博士論文

Study on the usefulness of tannin-related substances as feed additives in pig farming

（養豚におけるタンニン関連物質の飼料添加物としての有用性に関する研究）

Min MA

馬 敏

2022

Abstract

There are many vulnerable stages of pig growth that put pig farming at great risk, especially the weaning stage of piglets. Stress from weaning can lead to growth check, diarrhea or even death, causing serious economic losses. Adding antibiotics to the diet can protect them from intestinal pathogens. But with the increasing importance of antibiotics in the farming industry, drug-resistant strains have been triggered. Since 1986, the use of antibiotics in feed has been gradually banned in some countries, and the pork production efficiency has been influenced. Other feed additives or management programs such as probiotics, organic acids, mineral salts, and vaccines have emerged with more or fewer deficiencies in terms of efficacy, stability, and economic applicability. Therefore, a sustainable and effective antibiotics alternative is urgently needed to prevent diarrhea in early-weaned piglets and to promote growth performance.

Tannins have great potential for development as natural antimicrobial agents, due to their structural complexity, and the ability to bind indiscriminately to proteins which makes it more difficult for microorganisms to develop resistance. In addition, it has antioxidant, anti-inflammatory, and antiparasitic activities, and so on. Notably, its ability to bind to proteins allows them to also bind to feed proteins or digestive enzymes, interfering with the digestion and absorption of nutrients, thus creating anti-nutritional properties. Therefore, how to strike a delicate balance between anti-nutritional properties and beneficial biological activity is our research focus.

Due to the instability of hydrolyzed tannins, and considering the effect of tannins in complex environments, such as feed and intestine, we finally chose more stable condensed tannin (quebracho tannin) for the study. Past experience shows that the anti-nutritional properties of tannins are usually associated with high dosage. In view of this, we started with a small amount to determine the feasibility of quebracho tannin as a piglet feed additive, and tried various addition levels to determine the appropriate

dosage and the possibility of replacing antibiotics. In experiment 1 (**Chapter 2**), the feasibility of using low-levels MGM-P (quebracho tannin product) addition as feed additives for weaned piglets was assessed; it investigated MGM-P effects on growth performance, diarrhea, and overall health in early-weaned piglets. In total, 24 piglets (Duroc × Landrace × Yorkshire, 6.51±0.17 kg) were allocated to three treatment groups fed basal diets supplemented with 0, 0.2%, or 0.3% MGM-P for 20 days. The addition of 0.3% MGM-P to the diet of early-weaned piglets improved diarrhea incidence, hematological parameters, and intestinal mucosa structure. Furthermore, the addition of 0.2% or 0.3% MGM-P to the diet of early-weaned piglets did not affect their growth performance. In experiment 2 (**Chapter 3**), higher additive amounts were used, and an antibiotic control group was set up. 36 early-weaned piglets (Duroc × Landrace × Yorkshire, 5.84±0.21 kg) were allotted to one of four treatments and given either a control diet free of MGM-P (NC, negative control) or diets containing 0.5% (LT, low-level treatment), 1.0% (HT, high-level treatment) MGM-P, or antibiotics (PC, positive control). Growth performance and blood parameters were monitored during the feeding period. The results showed that in the first 14 d after weaning, the 0.5% MGM-P group had the highest feed conversion ratio (FCR) and higher ($p < 0.05$) than that in the 1.0% MGM-P group. Although there was no diarrhea, the average fecal scores in the whole experimental stage were HT > NC > PC > LT. Therefore, in combination with experiment 1, it can be concluded that around 0.5% is probably the appropriate additive amount of the MGM-P. To further validate the effect of 0.5% MGM-P supplementation in promoting growth in pigs, we conducted a trial for grow-finish pigs. In experiment 3 (**Chapter 4**), a total of 24 pigs (Duroc × Landrace × Yorkshire, 37.60 ± 0.86 kg) were divided into two groups (3 replicates of 4 pigs per treatment), and they were fed a control diet or 0.5% MGM-P supplementary diet for 16 weeks. Our results indicated that the average daily gain (ADG) of pigs in the MGM-P supplementary group was significantly higher ($p < 0.01$) compared to the control group at 8-12 w. Pigs received MGM-P had lower ($p < 0.05$) glutamic

oxaloacetic transaminase (GOT) levels and higher kidney weight ($p < 0.05$) at the end of the experiment compared with the control group. All these reasons could potentially promote growth performance in pigs.

The use of tannins in pig feed dates back centuries, Iberian pigs in the Mediterranean region, known for their high-quality meat, were often foraged for tannin-rich acorns or chestnuts. This may be due to the antioxidant activity of tannins, which helped improve the antioxidant capacity and unsaturated fatty acid ratio of the pork. And chestnut shells, which are discarded in chestnut food production, are very rich in tannins. Therefore, we tried to reuse these shells to explore the possibility of using the waste as feed to ensure the quality of meat and improve economic profitability.

Experiment 4 (**Chapter 5**) investigated the availability of chestnut by-products in finishing pig production. In the 110-day experiment, 6 Duroc finishing pigs (103.37±2.03 kg, males castrated) were selected as experimental animals and evenly divided into control and treatment groups (three pigs each). The control group was fed a basal diet (C) and the treatment group received a basal diet containing 60% chestnut shell meal (T). The assessment of pork quality revealed that the addition of chestnut shells effectively improved the sensory evaluation and color stability of the meat and increased the content of free amino acids. In order to maximize the use of chestnut by-products, the development of reasonable storage means is necessary to overcome the limitations imposed by the extremely seasonal nature of chestnut harvesting, so the feasibility of silage chestnut shells, and its effects on growth, health, and meat quality of finishing pigs was studied in experiment 5 (**Chapter 6**). Two 110-day feeding trial was conducted with total 12 Duroc finishing pigs (105.62±2.85 kg). Each trial used 6 pigs from the same sow and was assigned into two diet dietary treatments (3 pigs each). The pigs in trial 1 were fed with basic diet (S3C) or basic diet plus 60% chestnut shells silaged for 3 months (S3T), and in trial 2 were fed with basic diet (S12C) or basic diet plus 60% chestnut shells silaged for 12 months (S12T).

The results showed that silage chestnut shells had no effect on the growth performance of pigs. However, the tannin content in chestnut shells decreased with increasing silage time, and its effect on pork changed. The addition of short-term (3 months) silage chestnut shells to feed significantly improved pork tenderness, while the effect on free amino acids is not obvious. In contrast, the feeding of chestnut shells with long-term (12 months) silage had a positive effect on the free amino acid content of pork, but resulted in poor tenderness.

Collectively, quebracho tannin has shown effectiveness as a swine feed additive. 0.5% MGM-P supplementation can provide pigs with the right amount of quebracho tannin to improve health and growth performance, and has the potential to contribute to the mitigation of drug resistance by replacing antibiotic additives in today's pig industry. As for the substitution of some feeds by chestnut shell by-products, this waste food reuse method can improve the quality of pork on the one hand and reduce the cost of breeding on the other. Continuing to promote this kind of waste food reuse research in pig farming in the future will not only help to promote the sustainable development of pig farming to increase economic benefits but also help to establish an environmentally friendly image for pig farming. For example, making the pig industry less involved in competition for natural resources compared to other livestock industries; enabling the pig industry to help reduce food waste, etc., ultimately increasing the competitiveness and market share of the pig industry in the livestock sector.

Table of contents

STUDY ON THE USEFULNESS OF TANNIN-RELATED SUBSTANCES AS FEED ADDITIVES IN PIG FARMING I

（養豚におけるタンニン関連物質の飼料添加物としての有用性に関する研究） I

ABSTRACT II

TABLE OF CONTENTS VI

LIST OF TABLES XI

LIST OF FIGURES XIII

CHAPTER 1 INTRODUCTION 1

1.1. ANTIBIOTIC ALTERNATIVES 3

1.1.1. Vaccines 4
1.1.2. Zinc oxide 4
1.1.3. Probiotics 5
1.1.4. Acidifiers 6
1.1.5. Antimicrobial peptides 6
1.1.6. Enzyme preparations 7
1.1.7. Plant extracts 8
1.1.8. Other alternatives and policies 9

1.2. TANNIN 9

1.2.1. Tannin classification 10
1.2.2. Tannin source 10

1.3. BIOLOGICAL PROPERTIES OF TANNINS 11

1.3.1. Antiviral 11
1.3.2. Aantibacterial 12
1.3.3. Anti-oxidation 15
1.3.4. Anti-inflammatory 16
1.3.5. Antiparasitic 17

1.4. THE ADVANTAGES AND LIMITATIONS OF TANNINS VERSUS ANTIBIOTICS 18

1.4.1. Main function and defect of antibiotics 18
1.4.2. Advantages of tannin as an alternative to antibiotics 19
1.4.2.1. Bactericidal activity 19
1.4.2.2. Anti-drug resistance 20
1.4.2.3. Multiple biological activities 20
1.4.2.4. Low residue 20
1.4.3. Limitations of tannins 21

1.4.3.1. Anti-nutritional *21*
1.4.3.2. Toxicity *22*
1.4.3.3. Complexity *22*

1.5. QUEBRACHO TANNIN 22

1.6. FEEDING VALUE OF TANNIN-RELATED BY-PRODUCTS 24

1.7. SUMMARY AND RESEARCH OBJECTIVES 26

CHAPTER 2 EVALUATION OF THE EFFECT OF LOW-DOSE QUEBRACHO TANNIN ADDITION IN EARLY-WEANED PIGLET DIETS 31

2.1. INTRODUCTION 31

2.2. MATERIALS AND METHODS 32

2.2.1. MATERIALS 32
2.2.2. ANIMALS, TREATMENTS, AND EXPERIMENTAL DESIGN 32
2.2.3. DIET AND ANIMAL MANAGEMENT 33
2.2.4. GROWTH PERFORMANCE 33
2.2.5. DIARRHEA INCIDENCE 33
2.2.6. BLOOD SAMPLING 33
2.2.7. BLOOD HEMATOLOGY ANALYSIS 34
2.2.8. PLASMA COLLECTION AND IMMUNOGLOBULIN ANALYSIS 34
2.2.9. BLOOD BIOCHEMICAL EXAMINATION 34
2.2.10. PLASMA FREE AMINO ACIDS 34
2.2.11. CORTISOL MEASUREMENT 35
2.2.12. ACTUAL AND RELATIVE WEIGHTS/LENGTHS OF ORGANS AND INTESTINES 36
2.2.13. INTESTINAL MORPHOLOGY 36
2.2.14. STATISTICAL ANALYSIS 37

2.3. RESULTS 37

2.3.1. GROWTH PERFORMANCE 37
2.3.2. DIARRHEA INCIDENCE 37
2.3.3. BLOOD HEMATOLOGY ANALYSIS 37
2.3.4. BLOOD IMMUNOGLOBULIN ANALYSIS 38
2.3.5. BLOOD BIOCHEMICAL ANALYSIS 38
2.3.6. PLASMA FREE AMINO ACIDS 38
2.3.7. CORTISOL ANALYSIS 38
2.3.8. ACTUAL AND RELATIVE WEIGHTS/LENGTHS OF ORGANS AND INTESTINES 39
2.3.9. INTESTINAL MORPHOLOGY 39

2.4. DISCUSSION 39

2.5. CONCLUSIONS 44

CHAPTER 3 FEASIBILITY OF REPLACING ANTIBIOTIC ADDITIVES WITH QUEBRACHO TANNIN IN EARLY-WEANED PIGLETS 63

3.1. INTRODUCTION 63

3.2. MATERIALS AND METHODS 64

3.2.1. Materials 64
3.2.2. Animals, Treatments, and Experimental Design 64
3.2.3. Diet and Animal Management 65
3.2.4. Growth Performance 65
3.2.5. Diarrhea manifestations 65
3.2.6. Blood Sampling 66
3.2.7. Blood Hematology Analysis 66
3.2.8. Plasma Collection and Biochemical Examination 66
3.2.9. Plasma free amino acids 66
3.2.10. Actual and Relative Weights/Lengths of Organs and Intestines 67
3.2.11. Statistical Analysis 68

3.3. RESULTS 68

3.3.1. Growth Performance 68
3.3.2. Diarrhea manifestations 68
3.3.3. Blood Hematology Analysis 69
3.3.4. Blood Biochemical Analysis 69
3.3.5. Plasma free amino acids 69
3.3.6. Actual and Relative Weights/Lengths of Organs and Intestines 70

3.4. DISCUSSION 70

3.5. CONCLUSIONS 74

CHAPTER 4 EFFECT OF LONG-TERM DIETARY QUEBRACHO TANNIN SUPPLEMENTATION ON GROWTH PERFORMANCE AND OVERALL HEALTH OF GROW-FINISH PIGS 87

4.1. INTRODUCTION 87

4.2. MATERIALS AND METHODS 88

4.2.1. Materials 88
4.2.2. Animals, Treatments, and Experimental Design 88
4.2.3. Diet and Animal Management 88
4.2.4. Growth Performance 89
4.2.5. Blood Sampling 89
4.2.6. Blood Hematology Analysis 89
4.2.7. Plasma Collection and Biochemical Examination 89
4.2.8. Plasma free amino acids 89
4.2.9. Actual and Relative Weights/Lengths of Organs and Intestines 90
4.2.10. Statistical Analysis 91

4.3. RESULTS 91

4.3.1. Growth Performance 91
4.3.2. Blood Hematology Analysis 91
4.3.3. Blood Biochemical Analysis 92
4.3.4. Plasma free amino acids 92
4.3.5. Actual and Relative Weights/Lengths of Organs and Intestines 92

4.4. DISCUSSION 92

4.5. CONCLUSIONS 95

CHAPTER 5 EFFECTS OF SUPPLEMENTATION WITH TANNIN-RICH CHESTNUT BY-PRODUCTS ON GROWTH PERFORMANCE, BLOOD PARAMETER, AND MEAT QUALITY IN FINISHING PIGS 108

5.1. INTRODUCTION 108

5.2. MATERIALS AND METHODS 110

5.2.1. Animal ethics 110
5.2.2. Materials 110
5.2.3. Animals and experimental design 111
5.2.4. Sample collection 111
5.2.5. Feed analysis 112
5.2.6. Fattening performance 112
5.2.7. Blood hematology 112
5.2.8. Meat Color 112
5.2.9. Meat quality evaluation 113
5.2.10. Statistical Analysis 113

5.3. RESULTS 114

5.3.1. Chemical compositions of diets 114
5.3.2. Fattening performance 114
5.3.3. Blood hematology 114
5.3.4. Meat Color 115
5.3.5. Sensory evaluation of muscle 115
5.3.6. Chemical composition of muscle 115
5.3.7. Fatty acids composition of muscle 115
5.3.8. Amino acids profile of muscle 115

5.4. DISCUSSION 116

5.5. CONCLUSIONS 121

CHAPTER 6 EFFECTS OF SUPPLEMENTATION WITH SILAGE CHESTNUT BY-PRODUCTS ON GROWTH PERFORMANCE, BLOOD PARAMETER, AND MEAT QUALITY IN FINISHING PIGS 133

6.1. INTRODUCTION 133

6.2. MATERIALS AND METHODS 134

6.2.1. ANIMAL ETHICS 134
6.2.2. MATERIALS 134
6.2.3. ANIMALS AND EXPERIMENTAL DESIGN 134
6.2.4. SAMPLE COLLECTION 135
6.2.5. FEED ANALYSIS 135
6.2.6. FATTENING PERFORMANCE 136
6.2.7. BLOOD PARAMETERS 136
6.2.8. MEAT COLOR 136
6.2.9. MEAT QUALITY EVALUATION 137
6.2.10. STATISTICAL ANALYSIS 138

