06/2016<br>20/06/2017 |

| | | |
|---|---|---|
| | • Workshop and Feed conference at Nottingham University.<br>• SCI Agri-sciences Young Researchers 2015 by SCI<br>• EAAP Annual Conference 2016, Belfast, UK.<br>• Academic Teaching Mentoring Scheme.<br>• PhD Plus Programme (1st session)<br>• PhD Plus Programme (2nd session) academic mentoring seminar<br>• Electron Microscopy Workshops (4 sessions)<br>• Electron Microscopy training courses (3 days)<br>• The Leadership Programme (at Reading University/ Graduate School & Henley Business School) three sessions<br>• AVTRW 2017 at Surrey University<br>• IFSTAL – Innovative Food Systems Teaching and Learning programme (2 terms)<br>• (ISO 17025 – workshop)<br>• How to Accredit your Laboratory Practical Aspects of Gaining Accreditation<br>• Food Forum Conference 2016 (attendance at School of Geography and the Environment, | 28Aug-3Sep 2016<br>JAN/March 2016<br>08/07/2015<br>17/05/ 2016<br>17/5/2017<br>OCT-NOV/ 2016<br>9-11 May 2017<br>Feb 2017<br>12-13 Sep 2017<br>Autumn- 2016<br>Spring- 2017<br>26-27/01/2016<br>25/02/2016<br>15/07/2016 |

| | | |
|---|---|---|
| | University of Oxford)<br>• The Iraqi Cultural and Scientific Conference at Reading/ ICSCR-2016 | |
| Health and safety courses | • Health and safety course/ HCl training/ Chemical lab in TOB section – at Agriculture school/ Reading University<br>• Health and safety course/ Microbial lab at Chemistry, Food and Pharmacy school/ Reading University | 01/10/2014<br>15/10/2014<br>14/12/2015 |
| How to deal with project planning and delivery the ideas | • Presentation skills<br>• Preparing poster - Theory (Part 1) & Practical (Part 2)<br>• How to write a thesis<br>• How to write a dissertation<br>• How to summarise your research in 3 minutes | 05/05/2015<br>27/04/2015<br>10/12/2015<br>24/01/2017<br>23/01/2018 |
| The Preparing to Teach programme by CQSD (Workshops run by Reading University) | • How to design, prepare and deliver a successful lecture<br>• Introduction to teaching and learning.<br>• Marking and feedback<br>• Laboratory demonstrating & leading small groups | 25/01/2016<br>26/01/2016<br>26/01/2016<br>27/01/2016 |
| Work as individual and group working with other students | • Time management<br>• Presentation skills<br>• Surviving the viva | 19/02/2018<br>05/05/2015<br>12/02/2018 |

| | | |
|---|---|---|
| Encourage to attend public activities and events | • TEDx in University of Reading by TED society<br>• Member in Iraqi Society at Reading University<br>• Member in Iraqi Student Society (IQSS) at UK<br>• Fair-brother Lectures (part1 and 2)<br>• Food Public Lecture (titled Maintaining the quality of fresh produce alongside sustainability)<br>• Food Public Lecture (titled Brain Training)<br>• Society fresher's week (at RUSU) with Iraq Society (2016 & 2017)<br>• Public Lecture 11th Hugh Bunting Memorial Lecture<br>• Public Lecture 'life tools' (titled Techniques for Increasing Concentration" | 26/02/2015<br>2014-2015<br>2015-2016<br>04/05/2016<br>14/03/2017<br>10/05/2016<br>14/09/2016<br>21/09/2016<br>23/09/2017<br>09/11/2016<br>02/01/2017 |