6.3. RESULTS 138

6.3.1. CHEMICAL COMPOSITIONS OF DIETS 138
6.3.2. FATTENING PERFORMANCE 138
6.3.3. BLOOD HEMATOLOGY 139
6.3.4. BLOOD BIOCHEMISTRY 139
6.3.5. MEAT COLOR 140
6.3.6. CHEMICAL COMPOSITION OF MUSCLE 140
6.3.7. SENSORY EVALUATION OF MUSCLE 140
6.3.8. FATTY ACIDS COMPOSITION OF MUSCLE 140
6.3.9. AMINO ACIDS PROFILE OF MUSCLE 141

6.4. DISCUSSION 141

6.5. CONCLUSIONS 147

CHAPTER 7 GENERAL DISCUSSION 162

CHAPTER 8 CONCLUSION 169

ACKNOWLEDGEMENT 170

REFERENCES 173

List of tables

Table 2.1. Technical specification of MGM-P. 45

Table 2.2. Experimental animal allotment. 46

Table 2.3. Ingredients and chemical composition of basal diet (as-fed basis). 47

Table 2.4. Chemical composition of basal diet. 48

Table 2.5. Effects of low-dosage MGM-P supplementation on growth performance in weaned piglets (throughout the experimental period). 49

Table 2.6. Effects of low-dosage MGM-P supplementation on blood biochemical parameters in weaned piglets. 50

Table 2.7. Effects of low-dosage MGM-P supplementation on plasma free amino acids in weaned piglets (µM). 51

Table 2.8. Effects of low-dosage MGM-P supplementation on actual organ weight/length in weaned piglets. 52

Table 2.9. Effects of low-dosage MGM-P supplementation on relative organ weight/length in weaned piglets. 53

Table 3.1. Experimental animal allotment. 75

Table 3.2. Effects of high-dosage MGM-P supplementation on growth performance in weaned piglets. 76

Table 3.3. Effects of high-dosage MGM-P supplementation on blood biochemical parameters in weaned piglets. 77

Table 3.4. Effects of high-dosage MGM-P supplementation on plasma free amino acids in weaned piglets (uM). 79

Table 3.5. Effects of high-dosage MGM-P supplementation on the actual organ weight/length of weaned piglets. 80

Table 3.6. Effects of high-dosage MGM-P supplementation on the relative organ weight/length of weaned piglets. 81

Table 4.1. Experimental animal allotment. 97

Table 4.2. Ingredients and chemical composition of basal diets (as-fed basis). 98

Table 4.3. Effects of long-term dietary MGM-P supplementation on growth performance in grow-finish pigs (throughout the experimental period). 99

Table 4.4. Effects of long-term dietary MGM-P supplementation on blood biochemical parameters in grow-finish pigs. 100

Table 4.5. Effects of long-term dietary MGM-P supplementation on plasma free amino acids in grow-finish pigs (uM). 101

Table 4.6. Effects of long-term dietary MGM-P supplementation on the actual weights/lengths of organs and intestines of grow-finish pigs. 102

Table 4.7. Effects of long-term dietary MGM-P supplementation on the relative weights/lengths of organs and intestines of grow-finish pigs. 103

Table 5.1. Ingredients of basal diets (as-fed basis). 122

Table 5.2. Chemical compositions of different experimental diets (%). 123

Table 5.3. Effect of supplementing 60% chestnut shells in the diet on the fattening performance of finishing pigs (throughout the experimental period). 124

Table 5.4. Effects of supplementing 60% chestnut shells in the diet on the blood hematology of finishing pigs. 125

Table 5.5. Effects of supplementing 60% chestnut shells in the diet on the meat color of finishing pigs. 126

Table 5.6. Effects of supplementing 60% chestnut shells in the diet on the sensory evaluation of muscle in finishing pigs. 127

Table 5.7. Effects of supplementing 60% chestnut shells in the diet on the chemical composition of muscle in finishing pigs (g/100g). 128

Table 5.8. Effects of supplementing 60% chestnut shells in the diet on the fatty acids content of muscle in finishing pigs. 129

Table 5.9. Effect of supplementing 60% chestnut shells in the diet on the amino acids profile of muscle in finishing pigs (mg/100g). 130

Table 6.1. Chemical compositions of different experimental diets (%). 149

Table 6.2. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the fattening performance of finishing pigs (throughout the experimental period). 150

Table 6.3. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the blood biochemical parameters of finishing pigs. 151

Table 6.4. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the meat color of finishing pigs. 153

Table 6.5. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the chemical composition of muscle in finishing pigs (g/100g). 154

Table 6.6. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the sensory evaluation of muscle in finishing pigs. 155

Table 6.7. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the fatty acids content of muscle in finishing pigs. 156

Table 6.8. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the amino acids profile of muscle in finishing pigs(mg/100g). 157

List of figures

Figure 1.1. Representative tannins molecular structure. ... 28
Figure 1.2. The complexation of tannins with amino acids in protein. ... 29
Figure 1.3. Antibiotics resistance and tannins antimicrobial properties schematic diagram. ... 30
Figure 2.1. Effects of low-dosage MGM-P supplementation on growth performance in weaned piglets. ... 54
Figure 2.2. Effects of low-dosage MGM-P supplementation on diarrhea incidence in weaned piglets. ... 55
Figure 2.3. Effects of low-dosage MGM-P supplementation on blood hematology parameters in weaned piglets. ... 56
Figure 2.4. Effects of low-dosage MGM-P supplementation on blood immunoglobulin levels in weaned piglets. ... 57
Figure 2.5. Effects of low-dosage MGM-P supplementation on blood biochemical parameters of weaned piglets. ... 58
Figure 2.6. Effects of low-dosage MGM-P supplementation on plasma free amino acids of weaned piglets. ... 59
Figure 2.7. Effects of low-dosage MGM-P supplementation on the blood cortisol concentration of weaned piglets. ... 60
Figure 2.8. Effects of low-dosage MGM-P supplementation on morphology of the duodenum in piglets at 20 days post-weaning. ... 61
Figure 2.9. Effects of low-dosage MGM-P supplementation on the intestinal morphology of weaned piglets. ... 62
Figure 3.1. Effects of high-dosage MGM-P supplementation on growth performance in weaned piglets. ... 82
Figure 3.2. Effects of high-dosage MGM-P supplementation on fecal scores in weaned piglets. ... 83
Figure 3.3. Effects of high-dosage MGM-P supplementation on blood hematology parameters in weaned piglets. ... 84
Figure 3.4. Effects of high-dosage MGM-P supplementation on blood biochemical parameters of weaned piglets. ... 85
Figure 3.5. Effects of high-dosage MGM-P supplementation on the blood free amino acid concentration of weaned piglets. ... 86
Figure 4.1. Effects of long-term dietary MGM-P supplementation on growth performance in grow-finish pigs. ... 104
Figure 4.2. Effects of long-term dietary MGM-P supplementation on blood hematology parameters in grow-finish pigs. ... 105
Figure 4.3. Effects of long-term dietary MGM-P supplementation on blood biochemical parameters of grow-finish pigs. ... 106

Figure 4.4. Effects of long-term dietary MGM-P supplementation on the blood free amino acids concentration of grow-finish pigs. 107
Figure 5.1. Effect of supplementing 60% chestnut shells in the diet on the fatty acids composition of muscle in finishing pigs. 131
Figure 5.2. Effects of supplementing 60% chestnut shells in the diet on the amino acids content of muscle in finishing pigs. 132
Figure 6.1. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the blood hematology of finishing pigs. 158
Figure 6.2. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the blood biochemistry of finishing pigs. 159
Figure 6.3. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the fatty acids composition of muscle in finishing pigs. 160
Figure 6.4. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the amino acids content of muscle in finishing pigs. 161

Chapter 1 Introduction

Pork is one of the richest dietary sources of protein. According to the Food and Agriculture Organization of the United Nations (FAO), in 1979-2015, pork production continued accounted for more than 35% of the world's total meat production, much higher than other meat. (Ritchie and Roser 2017). As a constant increasing of the total global population and the global economy recovers in the late COVID-19, pork demand is believed to continue to increase. Therefore, it is essential to make the pig industry capable of dealing with various risks and challenges for stable and sustainable development.

Weaning is one of the biggest challenges for pig farming. Piglets weaning is a gradual process occurring naturally between 60-100 days after birth. However, in many places, pig industry weans piglets at the ages of 19–25 days to increase sow reproductive efficiency, thus improving annual productivity (Newberry and Wood-Gush 1985; Li 2017). Piglets' immune status is very low at this age because the level of antibodies obtained through maternal colostrum has already halved, while active immunity is just beginning to develop (Gaskins and Kelley 1995). Therefore, it is hard for piglets to adapt quickly to all the various changes brought by this stage, including abrupt separation from sow, exposure to unfamiliar piglets, establishment of a new social hierarchy, different housing conditions, and changes in feed sources, etc. (Campbell et al. 2013; Li 2017). Under the combined effect of these stresses, piglets will face two great challenges: first is the damage to the integrity of the intestinal mucosa, which disturbs digestive and absorptive capacity, and increases the susceptibility of intestinal oxidative stress and harmful microorganisms (Xiong et al. 2019); secondly, it will induce a disorder of the intestinal microbial balance in a short time (Etheridge et al. 1984). Both of them are key factors causing diarrhea at this most vulnerable stage. As the research by Montagne et al. (2007), the intestinal structure of the 21-day-old weaned piglets changed dramatically from day 0 to day 5 after weaning.

The relative weight of the small intestine decreased by 18%, and the villus height of proximal jejunum decreased by 40%. Such changes lead to a decrease of intestinal digestive ability (Montagne et al. 2007). On the third day postweaning, Franklin et al. (2002) found a significant increase in *Escherichia coli* (E. coli) populations averaged. This may lead to intestinal diseases, because during the first two weeks after weaning, E. coli plays a significant role in diarrhea (Laine et al. 2008). Under the combined influence of these factors, piglets are likely to suffer from the "so-called" post-weaning diarrhea (PWD), which seriously impacts the growth performance and survival rate of piglets, resulting in significant economic losses in the pig industry.

In 1950, scientists found that the addition of antibiotics to livestock feed accelerated animal growth and was less costly (Li 2017). During the later 35 years, it was observed that under the function of antibiotics, the growth rate of 7-25 kg piglets increased by 16.4% on average, and the feed utilization rate increased by 6.9% (Cromwell 2002). In addition, feeding pigs with antibiotics could help prevent diarrhea, porcine hemorrhagic enteropathy, and other intestinal diseases (M. Ellin Doyle 2001). Consequently, antibiotic supplements in feed can effectively control the risk of weaning. Although piglets may respond more to antibiotic additives compared to older pigs, many farmers apply antibiotics to most stages of pigs in order to maximize economic benefits (Li 2017). Antibiotics were widely used in the pig industry to improve weight gain of piglets and grow-finish pigs, feed conversion, and sows reproductive performance; to reduce the diarrhea and the mortality rate (Li 2017; Wang et al. 2020a). In 2010, the consumption of antimicrobials in food animal production worldwide was conservatively estimated to be 63,151 tons, among which the five countries with the largest consumption were China (23%), the United States (13%), Brazil (9%), India (3%) and Germany (3%), and it is expected to increase by 67% by 2030 (Van Boeckel et al. 2015). The abuse of antibiotics has aroused an alarming public health threat, such as the development of resistant pathogenic bacterial strains and the residual antibiotics in environment and food (Levy and

Marshall 2004; Mehdi et al. 2018). It is estimated that globally by 2050 there will be 10 million deaths per year if we do not respond aggressively. And fighting drug-resistant microbes will also require more medical resources, which will impose a huge economic burden on society (Gupta and Birdi 2017).

With the increasingly serious problem of bacterial resistance in the world, it has become a general trend to ban the use of antibiotics. Sweden became the first country to ban the use of antibiotics in all feeds as early as 1986 (Adjiri-Awere and Van Lunen 2005). Subsequently, the European Union has also banned the use of antibiotic in animal husbandry since January 2006 (Papatsiros et al. 2012). Since January 1, 2017, the US Food and Drug Administration (FDA) has banned the use of antibiotics as feed supplements to help livestock and poultry grow faster (Ekakoro et al. 2019). China's Ministry of Agriculture and Rural Affairs issued Announcement No.194, which stated that from January 1, 2020, antibiotics should be completely banned in feed.

The removal of antibiotics doubled the incidence of diarrhea and the number of subsequent medical treatments for diarrhea, and increased the death rate by about 1.5% (Riksdagsförvaltningen 1997). Cromwell et al. (2002) also reported that the survival rate of pigs from birth to weaning decreased from the previous 90% to 81% after the discontinuation of antibiotics. The best way to avoid these losses may be to increase the weaning age, as this allows piglets to have a more mature gastrointestinal tract and immune system, so as to be better able to cope with weaning stress (Rhouma et al. 2017). But the extending lactation or nursery period is detrimental to production, not only does it affect the sow's reproductive capacity, but it also requires more feeding space, and other additional expense (Hayes et al. 2000). So we need to seek effective measures and approaches to replace antibiotics.

1.1. Antibiotic alternatives

The best potential antibiotic alternatives were discussed at the 3rd Symposium on Alternatives to Antibiotics 2019 in Bangkok, Thailand. These included: (1) vaccines;

(2) microbial-derived products; (3) innovative medicines, chemicals, and enzymes; (4) phytochemicals; and (5) immune-related products ("Alternatives to Antibiotics" 2019).

1.1.1. Vaccines

Since the commercial weaning is carried out at a time when the passive immunity of piglets is declining, vaccination of piglets at this time seems to be a feasible scheme to improve their immunity and overcome stress during weaning. Enterotoxigenic E. coli (ETEC) is an important cause of diarrhea in weaned piglets, because F4 or F18 fimbriae expressed by it, can help bacteria colonize the small intestine and secrete enterotoxins that cause diarrhea (Melkebeek et al. 2013). Several oral vaccines have now been shown to induce the production of secretory IgA in piglets, which can react specifically with F4/F18 to prevent diarrhea (Melkebeek et al. 2013). However, there are still some problems with antigen degradation and intestinal malabsorption when using oral subunit vaccines in large animals (Potter et al. 2008). Parenteral vaccines could mitigate the attack of ETEC to some extent as well (Adjiri-Awere and Van Lunen 2005). Van der Stede et al. (2003) demonstrated that intramuscular administration of F4 fimbriae to pigs would reduce the F4 ETEC excretion upon challenge. However, this kind of injectable vaccine product will consume excessive human and financial resources in the process of use and bring additional stress to piglets, so it cannot be universally applied in intensive pig farming.

1.1.2. Zinc oxide

From a nutrition standpoint, zinc deficiency predisposes to diarrhea because it makes the body more sensitive to intestinal pathogens and may also limit the absorption of water and electrolytes (Wapnir 2000). High doses of zinc oxide dietary supplementation (2000-3000 mg/kg) have been claimed to be beneficial in relieving diarrhea in weaned piglets and therefore widely used in contemporary livestock operations (Xia et al. 2017). Poulsen et al. (1995) reported that, for two weeks after

weaning, supplementing 2500 ppm zinc oxide in piglets could reduce the incidence of diarrhea by 50%. In addition, some findings by Carlson et al. (1999) suggested that early-weaned piglets (< 14 days) fed 3,000 ppm zinc oxide for two weeks after weaning had a 32.48% increase in ADG compared to those piglets fed adequate zinc. Although zinc oxide is very effective in the treatment of diarrhea in piglets, there is a risk of promoting the emergence of drug-resistant strains of E. coli if used in excess, and it can also lead to metal contamination of the environment. Therefore, on 26 June 2017, the European Commission decided to ban the use of zinc oxide after a five-year transition (Juhász et al. 2019).

1.1.3. Probiotics

Based on the important influence of intestinal microbial ecology on the nutrition and pathology of weaned piglets, probiotics can be an effective alternative to antibiotics as intestinal flora regulators. This is because probiotics are believed to have the ability to fight pathogenic microorganisms. It can adhere to the intestinal mucosa to produce antimicrobial compounds, such as bacteriocins and organic acids, thus effectively stimulating the intestinal immune response and improving the intestinal flora (Heo et al. 2013; Cheng et al. 2014). Currently, probiotic products used as an alternative to antibiotics fall into three main categories: yeast, lactic acid producing bacteria (*Lactobacillus*, *Bifidobacterium*), and Bacillus (Gram-positive spore-producing bacteria) (Stein and Kil 2006). Huang et al. (2004) added 0.1% complex *Lactobacilli* preparation to the diet of weaned piglets and found that average daily feed intake（ADFI) and ADG was significantly improved from day 8 to 14 compared with the antibiotic addition group. However, HARPER et al. (1983) added 0.1% *Lactobacilli* preparation to the weaned piglets' diet and found that it did not bring any benefits to the growth of piglets. Such inconsistent information makes it difficult to evaluate the effect of probiotics. The possible negative effects of probiotics on the organism should not be marginalized as well, for example, *Lactobacillus* and *Bacillus* can also affect the normal gut flora in the intestine and may even lead to diseases such

as urinary tract infections (Cheng et al. 2014). Therefore, Chen et al. (2014) argued that stronger supervision and management is needed in the production and use of probiotics. They also pointed out another issue that we should be aware of how to make probiotics remain active during processing and storage as well as after entering the intestine.

1.1.4. Acidifiers

Acidifiers have also been studied extensively as alternatives to antibiotics, because of their potential to reduce the pH value of the gastrointestinal tract, thereby improving the digestibility of nutrients in weaned piglets and inhibiting the activity of pathogenic bacteria (Kil et al. 2011). Acidifier additives available in the diet include mainly organic acids, mineral acids, and acid salts (G. Papatsiros and Billinis 2012). Supplementation of 30 g/kg of citric acid in the diet of weaned piglets at 10 days of age significantly improved their ADG, but at the same time another problem was identified by the authors, the acidification of the diet led to a decrease in palatability (Henry et al. 1985). Even though the enhancement of organic acid supplements for weaned piglet's performance has been proven in many studies, the high manufacturing cost of organic acids makes it difficult to achieve the ideal amount when used in most feed products. Inorganic acids, on the other hand, are more advantageous in terms of price, but many previous studies have indicated that they are not as effective as organic acids (Kil et al. 2005; Dahmer and Jones 2021). Furthermore, regarding the results of acidifier application, there are serious inconsistencies. Partanen and Mroz (1999) summarized 20 trials and found that only 6 trials can prove that dietary acidifiers significantly reduce the pH of the gastrointestinal tract.

1.1.5. Antimicrobial peptides

Antimicrobial peptides is a type of peptide, also regarded as host defense peptides, have been extensively studied in recent years as potential alternatives to antibiotics (Xiong et al. 2019). Antimicrobial peptides, as part of the non-specific defense system

of the organism, are small cationic molecules produced naturally by animals, plants, and bacteria (Wu et al. 2012). Antimicrobial peptides can cross the outer cell membrane of Gram-negative bacteria and the thick cell walls of Gram-positive bacteria, interacting with the bacterial cytoplasmic-membrane to form micelle-like aggregates and finally affecting the normal physiological functions of bacteria. And it is difficult for bacteria to develop resistance to cationic peptides due to the charge-charge and hydrophobic interactions (Hancock 2001). Yi et al. (2015) found that the ADG and ADFI of 21-day-old weaned piglets were dramatically increased, while their diarrhea index was also significantly reduced, and all these changes were attributed to the addition of cathelicidin-BF (C-BF), an antimicrobial peptide derived from the snake venoms of *Bungarus fasciatus*. Peng et al. (2016) investigated the effect of antimicrobial peptide recombinant porcine β-defensin 2 (rpBD2) as an additive for 21-day-old weaned piglets, and ultimately found that dietary supplementation with 5 PD of β-defensin 2 significantly increased their final body weight, reduced the diarrhea index, and helped increase the length of the duodenal villi.

Although the antimicrobial peptide has superior effects, Hancock et al. (2006) identified the following limitations in its clinical use: 1) potential toxicity; 2) potential instability of proteases; 3) high manufacturing costs.

1.1.6. Enzyme preparations

In order to enhance the digestibility of feed nutrients by animals, thereby improving the performance of livestock on the same diet and reduce the cost of the diet, enzyme preparations are added to feeds as an alternative to antibiotics. (Wu et al. 2020). de Lange et al. (2010) reviewed that in addition to improving nutrient utilization, enzymes can also regulate the growth of microorganisms in the gastrointestinal tract by producing a variety of polysaccharide hydrolysis products, and finally improving performance of piglets. But compared to the weaning phase, enzyme preparations may achieve more desirable results during the growth and finishing phase. As in the study by Pettey et al. (2002), they observed a significant

increase in ADG and final body weight after supplementing the diets of grow-finish pigs with a β-mannanase preparation, but the same effect was not observed when added to the diets of piglets weaned at 17 to 23 days of age. Furthermore, when Baas et al. (1996) evaluated the effect of 10 commercially available enzyme preparations added to pig feeds, they found that enzyme preparations in five of these feeds did not play a role, which was suspected of being related to the damage of enzyme activity caused by low pH in the pig stomach.

It is easy to see that these antibiotics alternatives and management options listed above are more or less flawed in terms of efficacy, stability, cost, or environmental safety.

1.1.7. Plant extracts

Plant extract is a product obtained from plant material using different solvents and extraction methods (Shah et al. 2014). These extracts are derived from secondary metabolites produced by plants and their main components are polyphenolic compounds (Liu et al. 2018). Due to its multiple biological activities, such as antibacterial activities, antioxidant activities, and immunomodulatory activities, plant extracts as a strong alternative to antibiotic are worth investigating (Scalbert 1991; Rice-Evans et al. 1996; Chen et al. 2019a, 2021; Su et al. 2018; Xie et al. 2019). Li et al. (2012) reported that supplementing 0.01% mixed essential oil products which contained thymol and cinnamaldehyde in diet can improve the digestibility of dry matter and crude protein in piglets, increase the ratio of villus height to crypt depth in the jejunum, and reduce the number of total aerobic bacteria in the rectum. After supplementing the diet of 21-day-old weaned piglets with red pepper essential oil for 14 days, Cairo et al. (2018) found that when the essential oil was used at 0.15%, it significantly reduced *Enterobacteriaceae* in the jejunum and cecum, and effectively alleviated diarrhea. The other advantage of using plant extracts as feed additive is that it is not only less toxic and residue-free, but also less likely to make bacteria resistant to drugs (Nostro 2006; Hashemi and Davoodi 2011). This is partly due to the fact that

plant extracts are a mixture of many bioactive components, increasing the difficulty of inducing resistance in microorganisms (Redondo et al. 2014). However, this complexity also limits the widespread use of plant extracts themselves, as their internal composition is very susceptible to the type and site of the extracted plant, its harvest time, growth environment and geographical location, storage conditions and extraction methods. Make it difficult to precisely apply in the required occasions (Liu et al. 2018).

1.1.8. Other alternatives and policies

In addition to the antibiotic alternatives mentioned above, there are also prebiotics, synbiotics, amino acids/proteins, copper, vitamins, clay minerals, etc. In recent years, many new antibiotic alternatives have emerged, including nucleotides, thymine, phages, etc. However, because of the relatively small amount of research data on these above antibiotic alternatives, there is currently not enough to support their development to the level of practical applications.

Some scientists also recommended that the best course to the de-antibiotic policy governing pork production might be to improve management equipment and the farm environment to lessen the exposure of pigs to infectious diseases (Adjiri-Awere and Van Lunen 2005). Although animal welfare legislation has prompted the pig industry to improve the quality of flooring, living space, clean water, etc. in the farming process over the past few decades (Li 2017); however, further improve the living environment of pigs requires the application and improvement of a wide range of modern equipment such as flooring equipment, piggery ventilation and cooling systems, automatic pig feeders, barn heating equipment and even pig gestation crates, farrowing crates, piglet nursery boxes, etc., which will undoubtedly put a huge economic pressure on farm owners (Li 2017). In this regard, research on green and efficient alternatives to antibiotics is still very necessary.

1.2. Tannin

Tannins are astringent polyphenols that constitute one of the most abundant and widely distributed groups of natural products in the plant kingdom (Tsao 2010). It is widely used in human traditional medicine against chronic diarrhea because of its abilities to protect against intestinal bacteria and parasites (Lewis and Elvin-Lewis 2003; Parisi et al. 2018). The unique structures and mechanisms of tannins provide beneficial effects in pig farming, which are related to their antimicrobial, antioxidant and radical scavenging, and anti-inflammatory activities (Huang et al. 2018). Hence, tannin has potential as an alternative to antibiotic.

1.2.1. Tannin classification

The nutritional effects of tannins will vary with structural changes (Barry 1989). But this concept has not been taken seriously and clearly mentioned in many feeding trials (Mueller-Harvey 2006). Already in 1920, Freudenberg (1920) had classified tannins into two categories based on their structure, hydrolysable tannins and condensed tannins (**Figure 1.1**). Hydrolysable tannins are formed by esterification of a polyol carbohydrate core (usually d-glucose) with phenolic acids (such as gallic acid or ellagic acid) (Meyers et al. 2006). Which can be hydrolyzed under certain conditions, such as with hot water or with enzyme (e.g. tannase) (Khanbabaee and Ree 2001). Condensed tannins are hardly hydrolyzed, they are formed by linking C-4 of a catechin with C-8 or C-6 of the next monomer catechin (Khanbabaee and Ree 2001).

1.2.2. Tannin source

Tannin is the most abundant compounds after cellulose, hemicellulose and lignin in plants and widely distributed in animal feeds and forages such as bean pods, acorns, oak leaves, seed meal, sorghum grains, etc. (Kumar and Singh 1984; Arbenz and Avérous 2015). Condensed tannins are commonly found in some forage legumes, trees and shrubs; hydrolysable tannins are less common in forage grasses and more common in some fruits and in the bark, wood and leaves of trees (Sharma et al. 2019;

Min et al. 2003). Hydrolyzed tannins and condensed tannins may be present in the same plant as well, such as grapes and tea.

Brillouet et al. (2013) found that tannins may be formed by polymerization in new chloroplast-derived organelle, which are then bound by two chloroplast envelopes and then stored by admixture into the vacuoles through vacuolar invaginations. Therefore, tannins may be found in most parts of plant tissues, including branches, bark, roots, seeds and fruit, etc. (Haslam 1989). However, the number of tannins contained in the plant varies greatly depending on the plant part. For example, the tannins of pine, oak, or mimosa are mainly found in the bark, while the tannins of quebracho and chestnuts tend to accumulate in wood (Arbenz and Avérous 2015).

1.3. Biological properties of tannins

The ability to complex with proteins is the most important biological property of tannins. The word "tannin" comes from the French word “tan”, first used by the French chemist Séguin to describe the organic material used to transform skin into leather (Falcão and Araújo 2018). In the tanning process, tannins interacts with collagen and other proteins in the leather under the control of hydrophobic interaction, forming hydrogen bonds and creating complexes, making the leather stable and non-decaying (**Figure 1.2**) (China et al. 2021). This property is also common in our lives, when drinking red wine, the proteins in the saliva will combine with the tannins in the grapes or oak barrels to make the tongue feel astringent (Soares et al. 2017).

Plants produce tannins, originally as a defense mechanism to protect themselves from insects, bacteria, fungi and viruses (Dubreuil 2013). These defensive actions are based in many cases on the ability of tannins to bind to proteins.

1.3.1. Antiviral

Tannins have been found to have the ability to inhibit a variety of viruses, such as influenza A virus, human immunodeficiency virus, herpes simplex virus,

adenovirus, feline calicivirus, and so on (Vilhelmova-Ilieva et al. 2019). The antiviral activity of tannins is affected by molecular weight and extraction method. According to the study of Theisen et al. (2014), comparing the antiviral activities of different tannins, it was found that high molecular weight tannin extracts, such as tannic acid with a molecular weight of 1702 g/mol, was effective in inhibiting influenza A virus receptor binding and neuraminidase activity, and when the molecular weight was less than 500 g/mol, such as gallic acid inhibits neuraminidase but did not inhibit blood coagulation and which suggesting lower resistance activity. The inhibition of viruses by tannins may be accomplished by inhibiting viral replication, as found in a study of proanthocyanidins against herpes simplex type 1 (HSV-1) and type 2 (HSV-2) infections, it could inhibited the replication of HSV-1 and HSV-2 in vitro by altering the functions of the envelope glycoproteins gD and gB (Terlizzi et al. 2016). In addition, tannins inhibit viruses by limiting their adsorption to cells. Ueda et al. (2013) found that tannins from persimmon could inhibit the infectivity of 12 viruses, including norovirus, to below the detection limit, and suggested that the mechanism of inhibition is likely to be the interaction of tannins with viral particle proteins to hinder their adsorption to target cells.

1.3.2. Aantibacterial

The production of tannins by plants also helps them to fight against bacteria, even in the spoilage stage after plant death, the tannins could interact with proteins and membranes to prevent them from being degraded by microorganisms (Mehansho et al. 1987). The antibacterial activity of tannins is achieved by adhering to bacterial membrane to produce competitive inhibition of enzymes (Upadhyay 2011). Tannins are also capable of inactivating microbial adhesin, cell envelope transport proteins, etc. (Cowan 1999). Banso et al. (2007) studied the antibacterial effect of tannin from *Dichrostachys cinerea*, found whether it is E. coli, *Staphylococcus aureus*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Shigella boydii*, tannins all show antibacterial activity, and the ability increased with increasing dose. Reggi et al. (2020) found that

when quebracho tannin and chestnut tannin extracts were mixed in a 1:1 ratio, they could produce faster and more sustained inhibition of ETEC samples in vitro. This may be hopeful to be one of the treatments of piglet diarrhea.

Another remarkable thing is that tannins also have a good inhibitory effect on drug-resistant bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to many antibiotics, showed bactericidal effects when treated with tannins (MIC=0.78 mg/mL). Meanwhile, morphological observations revealed that tannin disrupted the integrity of the cell wall, allowing the release of cytoplasmic contents (Adnan et al. 2017). Dong et al. (2018) demonstrated the antibacterial effect of tannin against MRSA as well, during culture, tannin may bind directly to the peptidoglycan of the cell wall of resistant bacteria, reducing biofilm formation. According to Kusuda's research results, proanthocyanidins purified from an extract of the fruit peels of *Zanthoxylum piperitum* decreased the activity of β-lactamase in MRSA, and greatly reduce the stability of bacterial cell membrane of MRSA (Kusuda et al. 2006).

The ability of tannins to combat drug-resistant strains is attributed to an antibacterial mechanism different from that of antibiotics (**Figure 1.3**). The antibacterial pathways of antibiotics are outlined by Kohanski et al. (2010) and mainly include inhibition of DNA replication, inhibition of RNA synthesis, inhibition of protein synthesis, and inhibition of cell wall synthesis. It is not difficult to find that most situations require antibiotics to get inside the bacteria in order to work. First, bacteria will try to prevent the entry of antibiotics, such as dealing with polymyxin which can penetrate and cause membrane damage, by replacing the negatively charged phosphate on each lipid A in the outer membrane with a small neutral ethanolamine to reduce the negatively charged groups on the outer membrane, greatly reducing the affinity of the cationic polymyxin for the membrane and preventing its entry (Khondker and Rheinstädter 2020). Secondly, bacteria will try to eliminate antibiotics entering the cell, for example, Gram-negative bacteria are more likely to select and express efflux pump genes than Gram-positive bacteria, forming an efflux

pump on the extracellular mold to exclude drugs from the cell (Webber and Piddock 2003). As a result, it is difficult for the antibiotics to work again.

In contrast, tannins have the characteristic of binding extensively to proteins under certain conditions, so they can react with proteins on the membrane without entering the cell, reversibly or irreversibly disrupting the membrane structure as well as its function to achieve antibacterial activity. As reported by Tintino et al. (2016), tannins can inhibit the formation of the *Staphylococcus aureus* efflux pump. It is able to bind to the efflux pump on the cell membrane as well as membrane proteins alters their permeability. On the other hand, iron is required to establish the efflux pump and tannins can chelate with iron to reduce its supply. In addition, tannins can inhibit biofilm formation and altered the conformation of surface fimbriae, to prevent the adhesion of bacteria to the intestinal epithelium (Girard and Bee 2020). This is important for the prevention of intestinal diseases.

After the antibiotics enters the cell, bacteria produce enzymes that destroy the antibiotics structure by hydrolysis, oxidation, Lyases, etc., or modify the antibiotics, thereby inhibiting or deactivating it (Wright 2005). Bacterial resistance can also be achieved through modification of the intracellular target (including ribosomes, and enzymes related to biological metabolism or DNA replication) (Levy and Marshall 2004). Therefore, tannins may be more likely to act against these enzymes in common bacteria or even drug-resistant bacteria than antibiotics, thus achieving antibacterial effects.

It is worth mentioning that bacterial toxins are an important cause of diarrhea in weaned piglets. Such as the toxins produced by the ETEC and the α- and β- toxins produced by *Clostridium perfringens* type C, which enter the intestine will easily cause diarrhea (Mesonero-Escuredo et al. 2018). As a kind of protein, the toxin may be released by bacteria in the feed or in the intestine of the animal. If the feed is supplemented with tannins, the toxin may bind to it and thus its ability to damage the intestine is attenuated. According to the research of Elizondo et al. (2010) , the

quebracho and chestnut tannins not only inhibit the activity of C. perfringens, but also inhibit the toxicity of alpha and epsilon toxins. Tannins may also directly regulate the transcription of toxin genes to reduce toxin production (LEE et al. 2009).

1.3.3. Anti-oxidation

Tannin can function as antioxidants by providing hydrogen atoms or electrons (Quideau et al. 2011). It can also chelate metal ions such as Fe^{2+} and Cu^{2+} to interfere with the production of the Fenton reaction, thereby inhibiting the production of the hydroxyl radicals to act as antioxidants (Yin et al. 2015), meanwhile the antioxidant capacity of tannins is further enhanced by the increase in molecular aggregation (Sieniawska 2015).

The proanthocyanidins from quebracho have been shown to have potent activity in scavenging free radicals, and their addition to beef patties would improve the stability of lipids in meat (Fruet et al. 2020). Liu et al. (2012) also found that adding chestnut tannin to rabbit diets could significantly reduce thiobarbituric acid reacting substances values in meat and inhibit lipid peroxidation of rabbit meat. Additionally, piglets are sensitive to oxidative stress during weaning. Some studies have shown a significant decrease in superoxide dismutase activity and inhibition of antioxidant systems, as well as a significant increase in malondialdehyde, NO and H_2O_2 concentrations after weaning (Zhu et al. 2012). An imbalance between these reactive substances and the biological ability to scavenge reactive intermediates can damage cellular macromolecules, leading to irreparable oxidative damage and cell death (Yin et al. 2014). Liu et al. (2020) examined the effect of chestnut tannin on the antioxidant status and immune function of 28 days of age weaned piglets and observed that 0.1% tannin supplementation significantly increased the concentration of total antioxidant capacity and IgM in piglets' serum, and an improvement in intestinal villi morphology was observed. However, Gessner et al. (2017) also did not find any antioxidant effect of tannins from grapes in their experiments on piglets, and therefore speculated that

these bioactive substances would have antioxidant or anti-inflammatory effects only after the animals had received pro-oxidant treatments.

1.3.4. Anti-inflammatory

In traditional medicine, many tannin-rich plants are used as medicinal herbs to treat inflammatory diseases such as rheumatism, cholecystitis, gastroenteritis and urogenital tract inflammation-associated diseases (Piwowarski et al. 2014). Inflammation is a pathological process closely connected with oxidative stress, and in most cases they occur together and are mutually induced and synergistic, being the pathogenesis of many chronic diseases (Biswas 2016). The anti-inflammatory properties of tannins are therefore inextricably linked with their antioxidant properties. Zhang et al. (2006) showed that procyanidin dimer B2 can blunt cyclooxygenase 2 (COX-2) expression by stabilizing IκB protein and inhibiting NF-κB activation, while COX-2 has a potential role in a variety of inflammatory diseases. Active substances such as tannins are able to downregulate COX-2 expression, reducing will inhibit the stimulation of macrophages by lipopolysaccharides, resulting in a decrease in NO levels and alleviating inflammation. Just like the study of Park et al. (2014), both ellagitannins extracted from black raspberry seeds and proanthocyanidins extracted from grape seeds showed inhibition of NO production. Myeloperoxidase is thought to be associated with inflammation as well as oxidative stress in the body. Elevated activity may be associated with polymorphonuclear leukocytosis in inflammatory conditions, and tannins have been shown to reduce the activity of this enzyme in mice (Soyocak et al. 2019). Kitabatake et al. (2021) recently investigated the therapeutic effect of persimmon tannin on colitis in mice and found that the tannin was able to inhibit the expression of inflammatory cytokines (IL-1β) and chemokines (CXCL1) and improve the immune response to treat colon inflammation in mice. Fiesel et al. (2014) supplemented the diets of piglets at 5 weeks of age with 1% grape seed and grape pomace powder extracts, which significantly reduced the expression of ICAM1

and IL8 in the duodenum and ICAM1, IL1B, IL8 and TNF in the colon, exerting a significant anti-inflammatory effect.

1.3.5. Antiparasitic

Traditionally, some tannin-rich plant groups are effective anthelmintics against parasites. For example, in Africa, the tannin-rich *Vernonia elaeagnifolia DC.* (Asteraceae) is used orally as a folk medicine against leeches (de Boer et al. 2010). *Ascaris suum* are the most frequently parasitic disease in pig herds and are a challenge that many farms must face because infection can affect growth or cause diarrhea. Williams et al. (2014a) found that Prodelphinidins (a condensed tannin) were able to inhibit motility and migratory activity of newly hatched Ascaris larvae. In addition, tannins have been shown to have inhibitory effects on helminth parasites. Exposure of *Oesophagostomum dentatum* to 1 mg/mL of tannins extracted from hazelnut peel for 12 h resulted in a virtual loss of motility and significant damage to the cuticle of the worm, in addition, tannins effectively inhibited the molt of the worm from the L3 to L4 stage (Williams et al. 2014b). The ellagitannins (punicalagins and punicalins) in immature pomegranate peels were found to have possible in vitro antiplasmodial activity by Dell'Agli et al (2009).

It is speculated that the anthelmintic effect of tannins may be related to the strong binding capacity to proteins, and it must be noted that the cuticle of Ascaris lumbricoides is an important defense tissue, and the collagen in it contains higher levels of proline than vertebrate collagen, which increases the affinity for tannins binding (Fujimoto and Kanaya 1973). For adult parasites, aside from the cuticle, tannins are a potential threat to the intestine and uterus, and these effects may affect the normal physiological functions of Ascaris (Hoste et al. 2006). Moreover, there is a possible speculation that tannins inhibit parasites by binding to functional enzymes released by the parasite and interfering with metabolic and other pathways (Hoste et al. 2006).

At present, not only bacterial resistance to antibiotics but also animal parasite resistance to anthelmintics has become a problem that we need to face squarely (Shoop 1993). Ivermectin, originally a highly effective anthelmintic, has lost its efficacy in many areas with the emergence of drug resistance. The mechanisms of parasite resistance to ivermectin are manifested in two main ways: increased excretion and metabolism of the drug by parasite cells, and alteration of the receptor sites for the drug or reduction in receptor expression (Prichard 2007). Although the antiparasitic mechanism of tannins has not been studied conclusively, the ability of tannins to bind extensively to proteins makes them promising as novel antiparasitic agents.

1.4. The advantages and limitations of tannins versus antibiotics

Over the past several decades, various alternatives to antibiotics and additional measures have been tried to reduce the use of antibiotics (Callaway et al. 2021). Despite the wide variety of projects being studied, there are actually few alternatives that can rival antibiotics due to a variety of reasons. To find a more high-quality alternative, which requires a deeper understanding of the advantages and disadvantages of antibiotics.

1.4.1. Main function and defect of antibiotics

The most important function of antibiotics that can play a role in several critical stages of high risks, such as piglet weaning, is the inhibition of microorganisms. Whether added to feed to inhibit the activity of microorganisms that may be present in the feed or entering the animal's intestine to act on most intestinal microorganisms, antibacterial is a central factor. On the other hand, it is not clear why antibiotics promote animal growth, but it is plausible to infer that it is also related to the effect of antibiotics on intestinal flora (Low et al. 2021). Firstly, a strong evidence is that the use of antibiotics in germ-free animals does not promote their growth (Coates et al. 1963; Freeman et al. 1975). Next, exogenous injectable administration also promotes

the growth of animals, and these antibiotics all can reach the intestine (McLaren et al. 1990; Zhang et al. 2013). When the number of gut microorganisms decreases, some of the nutrients previously used by them can be used by the intestine to promote animal growth. Furthermore, animals fed antibiotics are said to have thinner small intestinal walls, more elongated and regular intestinal villi, and less connective and lymphoid tissue. This allows for easier absorption of nutrients and reduced energy expenditure, further helping animals to improve performance (Gaskins et al. 2002). Although it is unclear whether there is an association between microbial and gut changes, Coates et al. (1955) noted that there may be no relationship between decreased gut-weight due to penicillin feeding and decreased gut microbial load.

The main disadvantages of antibiotics are bacterial resistance, body residues, and environmental contamination, etc., which have been known to be safety issues (McEwen and Fedorka-Cray 2002). But another point that is easily overlooked is that antibiotics can kill some of the beneficial microorganisms in the gut that prevent harmful bacteria from attacking and stimulate the renewal of intestinal cells and the formation of more intestinal villi (Low et al. 2021).

From this point of view, the development of ideal antibiotic alternatives, attention must be paid to non-polluting, residue-free and non-toxic side substances that do not induce bacterial resistance, exert antimicrobial activities similar to those of antibiotics, but at the same time, it does not kill intestinal bacteria indiscriminately and are natural, safe and inexpensive.

1.4.2. Advantages of tannin as an alternative to antibiotics

1.4.2.1. Bactericidal activity

The most important advantage of tannins is their bactericidal effect. Callaway et al. (2021) suggested that an effective alternative to antibiotics needs to replicate the benefits of antibiotics and have a bactericidal effect similar to that of antibiotics. Tannins have a desirable bactericidal effect, and based on their bactericidal

mechanism, it is possible that only one type of tannin can fight against a wide range of bacteria, which is an advantage over antibiotics.

1.4.2.2. Anti-drug resistance

This is the most notable advantage of tannins. The increase in bacterial resistance caused by the use of antibiotics as feed additives has begun to threaten humans, especially in Gram-negative bacteria (*Salmonella spp.* and E. coli) (Butaye et al. 2003). As above mentioned, bacteria develop antibiotic resistance through mutations in genetic material leading to the modification of specific targets or the formation of efflux pumps or biofilms that prevent antibiotics from entering the cell and reaching the target (Gupta and Birdi 2017). By acting on various functional proteins, tannins can kill or inhibit bacteria, even drug-resistant bacteria by a more direct mean. Moreover, the inhibitory effect of tannins on toxins makes them potentially more potent against harmful bacteria. At the same time these mechanisms and the complexity of the tannin extract composition are also more difficult to produce resistant bacteria.

1.4.2.3. Multiple biological activities

The presence of multiple bioactivities may make tannins more capable of controlling the risk of disease in piglets during the early weaning phase (Wei et al. 2017). Considering the complex mechanisms that cause PWD, tannins not only regulate intestinal flora, but they may also act in multiple ways to prevent PWD by improving oxidative, inflammatory, and immune status of the livestock intestine to enhance intestinal health and mucosal integrity, and even by preventing parasitic infections.

1.4.2.4. Low residue

In addition, tannin additives also have the advantage of low residue. Tannin absorption rate in the intestine is very low, only between 5-10% of the total, absorbed tannins or phenolic small molecules will also enter the liver through methylation,

glucuronidation and other metabolic pathways into hydrophilic substances and then eliminated from the body with urine (Gessner et al. 2017). Drinking tea is a good example. Most of the polyphenols in green tea are metabolized into small molecules in the intestine and excreted through the kidneys, and the rest absorbed by the body in small portions still be able to play a potentially protective role (Clifford et al. 2013). Of course, the possible risk of toxicity of these small molecules cannot be ruled out, so it is necessary to carefully identify the type of tannins and the dosage to be used.

Antibiotics, however, tend to remain in internal organs and muscles, and more importantly, they have a cumulative effect, and consumption of animal products fed with antibiotic-containing feeds carries the risk of increased drug-resistant bacteria (Zhang et al. 2021).

1.4.3. Limitations of tannins

1.4.3.1. Anti-nutritional

As a defense mechanism in plants, tannins can produce astringency to reduce palatability and thereby protect plants from animals (Barbehenn and Peter Constabel 2011). This astringency is caused by the precipitation of tannins bound to salivary proteins, especially proline-rich proteins, in addition, tannins have the ability to interact with dietary proteins, forming covalent or non-covalent bonds that shield proteins from hydrolysis by enzymes, then dissociated under certain circumstances, tannins may also bind to various digestive enzymes and inhibit their activity, such as α-amylase, α-glucosidase and pancreatic lipase associated with starch and lipid digestion, and pepsin and pancreatic protease related to protein digestion (Cirkovic Velickovic and Stanic-Vucinic 2018). Additionally, it is reported that except for protein, tannins can form reversible and irreversible complexes with polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals, in this way the presence of tannins may lead to more nutrient loss (Kumar 2011). Therefore, the presence of these properties makes tannins generally considered to have anti-nutritional properties.

1.4.3.2. Toxicity

Tannins in plants are also thought to be one of the causes of livestock poisoning. It may be broken down and absorbed in the gastrointestinal tract and may cause lesion in the liver and kidneys of livestock after entering blood circulation (Filippich et al. 1991). Smeriglio et al. (2017) suggested that hydrolyzed tannins could produce toxic substances to the liver when hydrolyzed in the intestine, while condensed tannins is difficult to hydrolyze because of the C-C (4–8 or 6–8) bond linkage. Therefore, the toxicity of condensed tannins is theoretically low. However, the mechanism of this toxic effect is currently unknown (Jouany and Morgavi 2007).

1.4.3.3. Complexity

The composition and concentration of tannins extracted from plants can be affected by various factors, such as the type of plants extracted, the site of extraction, the region of growth, the period of growth, and the storage and processing of the tannin products (Liu et al. 2018). In addition, the molecular weight of tannins ranges from 500 to 30,000 Da and contains a wide variety of structural elements and different degrees of polymerization, making the structure very complex. Highly polymerized tannins are difficult to analyze, and the number of possible isomers increases with the degree of polymerization (Serrano et al. 2009). The structure of tannins, especially their degree of polymerization, in turn determines to some extent their properties (Jerez et al. 2007). It follows that this complexity makes it difficult to determine the effective types, the correct dosages, and the specific action mechanisms of tannins in practical applications.

1.5. Quebracho tannin

Quebracho tannin (QT) was selected as the experimental object in this study, which is very abundant in the wood of Kenwood (*Schinopsis lorentzii*) from South America (Buccioni et al. 2017). QT was chosen first because of its low cost and easy extraction through hot water, so it is one of the most popular tannin products on the

market today (Bellotti et al. 2012; Redondo et al. 2014). Secondary, condensed tannins usually have a stronger antimicrobial capacity compared to hydrolyzed tannins (Krzyzowska et al. 2017). Finally, the stability of concentrated tannins is also an important aspect we consider. Concentrated tannins can maintain a relatively stable structure in a complex environment, it can not only inhibit the growth of mold and toxin production in the feed, but also maintain a strong biological activity for a long time even after entering the intestinal tract. Also, it is not easy to break down into small molecules into the blood, causing stress to the liver and kidneys. On the contrary, hydrolysable tannins are easily degraded or hydrolyzed by microorganisms in feeds and in complex environments such as the gastrointestinal tract: on the one hand, the phenolic small molecules generated by degradation no longer have the same strong ability to precipitate proteins as tannin macromolecules; on the other hand, the presence of polyphenols in feeds as well as hydrolyzed phenolic acid, gallic acid and ellagic acid may affect the action of hydrolysable tannin additives, causing a misjudgment of the experimental misinterpretation of the results.

QT has been extensively used in the ruminants feed because it can reduce the degradation of feed protein by rumen bacteria to increase the amount of rumen bypass protein, thus improving the digestion efficiency of nitrogen and reducing the generation of methane (Piñeiro-Vázquez et al. 2018; Frutos et al. 2000; Beauchemin et al. 2007; Piluzza et al. 2014). The study has shown that adding QT increases the amount of crude protein flowing out of the rumen and into the duodenum, mainly by binding to the dietary crude protein to prevent it from being degraded by rumen bacteria (Castro-Montoya et al. 2018). But there are few reports on applying QT to piglet diet. Su et al. (2016) studied the effects of adding QT on nursing pigs and found that the addition of tannin at 0.1% level had no positive effect on the growth and diarrhea of pigs. Caprarulo et al. (2020) added a 1.25% QT and chestnut tannin mixture to the diet of weaned piglets, but did not find any effect on piglet growth performance.

In contrast, there are many studies on the application of QT in rabbit nutrition. Zotte et al. (2009) added 1% and 3% of QT extracts to the diet of fattened rabbits and found that it had a significant beneficial effect on live performance. It was also found that the addition of QT, especially at 3%, considerably increased the weight gain and feed efficiency of rabbits, and the slaughter weight was significantly higher. As well, the number of E. coli in the cecum decreased significantly, demonstrating the antibacterial efficacy of QT. Mancini et al. (2019) also studied the efficacy of QT in rabbits and found that lower levels of addition (0.3% and 0.6%) did not affect growth performance, feed digestibility or fecal microorganisms, but an increase in the concentration of catalase and glutathione peroxidase in plasma was observed, suggesting a trend towards increased antioxidant capacity. Parisi et al. (2018) similarly studied the effects of QT at 0.3% and 0.6% spiked levels on growth and health in rabbits, confirming that QT was not toxic and contributed to the reduction of *Eimeria spp.* oocysts excretion. It was also found that 0.6% was also effective against E. coli to some extent.

Based on these evidence, QT in concentrated tannins has the potential to function as a pig feed additive and even replace antibiotics, which requires urgent research and development. Therefore, the usefulness of QT in pig farming was investigated in this study.

1.6. Feeding value of tannin-related by-products

In fact, there are precedents for the use of tannins in pig nutrition. The Iberian pig is indigenous to the forests of the Iberian Peninsula and has been active for centuries in a large part of the southwestern Iberian Peninsula (Bottos 2019). The area where they live is covered with large acorns and chestnut forests, so these acorns and chestnut fruits have become part of the diet of these Iberian pigs. The maturation of acorns occurs from the beginning of November to the end of February. Iberian pigs use a lot of mature acorns as feed in the later fattening stages, which allows them to

produce high quality pork with very specific characteristics (Lopez-Bote 1998). It is said that these pigs were so fond of acorns that can even consume 7-10 kg per day (Rey et al. 1998). And acorns are rich in tocopherols, proanthocyanidins, ellagitannins and other tannin-like substances (Bolling et al. 2010). The researchers did not find cases of poisoning in Iberian pigs due to excessive consumption of acorns during long-term foraging. In addition, these active substances play an important role in the regulation of lipid oxidation, and the high content of unsaturated fatty acids (UFA) in the meat and meat products of Iberian pigs may be related to this (Decker et al. 2000).

There are many similar examples of extensive or semi-extensive pig farming that has been used in some parts of Europe for a long time. For example, pigs are stocked in chestnut groves during the fattening period, which makes chestnuts easily available to pigs. Not only does it reduce feed costs, but it also produces higher quality pork. (Lebret 2008; Bělková et al. 2017; Echegaray et al. 2020a). Pugliese et al. (2013) in a study in pigs (*Cinta Senese*), pointed out feeding with chestnuts (90%) and bran during the last month of the fattening period can help increase content of intermuscular fat and the UFA. Jesús et al. (2016) supplemented commercial feed with 25% dried chestnuts and found an increase in slaughter rate and monounsaturated fatty acids in the muscle of Celta pigs.

With the reduction of forests and the privatization of land, these classic farming models have become very rare. Although high quality pork has been produced by imitating this farming model, it is very expensive. So it would make sense to find cheap, and tannin-rich feeds for the pig industry.

Chestnuts is a widely popular food in Japan, and according to statistics, Japan harvested 18,700 tons of chestnut kernels in 2017, however, this did not meet the domestic consumption demand and $1.8 millions of chestnut kernel were imported in the same year (Tridge, 2020). In the process of making chestnuts into food, a large number of chestnut shells are produced and discarded. It is well-known that chestnut fruits are rich in tannins. Interestingly, Vekiari et al. (2008) determined the

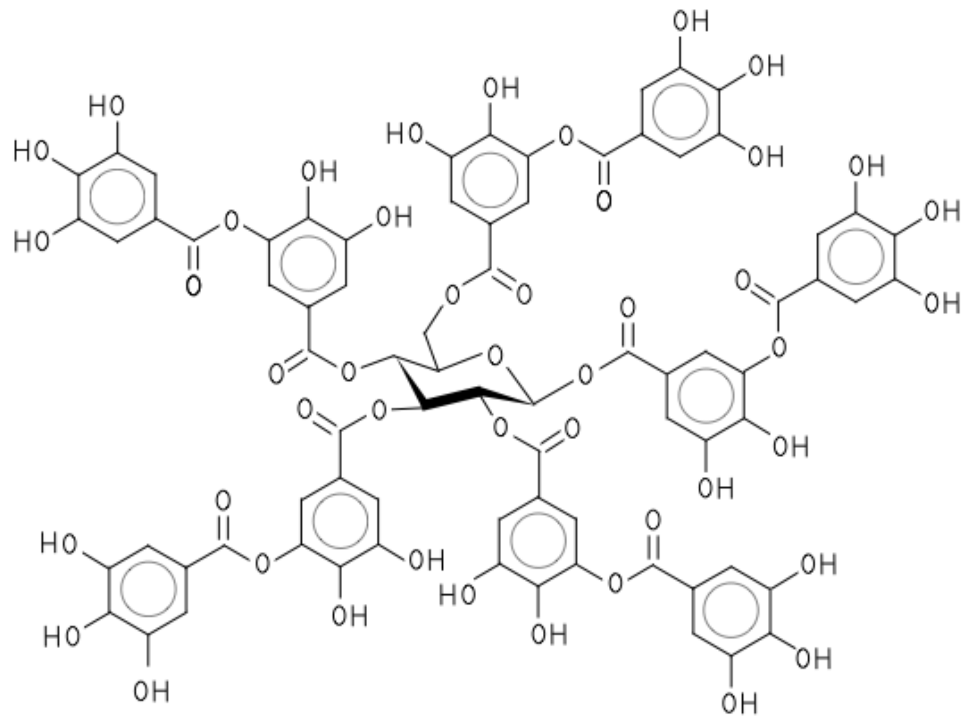
concentrations of tannins in different chestnut fruit tissues, and found that tannins were mainly concentrated in the integument (inner shell) and the pericarp (outer shell) and mainly in the form of ellagitannins. Ellagitannins belong to the hydrolysable tannins class of polyphenols, which are widely distributed in fruits, nuts and plant seeds (Landete 2011). Barreira et al. (2008) found that the presence of tannins in chestnut shells makes it have strong antioxidant capacity, especially for lipid oxidation. In addition, ellagitannins also have biological activities such as anti-inflammatory, anti-malarial and anti-microbial activities (Reddy et al. 2007; Bazylko et al. 2013; Granica et al. 2015). Tomažin et al. (2020) reported that supplemented with 3% chestnut tannin extract in diet helps delay the oxidation of pig ham. However, compared with extracting active ingredients through a complex process, feeding chestnut shells directly can save costs. Although there are some literatures mention the use of chestnuts in pig breeding, the research on the use of chestnut shells is very limited.

Thus, it is necessary to explore the potential of these tannin-rich by-products discarded during processing for use in pig farming. However, it is important to note that chestnuts are extremely seasonal and difficult to cover the long feeding stage, so it is urgent to develop a reasonable storage procedure. Freezing can preserve chestnut shells to prevent deterioration, but it is not suitable for large-scale farming because of its high cost. Silage is a common practice for preserving plant-based feeds and plays an important role in livestock production systems (Jayanegara et al. 2019). It should be noted that active substances in chestnut shells (e.g., hydrolysable tannins) may be significantly degraded by microorganisms during storage (He et al. 2018). In view of this, it is also necessary to assess the feasibility of chestnut shells silage to develop reusable uses of these wastes in large-scale farming operations.

1.7. Summary and research objectives

1. Tannins as a feed additive has been a hot topic in recent years as it has many biological activities and has advantages over antibiotics in terms of antimicrobial properties. However, relatively little research has been done on monogastric animals, especially pigs, we conjecture that tannin has the potential to become a feed additive or even a replacement for antibiotics in the pig industry. Considering that the hydrolysable tannins may undergo complex changes in the feed and gastrointestinal tract, and is easily absorbed by the intestine to burden the liver, while condensed tannins are relatively stable and may be present in the feed and intestine for a long time to play a role. Therefore, a condensed tannin product, MGM-P (containing more than 50% QT), was selected for this study in an attempt to determine its effects as a feed additive on the growth and health of pigs at various stages. In order to avoid the anti-nutritional properties of tannins and to maximize the antimicrobial properties of tannins, we have to find a more appropriate dosage of additive. Summing up past experiences, we began with a low-level addition trial. First, we tried its effect in weaned piglets' diets, then we tried a more reasonable amount and compared it with antibiotics. After obtaining the appropriate addition level, we tried to use it in other stages of pig breeding to provide data to support the possibility of QT as an alternative to antibiotics.
2. Supplementation of chestnut shell by-products to finishing feed to study its effects on growth, health and meat quality of pigs. On the one hand, the aim is to try to replicate the traditional farming experience to produce high-quality pork at a lower cost; on the other hand, it is to explore the possibility of reusing this industrial production waste in order to reduce the waste of resources. Finally, the experiment will be conducted to discover the feeding value of silage chestnut shells to find a reasonable storage mean to overcome the seasonality of these by-products and to make them suitable for today's large scale pork production contributing to the sustainability of the pig farming industry.

Gallotannin
(Hydrolysable tannins)



Proanthocyanidin
(Condensed tannins)

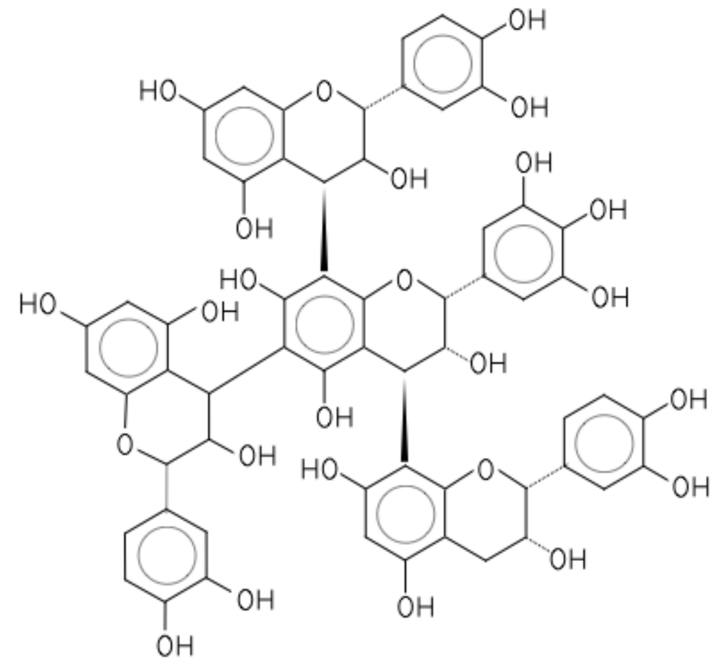


Figure 1.1. Representative tannins molecular structure. Gallotannin (hydrolysable tannins) and proanthocyanidin (condensed tannins).

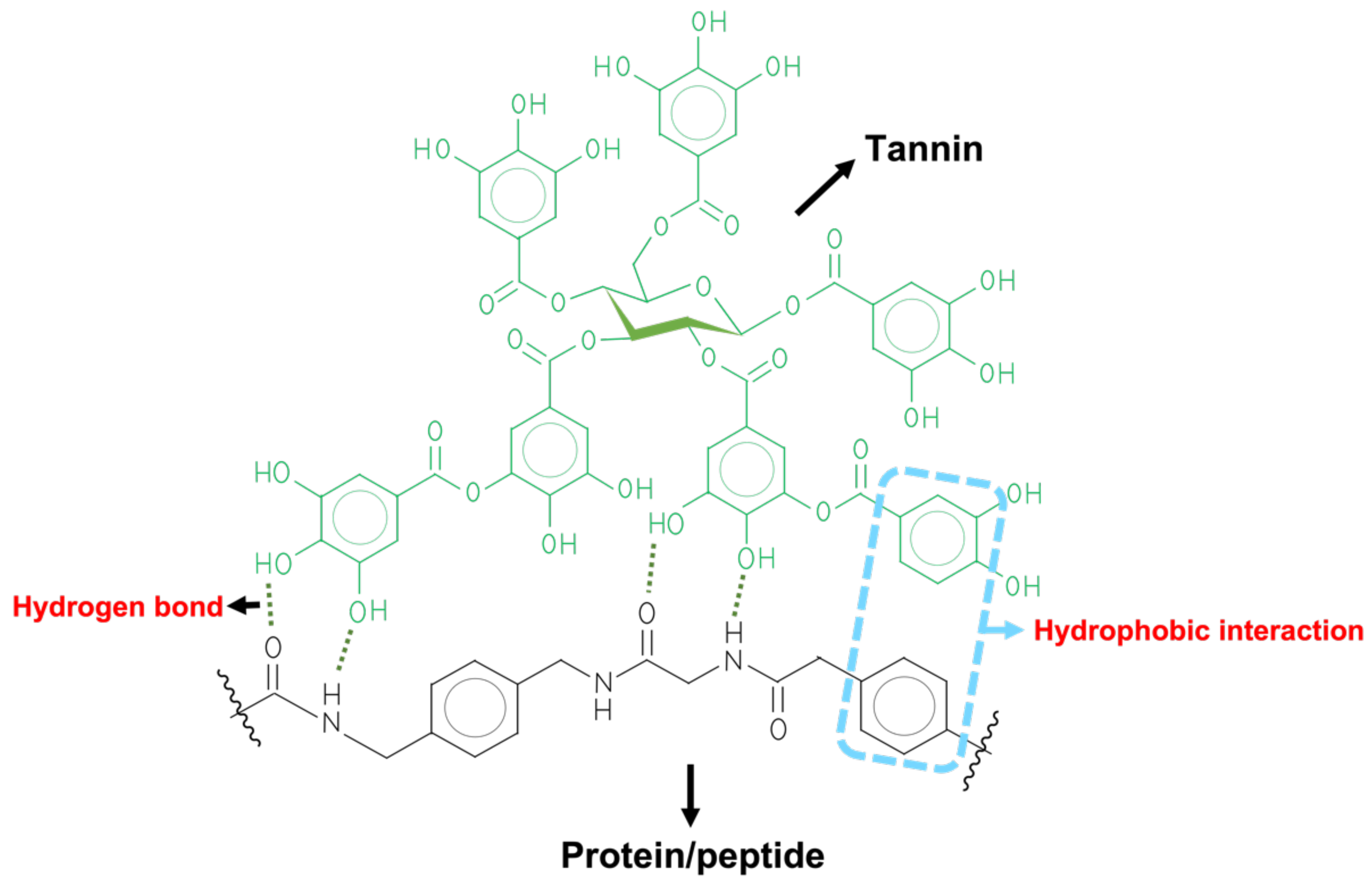


Figure 1.2. The complexation of tannins with amino acids in protein.

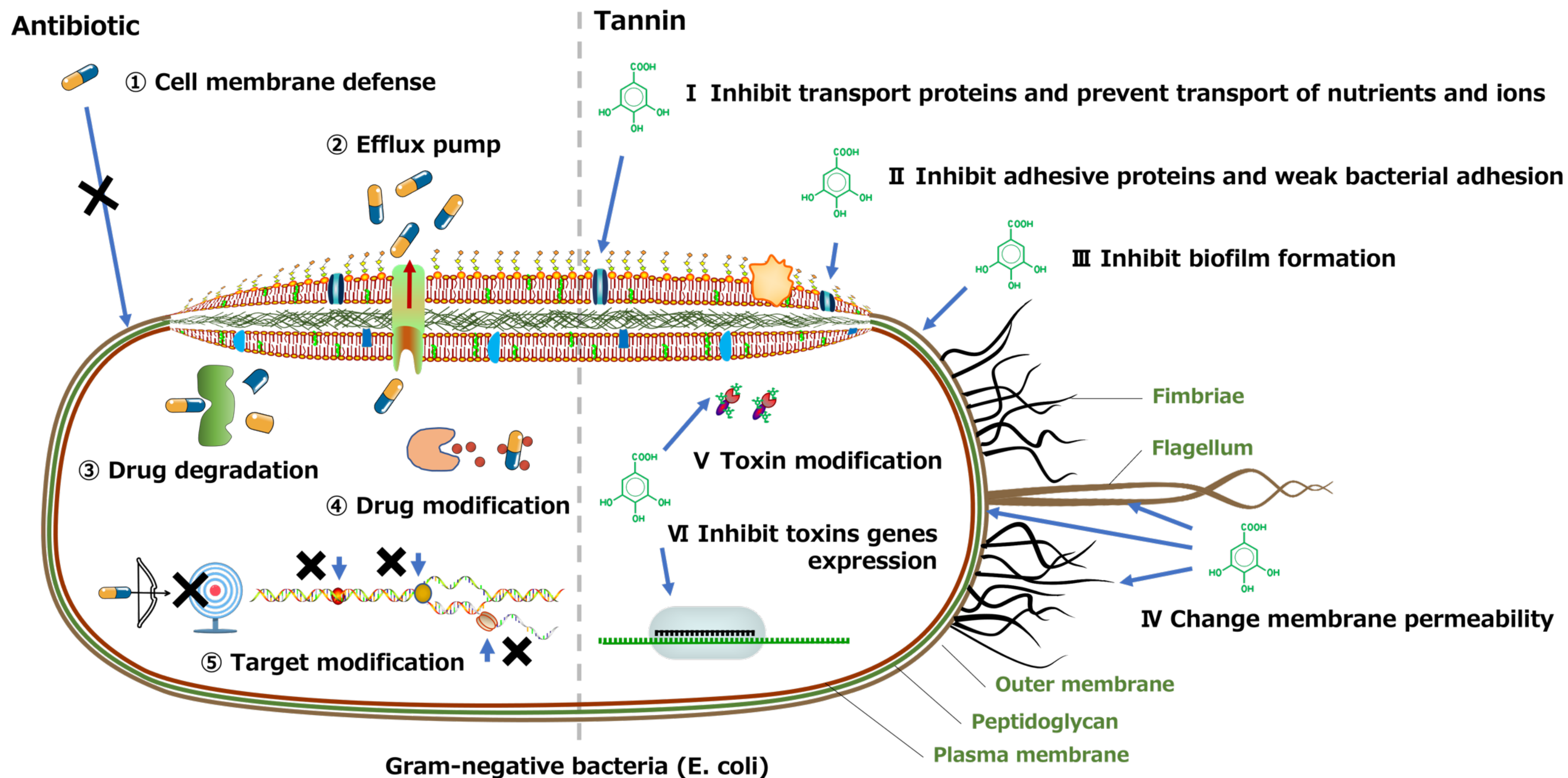


Figure 1.3. Antibiotics resistance and tannins antimicrobial properties schematic diagram. On the left is the schematic diagram of antibiotic resistance, ①-⑤ indicate the 5 main resistance mechanisms. On the right side is tannins antimicrobial properties schematic diagram, I-VI indicate 6 possible antibacterial mechanisms.

Chapter 2 Evaluation of the effect of low-dose Quebracho tannin addition in early-weaned piglet diets

2.1. Introduction

Anti-nutritional properties and possible toxicity are the priority when using tannin as a feed additive. The current research on the application of tannins, although mainly in ruminants, still provides us with many informative suggestions. Both antinutritional properties and toxicity are usually related to the concentration of tannins. Gxasheka et al. (2015) summarized previous studies showing that higher concentrations of tannins supplementation decreased the absorption efficiency of essential amino acids, while lower concentrations of tannins increased protein digestion in the abomasums and small intestine, resulting in greater intestinal absorption of amino acids. At tannins concentrations greater than 5%, feed intake is affected, and toxicity occurs. A similar review was done by Hoste et al. (2006) and found that growth was inhibited at concentrations of ingested condensed tannins above 7% (dry matter), while less than 6% (dry matter) increased weight gain and milk yield in cows. Bone and Mills (2013) similarly concluded that exposure to low levels of tannins is unlikely to affect health, while also recommending avoidance of long-term intake of hydrolyzed tannins. Therefore, the dose of tannins must be strictly controlled when it is used as an antibiotic substitute, to avoid anti-nutrition and toxicity.

To our knowledge, there are very few data regarding the use of QT (quebracho tannin) for post-weaned piglets. Su et al. (2016) reported that the addition of 0.1% QT to 15-kg body weight (BW) piglets significantly reduced feed intake and BW gain during the first week; both aspects recovered during the following week. However, Caprarulo et al. (2020) added a mixture of 1.25% QT and chestnut tannin in a 1:1 ratio to the diet of weaned piglets; it did not affect piglet growth performance, although the diarrhea incidence in the first 14 days post-weaning was higher in the tannin group (5.00%) than the control group (3.39%).

Therefore, in the present study, a relatively low level of 0.2% or 0.3% QT product (MGM-P) was added to the commercial diet of 21-day-old weaned piglets. Their possible anti-nutritional properties and toxicity were evaluated by assessing the effects on growth performance, diarrhea, and overall health status of piglets.

2.2. Materials and Methods

2.2.1. Materials

Commercial MGM-P was provided by Kawamura Ltd. (Tokyo, Japan); condensed tannins comprised more than 50% of the overall extract (**Table 2.1**).

2.2.2. Animals, Treatments, and Experimental Design

The experiment was conducted at the Animal Resource Science Center of the University of Tokyo (Kasama, Japan), and approved (P20-096) by the Animal Care and Use Committee of the Faculty of Agriculture, The University of Tokyo. Three pregnant specific-pathogen-free sows were purchased from Nakamura Chikusan (Ibaraki, Japan) at 1 week before delivery. Thirty piglets (Duroc × Landrace × Yorkshire) were born within 2 days. The lactation period was 21 days. The male piglets were castrated at 1 week of age. Concurrently, all piglets were numbered; from 2 weeks of age, they were provided Antibacterial-substance-free (ASF) early-stage fodder (Marubeni Nisshin Feed, Tokyo, Japan) as creep feed, and during the experimental period. The piglets were weighed at 21 days of age. Using the Experimental Animal Allotment Program (version 1.1) in accordance with the method established by Kim and Lindemann (Kim and M.D. Lindemann. 2007), 24 piglets (6.51±0.17 kg) were selected and divided into three groups (n = 8 per group) according to weight and sex. The animals in each group were divided into two identical pens containing four piglets each (**Table 2.2**).

The control group received ASF early-stage fodder without any added MGM-P; the 0.2 MGM (0.2% MGM-P) and 0.3 MGM (0.3% MGM-P) groups received ASF

early-stage fodder with 2 g/kg and 3 g/kg MGM-P, respectively. The experimental period was 20 days after weaning.

2.2.3. Diet and Animal Management

Table 2.3 lists the ingredients in ASF early-stage fodder, which meets the National Research Council standards (**Table 2.4**) (NRC 2012). All piglets were raised in the same high-bed nursery house, equipped with an air conditioner and mechanical ventilation, molded plastic pen floors, a feed hopper, and a SUEVIA water cup. Throughout the experimental period, piglet health status was checked and recorded twice daily.

2.2.4. Growth Performance

The piglets were weighed at the same time on 0 (weaning day), 7, 14, and 20 d after weaning; the amounts of fodder consumed in each pen were recorded. The average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were analyzed.

2.2.5. Diarrhea Incidence

To determine the incidence of diarrhea, piglet feces were observed twice per day (9:00 am and 3:00 pm) and classified into one of the following grades based on appearance; grade 1, hard cylinders; grade 2, soft cylinders; grade 3, thick and mushy feces; and grade 4, sloppy feces. In the present study, grade 4 was defined as diarrhea. The diarrhea incidence was the sum of piglets with diarrhea once or more throughout the experimental period, divided by the total number of piglets in each group.

2.2.6. Blood Sampling

Blood was collected from the jugular vein immediately after weighing on days 1, 7, 14, and 20. A 21-gauge needle (VENOJECT II; Terumo, Tokyo, Japan) was used to harvest blood for storage in 5-mL collection tubes containing EDTA-Na.

2.2.7. Blood Hematology Analysis

Blood hematology analyses, including white blood cell (WBC) count, lymphocyte count, neutrophil count, red blood cell (RBC) count, and platelet (PLT) count, were performed using a pocH-100iV Diff hematology analyzer (Sysmex Corp., Kobe, Japan).

2.2.8. Plasma Collection and Immunoglobulin Analysis

After hematology analyses, the blood was centrifuged for 20 min (3000 rpm) at 4 °C to obtain plasma. The plasma was immediately subjected to biochemical analyses, and the remaining plasma was stored at −80 °C for subsequent use.

Immunoglobulin A (IgA) and immunoglobulin G (IgG) levels were measured by enzyme-linked immunosorbent assay kits (COSMOBIO, Tokyo, Japan).

2.2.9. Blood Biochemical Examination

Blood biochemical examinations were performed using an automatic dry-chemistry analyzer (DRI-CHEM 3500s; Fujifilm, Tokyo, Japan). The analysis included glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), gamma glutamyl transferase (GGT), ammonia (NH_3), blood urea nitrogen (BUN), amylase (AMYL), glucose (GLU), total protein (TP), albumin (ALB) and triglyceride (TG).

2.2.10. Plasma free amino acids

In total, 18 amino acids were tested in this experiment, including 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), 2 semi-essential amino acids (cysteine, and tyrosine), and 6 non-essential amino acids (aspartic acid, serine, alanine, glycine, glutamic acid, and proline). The analysis was carried out using LC/MS/MS Method Package for Primary Metabolites version 2.0 (Shimadzu, Kyoto, Japan) with a Shimadzu LCMS-8030 system. In brief, each plasma sample (50 μL) was

deproteinized using a methanol-containing internal standard. The supernatant was filtered, dried, and dissolved; 20 μL of sample was then injected for liquid chromatography/mass spectrometry (LC/MS) analysis. The ultra-high-performance liquid chromatographic system in this study was equipped with a DGU-20 A_{3R} degasser, two LC-20 AD binary pumps, an SIL-20 AC_{HT} auto-sampler, a CBM-20A control module, an SPD-20A detector, and a CTO-20 AC column oven. It was then coupled with an LCMS-8030 triple quadrupole mass spectrometer (Shimadzu) with an electrospray ionization source. For liquid chromatography analysis, a Discovery HS F5-3 column (2.1 mm × 150 mm, 3 μm, Sigma-Aldrich, St. Louis, MO, USA) was used. The analysis was performed by means of a gradient system with two mobile phases: (A), a mixture of 0.1% formic acid (LC/MS grade; Wako Pure Industries, Osaka, Japan) and water (LC/MS grade; Wako Pure Industries); and (B), acetonitrile (LC/MS grade; Wako Pure Industries) containing 0.1% formic acid. The analysis began at 0% (B) for 2 min, with the concentration of (B) increasing to 25% at 5 min, 35% at 11 min, and 95% at 15 min, remaining at 95% until 20 min, and then decreasing to 0% at 20.1 min and remaining at this level until 25 min. The column temperature was 40°C, the flow rate was 0.25 mL/min, and the injection volume was 3 μL. For mass spectrometry analysis conditions, the nebulizer flow rate was 2 L/min and the drying gas flow rate was 15 L/min. The DL and heat block temperatures were 250°C and 400°C, respectively. 2-Morpholinoethanesulfonic acid (Dojindo, Kumamoto, Japan) and L-methionine sulfone (Wako Pure Industries) were used as internal standards in the analysis. Program monitoring and data acquisition were controlled using LabSolutions LCMS software (Shimadzu).

2.2.11. Cortisol Measurement

The cortisol concentration in plasma was determined using duplicate enzyme-linked immunosorbent assay kits (Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the manufacturer’s protocol.

2.2.12. Actual and Relative Weights/Lengths of Organs and Intestines

After the feeding trial, two piglets near the average BW for each pen underwent induction of deep anesthesia via thiopental sodium (Ravonal 0.5 g; Mitsubishi Tanabe Pharma, Osaka, Japan) injection into the jugular vein; they were then sacrificed. Necropsies were performed and the organs (liver, pancreas, spleen, kidney, stomach, small intestine, large intestine, and thymus) were carefully removed. The weights of all organs, including individual intestinal tract sections, were measured. The relative organ weights were calculated as the organ weight divided by BW (%). The lengths of individual intestinal tract sections were measured and the relative lengths of intestinal tract sections to piglet BW were also calculated (cm/kg). Specific parts of the small and large intestines were removed (described in next section) and fixed in 4% paraformaldehyde for the examination of intestinal morphology.

2.2.13. Intestinal Morphology

The following samples of intestinal tract were harvested immediately after measurement of the intestinal tract length: 2 cm of duodenum, 10 cm from the stomach; 2 cm of jejunum, 50 cm from the duodenal sampling site; 2 cm of ileum, 50 cm from the cecum; and 2 cm of colon, 50 cm from the cecum. The samples were rinsed in cold 0.1 M phosphate-buffered saline, then divided into two sections (1 cm each) and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The fixed tissues were then carefully oriented and embedded in Tissue-Tek OCT (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and sectioned into 6-μm slices using a Leica CM1850 cryomicrotome (Leica Microsystems Co., Ltd., Wetzlar, Germany). The tissue sections were stained with hematoxylin and eosin for morphological analysis. Cross-sectional slices were viewed with an Olympus IX71 microscope (Olympus, Tokyo, Japan) and images were produced using DP Controller (version 1.2.1.108, Olympus). One cross-section with 10 consecutive intact, well-oriented crypt–villus units was selected from each 1-cm section of intestinal sample; because villi in the

flat part between folds exhibited more regularity than did villi on the folds, only sections with flat parts were selected. The villus height and crypt depth were analyzed using the open-source software ImageJ (version 1.52 k, National Institutes of Health, Bethesda, MD, USA). Villus height was measured from the tip of the villus to the villus crypt junction, crypt depth was defined as the depth of the invagination between adjacent villi, and the ratio of villus height to crypt depth was calculated. Mucosal thickness was measured from the tip of the villus to the bottom of the muscularis mucosae.

2.2.14. Statistical Analysis

Data analysis was performed using JMP Pro software (version 15.2.0, SAS Institute Inc., Cary, NC, USA). One-way analysis of variance was used to compare differences among experimental groups. When the *p*-value from analysis of variance was < 0.05, pairwise differences were assessed using the Tukey–Kramer honestly significant difference test. *p*-values < 0.05 were considered to indicate statistical significance. Results are presented as the means ± standard errors of the mean.

2.3. Results

2.3.1. Growth Performance

As shown in **Table 2.5** and **Figure 2.1**, 0.2% and 0.3% MGM-P supplementation did not influence growth performance indices, including the ADG, ADFI, or FCR.

2.3.2. Diarrhea Incidence

As shown in **Figure 2.2** the diarrhea incidences were 12.5% and 12.5% in the control and 0.2% MGM groups, respectively, during the 20-day post-weaning period. However, no diarrhea was observed in the 0.3% MGM group.

2.3.3. Blood Hematology Analysis

As shown in **Figure 2.3**, there were large differences in WBC counts, particularly the neutrophil counts, between the treatment and control groups at 14 days post-

weaning; there were no differences in red blood cell count or platelet count. The WBC and neutrophil counts were significantly lower in the 0.3% MGM group than in the control group ($p < 0.05$).

2.3.4. Blood Immunoglobulin Analysis

As shown in **Figure 2.4**, there were no changes in plasma IgA or IgG concentrations throughout the experimental period.

2.3.5. Blood Biochemical Analysis

As shown in **Figure 2.5** and **Table 2.6**, MGM-P supplementation did not influence GPT or GOT concentrations in piglet plasma ($p > 0.05$). The concentrations of GGT were lower in the treatment groups than in the control group on day 20 post-weaning. Both glucose and ammonia concentrations tended to decrease in the treatment group; they were significantly lower in the 0.2% MGM group ($p < 0.05$) than in the control group on day 20 post-weaning. The amylase level exhibited a similar tendency, but the difference was not statistically significant ($p > 0.05$). The total protein level did not significantly differ among groups ($p > 0.05$).

2.3.6. Plasma free amino acids

To assess the effects of MGM-P supplementation on amino acid metabolism of monogastric animal, plasma free amino acids were analyzed as shown in **Table 2.7** and **Figure 2.6**. Most of the analyzed amino acids were unaffected by MGM-P treatment. Notably, lower levels of alanine were found on day 20 post-weaning in the 0.2% MGM-P group compared to the control group ($p < 0.05$). The plasma lysine concentration of the 0.3% MGM-P group was slightly lower than the other groups during the first week of the experiment, increased rapidly during the second week, then approached the other groups, and all changes were not statistically significant ($p > 0.05$).

2.3.7. Cortisol Analysis

As shown in **Figure 2.7**, the cortisol concentration on the day of weaning was significantly higher in the control group than in the treatment group ($p < 0.05$). Throughout the experimental period, the cortisol concentration in the 0.3% MGM group remained at a consistently low level.

2.3.8. Actual and Relative Weights/Lengths of Organs and Intestines

Pathological autopsy confirmed that no organ abnormalities were present. **Table 2.8** and **Table 2.9** show the effects of MGM-P supplementation on organ weight or length and relative weight or relative length in weaned piglets. All results revealed no significant deviations between the treatment and control groups ($p > 0.05$).

2.3.9. Intestinal Morphology

Figure 2.8 shows that, compared with piglets in the control group, piglets in the treatment groups had more complete villus structures (duodenum) on day 20 post-weaning; in particular, piglets treated with 0.3% MGM-P appeared to have denser and fuller villi. Whereas the villi of the control group were severely atrophied and a clear exposure of the lamina propria could be observed. Morphometric measurements are summarized in **Figure 2.9**. In particular, piglets in the 0.3% MGM group displayed increased jejunal villus height ($p < 0.05$) and decreased ileal crypt depth ($p < 0.05$), compared with piglets in the control group. Moreover, thinner colonic mucosae were detected in the 0.3% MGM group.

2.4. Discussion

It is controversial as a feed additive whether tannins have positive effects on early-weaned piglet growth. Several studies have indicated that tannin as an anti-nutrition factor that can reduce feed intake and nutrient digestibility (particularly protein), thus inhibiting piglet growth (Jansman 1993; Redondo et al. 2014). Lizardo et al. (1995) reported a reduction in the growth performance of weaned piglets fed a tannin-rich sorghum diet. However, the present study showed that neither

concentration of MGM-P (0.2% and 0.3%) had a negative impact on animal growth performance, ADFI, and FCR throughout the experiment. Few studies have investigated the effects of QT on intestinal health and growth in weaned piglets. The heterogeneous responses seen among studies may be related to the dose of tannins, type of tannin products, age of the animals, ingredients of the basal diet, and hygiene and storage status.

Diarrhea is an important cause of death in early-weaned piglets, and causes extensive economic losses worldwide (Fairbrother et al. 2005). Early weaning frequently triggers intestinal inflammation and disrupts intestinal barrier function, resulting in intestinal structures that are vulnerable to bacterial invasion (which is an important cause of diarrhea in piglets) (Verhelst et al. 2014). The present study showed that 0.3% MGM-P supplementation significantly reduced the incidence of diarrhea among early-weaned piglets by increasing jejunal villus height, decreasing ileal crypt depth, and reducing colonic mucosa. In some studies of intestinal inflammation, the WBC count dramatically increased soon after weaning (Davis et al. 2006; Sugiharto et al. 2014).

In the present study, diarrhea gradually appeared in the control and 0.2% MGM groups after weaning, but no diarrhea was observed in the 0.3% MGM group. Though the leukocyte counts of piglets in all groups were within the normal range (Czech et al. 2018), the rate of change in WBC count was lowest in the 0.3% MGM group, and showed significant group differences at 14 days post-weaning. Furthermore, the neutrophil count was significantly lower in the 0.3% MGM group than in the other groups. Neutrophils are closely associated with intestinal homeostasis and disease; they represent a key component of the innate response during an inflammatory reaction (Fournier and Parkos 2012) and have important roles in the defense against bacterial and fungal pathogen invasion (Mócsai 2013). Yi et al. (2016) reported that diarrhea in weaned piglets was accompanied by a substantial increase in neutrophil recruitment to the intestinal tract. The results of the present study are consistent with

the above finding, where the prevention of diarrhea in weaned piglets was associated with low levels of neutrophils.

Liu et al. (2020) demonstrated that the use of chestnut tannin as feed additives could increase immunoglobulin levels in pigs and broilers. However, the present study did not observe changes in immunoglobulin concentrations, suggesting that the results were related to tannins type; thus far, there have been few reports describing the effect of MGM-P supplementation on immunoglobulin levels in pigs.

Hydrolyzed tannins readily degrade in the gastrointestinal tract and enter the systemic circulation, causing liver toxicity. Filippich et al. (1991) showed that after oral administration of >7.5 mg/g BW of crude extract from yellow-wood leaves, obvious liver lesions occurred within 48 h; a hepatotoxic component known as punicalagin (hydrolyzed tannin) was subsequently isolated. However, little is known regarding the toxicity of condensed tannins. Plasma GPT, GOT, and GGT are important indicators of liver injury. When the liver is damaged, the plasma levels of GPT, GOT, and GGT increase (Huang et al. 2006; Zhang et al. 2016). In the present study, no changes were observed in GPT or GOT; the concentration of GGT was lower in the 0.3% MGM group. These findings demonstrated that MGM-P addition was safe, and the piglets' livers were in good condition. McDougall et al. (2005) reported that tannin-rich extracts were effective amylase inhibitors. In the present study, 0.2% MGM-P supplementation led to decreased amylase activity and glucose concentration. The 0.3% MGM group exhibited similar tendencies, but the differences were not statistically significant. These results suggest that further investigations are needed to evaluate the relationships of amylase activity and glucose concentration with growth performance and feed intake, according to additive levels. Furthermore, there were no differences in total protein levels among groups, consistent with the lack of any negative impact on animal growth performance, ADFI, or FCR throughout the experiment. The ammonia concentration tended to be lower in the treatment than control group. The hepatic urea cycle is the main route for

conversion of ammonia into blood urea nitrogen; therefore, the concentration of ammonia can serve as an indicator of liver function (Ramaiah 2007). Liver impairment increases the ammonia concentration in the blood, which exacerbates systemic inflammation and liver injury (Aldridge et al. 2015). MGM-P supplementation is beneficial for the liver. Tannins have been shown to exert a protective effect on the liver, presumably by enhancing resistance to oxidation and inflammation (Zhang et al. 2017; Sobeh et al. 2017).

Plasma amino acid levels are strongly associated with animal growth performance (Wu et al. 2014). The metabolism of amino acids, especially alanine, is closely related to glucose homeostasis (Felig 1973). Amino groups from amino acids are transferred to pyruvate from blood glucose or glycogen to form alanine in extrahepatic tissues, which is converted into urea and glucose in the liver; the glucose then returns to peripheral tissues (Exton et al. 1970 p. 1). This cycle reduces the production of ammonia in amino-acid metabolism while maintaining a degree of blood glucose stability. The blood concentrations of alanine, ammonia, and glucose on day 14 post-weaning were significantly lower in the 0.2% MGM-P group than in the control group, indicating that the addition of 0.2% MGM-P was beneficial for this cycle. In this study, MGM-P supplementation did not significantly influence plasma essential amino acids or semi-essential amino acids. Take lysine as an example, the lack of lysine in cereal-based diets makes it the first-limiting amino acid for pigs (Kuang et al. 2015). Although lysine concentrations decreased in piglets of the 0.3 MGM-P group during the first week after weaning, they increased rapidly to higher levels from the second week onwards, suggesting that higher QT supplementation did not consistently affect the metabolism of cereal proteins in the intestine. Thus, controlling tannins in the diet at appropriate doses does not appear to have an anti-nutritional effect.

Cortisol is a glucocorticoid produced by the adrenal cortex under stress, and thus is widely used to detect physiological stress in pigs (Bottoms et al. 1972). At the

beginning of our experiment, the cortisol concentration was higher in the control group than the other groups; we presumed that this was due to interindividual differences in reactions to bleeding, although the animals were randomly assigned to groups. Throughout the experimental period, 0.3% MGM-P supplementation decreased plasma cortisol levels, suggesting improved physiological condition; this might explain why tannin did not negatively affect growth and diarrhea (i.e., because stress is closely related to growth performance) (Campbell et al. 2013).

In the present study, detailed pathological examinations were performed during dissection, and revealed that 0.2% and 0.3% MGM-P had no pathological effects on the organs. These results are consistent with the findings of Wang et al. (2020c) that adding various plant extracts to the diet of 21-day-old weaned piglets had no pathological effects on organs after 14 days of supplementation. The intestinal development and digestive function of piglets are severely impaired during the weaning process (Xiong et al. 2019). Importantly, we found no changes in organ weight, relative organ weight, intestine length, or relative intestine length.

Integrity of the morphological structure is necessary to ensure good intestinal function; a complete mucosal structure is more protective against harmful substances. Weaning stress is associated with poorer intestinal barrier function (Dw et al. 1988); it causes villous atrophy and crypt hyperplasia in the intestinal tracts of piglets, resulting in dysfunctional nutrient digestion and absorption (Hedemann et al. 2003). Bilić-Šobot et al. (2016) reported that supplementation with 3% hydrolysable chestnut tannin significantly increased duodenal villus height in fattening boars. Our results were similar, in that 0.3% MGM-P significantly increased the jejunal villus height. Biagi et al. (2010) found that ileal crypt depths tended to decrease in animals supplemented with tannins (2.25 or 4.5 g/kg). The present study showed that supplementation with MGM-P, particularly 0.3% MGM-P, led to noticeably shallower ileal crypts. These shallower crypts may help to reduce the severity of post-weaning diarrhea in piglets because of robust secretory functioning in the small

intestine crypts (Biagi et al. 2010). The main functions of the colon are to absorb water and mineral salts from chyme. In this study, the piglets supplemented with 0.3% MGM-P had thinner colonic mucosa than those in the other two groups, which suggests that 0.3% MGM-P might have thinned the colonic mucosa, to help the piglets absorbing water from the stool and reducing the incidence of diarrhea.

2.5. Conclusions

The present study demonstrated that MGM-P supplementation helped prevent diarrhea in 21-day-old weaned piglets, particularly those in the 0.3% MGM group. This treatment positively influenced piglet health without adversely affecting growth performance.

Table 2.1. Technical specification of MGM-P.

Characteristic	Criterion
Polyphenols (mg catechin/g)	>500
Humidity (maximum)	15
pH	4.5 ± 1

Table 2.2. Experimental animal allotment.

Group	n			BW			
	Male	**Female**	**Total**	**Mean**	**SEM**	**p-value**	**CV (%)**
Control	5	3	8	6.56	0.33		
0.2 MGM	5	3	8	6.49	0.28	0.99	0.58
0.3 MGM	5	3	8	6.49	0.32		

Abbreviations: n, number of piglets; BW, body weight; SEM, standard error of the mean; CV, coefficient of variation.

Table 2.3. Ingredients and chemical composition of basal diet (as-fed basis).[1]

Ingredient	Content (%)
Corn	34.45
Defatted milk powder	18.00
Fatty powder	6.20
Sugar	10.00
Soybean meal	25.00
Fish meal	4.50
Calcium diphosphate	0.20
Calcium carbonate	0.65
Salt	0.20
B vitamins	0.15
Vitamins A, D and E	0.10
Trace minerals	0.15
L-lysine hydrochloride	0.06
DL-Methionine	0.09
L-Threonine	0.03
Copper sulphate	0.21
Vitamin K3	0.01
Total	100

[1] The other diets were based on this diet, to which MGM-P was added in different proportions.

Table 2.4. Chemical composition of basal diet.

Chemical Composition	Content (%)	Amino Acid	Content (%)
DM	90.50	Contained	
CP	22.60	Arginine	1.32
EE	6.60	Histidine	0.63
CF	1.10	Isoleucine	0.99
Ash	5.60	Leucine	2.03
NFE	54.60	Lysin	1.56
DE (Mcal/kg)	3.70	Methionine + cysteine	0.83
Ca	0.81	Phenylalanine + tyrosine	1.92
NpP	0.45	Threonine	0.96
Na	0.26	Tryptophan	0.28
Cl	0.36	Valine	1.15
K	0.99	Digestible	
Mg	0.14	Arginine	1.22
Fe (mg/kg)	182.18	Histidine	0.58
Zn (mg/kg)	105.32	Isoleucine	0.88
Mn (mg/kg)	87.51	Leucine	1.83
Cu (mg/kg)	125.29	Lysin	1.42
I (mg/kg)	1.95	Methionine + cysteine	0.74
Se (mg/kg)	0.30	Phenylalanine + tyrosine	1.47
Vitamin A (IU/kg)	100051.62	Threonine	0.85
Vitamin D (IU/kg)	2000	Tryptophan	0.25
Vitamin E (IU/kg)	20.04	Valine	1.01
Vitamin K (IU/kg)	0.57		
Thiamine (mg/kg)	5.15		
Riboflavin (mg/kg)	15.38		
Pantothenic acid (mg/kg)	27.83		
Nicotinic acid (mg/kg)	25.63		
Vitamin B6 (mg/kg)	5.93		
Choline (mg/kg)	1204.8		
Vitamin B12 (μg/kg)	21.88		
Biotin (mg/kg)	0.16		
Folic acid (mg/kg)	0.36		

Abbreviations: DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; NFE, nitrogen free extract; DE, digestible energy.

Table 2.5. Effects of low-dosage MGM-P supplementation on growth performance in weaned piglets (throughout the experimental period).

Item	Initial BW (kg)	Final BW (kg)	ADG (kg)	ADFI (kg)	FCR (kg/kg)
Control	6.56 ± 0.33	17.56 ± 0.65	0.55 ± 0.02	0.82	1.49
0.2 MGM	6.49 ± 0.28	17.46 ± 0.93	0.55 ± 0.04	0.81	1.47
0.3 MGM	6.49 ± 0.32	17.79 ± 0.82	0.57 ± 0.03	0.84	1.47

Values of BW and ADG are expressed as mean ± SEM; n = 8. There were no statistically significant differences among the three groups based on one-way analysis of variance.

Table 2.6. Effects of low-dosage MGM-P supplementation on blood biochemical parameters in weaned piglets.

Item	GPT (U/L)	GOT (U/L)	GGT (U/L)	NH_3 (μg/dL)	BUN (mg/dL)	AMYL (U/L)	GLU (mg/dL)	TP (g/dL)	ALB (g/dL)	TG (mg/dL)
0d[1]										
Control	46.88 ± 1.63	51.25 ± 4.81	32.88 ± 2.55	152.13 ± 26.13	3.59 ± 0.10	516.38 ± 27.77	152.13 ± 14.14	5.50 ± 0.11	3.40 ± 0.09	77.88 ± 15.22
0.2 MGM	46.50 ± 1.73	46.13 ± 4.30	27.75 ± 3.19	141.75 ± 10.31	3.45 ± 0.22	384.63 ± 48.38	134.50 ± 2.59	5.61 ± 0.15	3.56 ± 0.13	57.00 ± 10.39
0.3 MGM	42.88 ± 1.97	50.00 ± 3.34	31.13 ± 3.51	144.75 ± 8.07	3.69 ± 0.34	465.25 ± 73.69	132.63 ± 3.91	5.50 ± 0.11	3.48 ± 0.07	81.25 ± 13.24
7d										
Control	55.88 ± 4.34	45.75 ± 3.45	34.75 ± 2.95	191.75 ± 20.51	6.29 ± 0.29	352.38 ± 15.83	135.00 ± 4.75	5.24 ± 0.15	3.54 ± 0.10	27.75 ± 5.44
0.2 MGM	48.13 ± 3.82	41.88 ± 3.36	35.88 ± 3.88	141.75 ± 14.09	6.26 ± 0.71	274.25 ± 29.79	133.00 ± 9.30	5.39 ± 0.24	3.64 ± 0.12	23.63 ± 5.74
0.3 MGM	52.63 ± 2.96	54.25 ± 8.51	30.88 ± 1.68	190.00 ± 14.05	5.16 ± 0.34	368.13 ± 40.75	145.13 ± 4.77	5.15 ± 0.11	3.65 ± 0.09	34.75 ± 5.75
14d										
Control	50.88 ± 2.98	57.50 ± 8.17	36.25 ± 2.23	135.50 ± 15.20	11.00 ± 1.04	553.88 ± 39.36	148.75 ± 3.71	4.96 ± 0.10	4.00 ± 0.14	32.63 ± 5.04
0.2 MGM	49.43 ± 2.89	56.43 ± 15.31	32.00 ± 3.42	134.43 ± 19.83	12.73 ± 0.96	403.29 ± 52.81	145.14 ± 5.31	5.13 ± 0.09	4.29 ± 0.16	22.00 ± 3.55
0.3 MGM	47.63 ± 2.38	47.38 ± 4.17	30.38 ± 2.24	127.13 ± 5.73	10.98 ± 0.89	525.88 ± 62.53	145.38 ± 3.67	4.68 ± 0.23	3.81 ± 0.25	68.38 ± 40.27
20d										
Control	52.63 ± 5.06	50.00 ± 3.94	54.13 ± 5.57[a]	191.63 ± 25.79[a]	13.31 ± 1.21	509.38 ± 33.64	149.00 ± 3.86[a]	5.25 ± 0.13	4.69 ± 0.13	44.63 ± 4.65[ab]
0.2 MGM	47.14 ± 1.62	63.00 ± 7.20	48.14 ± 7.05[ab]	109.14 ± 6.72[b]	13.69 ± 1.17	390.00 ± 54.49	135.29 ± 2.39[b]	5.56 ± 0.18	4.77 ± 0.09	24.29 ± 2.08[b]
0.3 MGM	45.88 ± 2.35	54.13 ± 9.16	35.50 ± 1.45[b]	158.00 ± 20.41[ab]	14.46 ± 1.13	463.00 ± 45.61	145.88 ± 2.05[ab]	5.18 ± 0.08	4.68 ± 0.10	52.00 ± 10.21[a]

Abbreviations: GPT, glutamate-pyruvate transaminase; GOT, glutamate-oxaloacetate transaminase; GGT, gamma-glutamyl transpeptidase; NH_3, ammonia; BUN, blood urea nitrogen; AMYL, amylase; GLU, glucose; TP, total protein; ALB, albumin; TG, triglyceride. All data are expressed as mean ± SEM; n = 8. [1] Blood was collected before the provision of feed with MGM-P on the day of weaning. [a,b] Mean values within a row with dissimilar superscript letters are significantly different ($p < 0.05$).

Table 2.7. Effects of low-dosage MGM-P supplementation on plasma free amino acids in weaned piglets (μM).

Item	Cystine	Aspartic acid	Serine	Alanine	Glycine	Threonine	Glutamic acid	Proline	Lysine	Histidine	Arginine	Valine	Methionine	Tyrosine	Isoleucine	Leucine	Phenylalanine	Tryptophan
0d[1]																		
Control	27.20 ± 1.46	40.81 ± 2.26	244.45 ± 13.36	522.46 ± 48.96	856.74 ± 17.70^{ab}	196.29 ± 12.44	121.61 ± 12.89	154.82 ± 10.63	55.55 ± 3.80	20.47 ± 2.62	115.97 ± 6.01	188.12 ± 16.40	37.96 ± 1.70	187.16 ± 9.43	116.86 ± 4.08	232.57 ± 13.84	117.53 ± 13.08	73.64 ± 4.09
0.2 MGM	28.58 ± 1.43	40.82 ± 2.29	237.40 ± 17.76	466.06 ± 20.38	760.89 ± 40.00^{b}	188.16 ± 18.20	131.52 ± 14.84	139.63 ± 12.18	59.23 ± 4.45	15.51 ± 1.61	110.22 ± 13.65	162.4 ± 11.60	37.14 ± 2.93	170.16 ± 14.76	119.66 ± 8.91	231.36 ± 16.27	93.84 ± 6.63	68.13 ± 5.92
0.3 MGM	27.36 ± 2.82	46.32 ± 2.25	240.59 ± 13.89	458.38 ± 30.56	900.95 ± 40.54^{a}	218.11 ± 20.96	136.20 ± 12.23	141.77 ± 7.47	59.84 ± 4.40	20.33 ± 1.71	116.24 ± 5.82	188.62 ± 16.50	40.77 ± 2.59	194.47 ± 10.00	111.41 ± 9.33	223.79 ± 18.49	116.03 ± 9.30	93.86 ± 12.95
7d																		
Control	48.75 ± 4.97	66.90 ± 2.38	237.49 ± 16.21	595.62 ± 66.11	1041.99 ± 101.18	344.31 ± 31.54	196.85 ± 16.33	137.96 ± 14.08	103.55 ± 12.28	28.28 ± 3.63	91.64 ± 13.26	333.13 ± 32.91	103.85 ± 13.53	179.09 ± 19.32	153.13 ± 15.01	278.99 ± 30.74	136.96 ± 10.80	73.88 ± 6.53
0.2 MGM	50.58 ± 2.26	63.71 ± 1.85	236.17 ± 17.11	485.92 ± 38.21	993.84 ± 83.85	355.20 ± 39.83	195.35 ± 18.83	120.55 ± 11.56	100.15 ± 8.31	23.05 ± 2.21	78.28 ± 6.64	318.94 ± 22.47	93.67 ± 12.77	160.89 ± 15.77	144.48 ± 10.69	237.57 ± 18.29	131.25 ± 15.99	67.79 ± 6.20
0.3 MGM	45.47 ± 3.79	64.24 ± 2.78	193.93 ± 11.25	555.76 ± 19.96	840.73 ± 30.03	267.19 ± 28.29	204.07 ± 20.30	131.35 ± 5.64	78.41 ± 8.43	24.37 ± 1.87	61.71 ± 8.77	303.01 ± 26.04	91.91 ± 7.44	146.02 ± 9.21	134.09 ± 10.38	224.04 ± 13.28	113.31 ± 7.65	55.63 ± 4.26
14d																		
Control	37.12 ± 2.66	51.78 ± 2.39	269.71 ± 30.31	336.05 ± 28.91	774.42 ± 42.16	418.32 ± 34.85	125.08 ± 9.70	125.35 ± 11.33	144.16 ± 17.55	34.28 ± 3.81	142.16 ± 17.06	295.57 ± 28.79	166.48 ± 28.91	188.67 ± 19.96	146.34 ± 16.07	215.03 ± 28.82	91.24 ± 10.03	71.13 ± 6.91
0.2 MGM	41.47 ± 1.73	44.85 ± 2.45	247.91 ± 13.81	276.12 ± 16.75	840.46 ± 61.67	405.80 ± 16.08	115.29 ± 9.33	112.81 ± 14.21	130.52 ± 21.57	32.45 ± 3.87	126.36 ± 12.63	325.50 ± 23.80	159.37 ± 32.84	187.39 ± 10.04	137.22 ± 12.52	210.01 ± 16.93	91.81 ± 5.35	77.44 ± 4.30
0.3 MGM	46.43 ± 3.86	54.89 ± 8.47	255.08 ± 13.13	306.56 ± 11.80	842.92 ± 36.50	427.80 ± 22.33	106.50 ± 7.48	122.98 ± 6.00	173.20 ± 17.05	37.67 ± 1.88	131.86 ± 7.97	305.62 ± 14.01	207.68 ± 25.31	208.07 ± 7.99	143.22 ± 8.08	231.67 ± 15.90	100.00 ± 6.66	80.41 ± 3.34
20d																		
Control	38.03 ± 1.64	45.81 ± 2.32	216.64 ± 19.46	324.69 ± 14.51^{a}	682.26 ± 32.13	413.23 ± 28.05	121.48 ± 6.38	121.00 ± 7.28	142.71 ± 14.45	41.00 ± 2.81	119.93 ± 9.10	340.13 ± 40.88	100.80 ± 7.04	183.14 ± 12.98	148.04 ± 12.85	239.51 ± 21.96	94.25 ± 11.40	72.46 ± 6.55
0.2 MGM	39.16 ± 2.05	45.00 ± 1.98	206.41 ± 17.30	275.27 ± 11.65^{b}	656.26 ± 25.60	409.75 ± 14.86	117.19 ± 13.55	125.29 ± 6.88	141.86 ± 13.13	36.99 ± 2.24	93.45 ± 8.88	338.80 ± 13.32	80.82 ± 6.91	164.51 ± 6.34	140.78 ± 5.71	234.12 ± 7.08	101.56 ± 4.61	79.53 ± 4.78
0.3 MGM	36.94 ± 2.98	43.51 ± 4.17	196.61 ± 10.42	280.28 ± 13.93^{ab}	608.82 ± 29.45	397.10 ± 20.81	107.09 ± 10.51	129.07 ± 13.17	134.21 ± 12.23	40.91 ± 4.25	103.90 ± 5.80	330.10 ± 29.42	107.70 ± 12.95	181.42 ± 16.81	141.87 ± 11.70	230.59 ± 17.35	94.60 ± 8.07	73.15 ± 4.19

All data are expressed as mean ± SEM; n = 8. [1] Blood was collected before the provision of feed with MGM-P on the day of weaning. ^{a,b} Mean values within a row with dissimilar superscript letters are significantly different ($p < 0.05$).

Table 2.8. Effects of low-dosage MGM-P supplementation on actual organ weight/length in weaned piglets.

Item	Liver (g)	Pancreas (g)	Spleen (g)	Kidney (g)	Stomach (g)	Small intestine weight (g)	Small intestine length (m)	Large intestine weight (g)	Large intestine length (m)	Thymus (g)
Control	425.28 ± 39.64	31.45 ± 4.52	32.40 ± 2.26	97.90 ± 5.19	76.58 ± 6.28	598.73 ± 34.20	11.14 ± 0.40	189.75 ± 37.24	1.98 ± 0.37	35.73 ± 1.33
0.2 MGM	478.38 ± 45.16	35.70 ± 2.78	35.65 ± 2.26	115.28 ± 8.31	78.98 ± 7.53	804.40 ± 85.75	12.68 ± 0.47	230.03 ± 19.61	2.58 ± 0.02	40.83 ± 11.81
0.3 MGM	474.08 ± 31.26	34.73 ± 2.19	35.08 ± 2.93	103.50 ± 6.45	78.85 ± 11.64	590.50 ± 39.51	11.93 ± 0.79	187.63 ± 16.50	2.61 ± 0.31	41.43 ± 8.93

All data are expressed as mean ± SEM; *n* = 4. There were no statistically significant differences among the three groups based on one-way analysis of variance.

Table 2.9. Effects of low-dosage MGM-P supplementation on relative organ weight/length in weaned piglets.

Item	Liver (%)	Pancreas (%)	Spleen (%)	Kidney (%)	Stomach (%)	Small intestine weight (%)	Small intestine length (cm/kg)	Large intestine weight (%)	Large intestine length (cm/kg)	Thymus (%)
Control	2.35 ± 0.10	0.18 ± 0.02	0.18 ± 0.02	0.55 ± 0.01	0.43 ± 0.02	3.33 ± 0.09	62.45 ± 4.33	1.04 ± 0.15	11.04 ± 2.08	0.20 ± 0.01
0.2 MGM	2.58 ± 0.18	0.19 ± 0.01	0.19 ± 0.01	0.62 ± 0.02	0.43 ± 0.03	4.37 ± 0.48	68.63 ± 2.33	1.24 ± 0.07	14.00 ± 0.58	0.22 ± 0.05
0.3 MGM	2.60 ± 0.16	0.19 ± 0.01	0.19 ± 0.01	0.57 ± 0.02	0.42 ± 0.03	3.24 ± 0.18	65.96 ± 5.80	1.02 ± 0.05	14.36 ± 1.62	0.22 ± 0.04

All data are expressed as mean ± SEM; $n = 4$. There were no statistically significant differences among the three groups based on one-way analysis of variance.

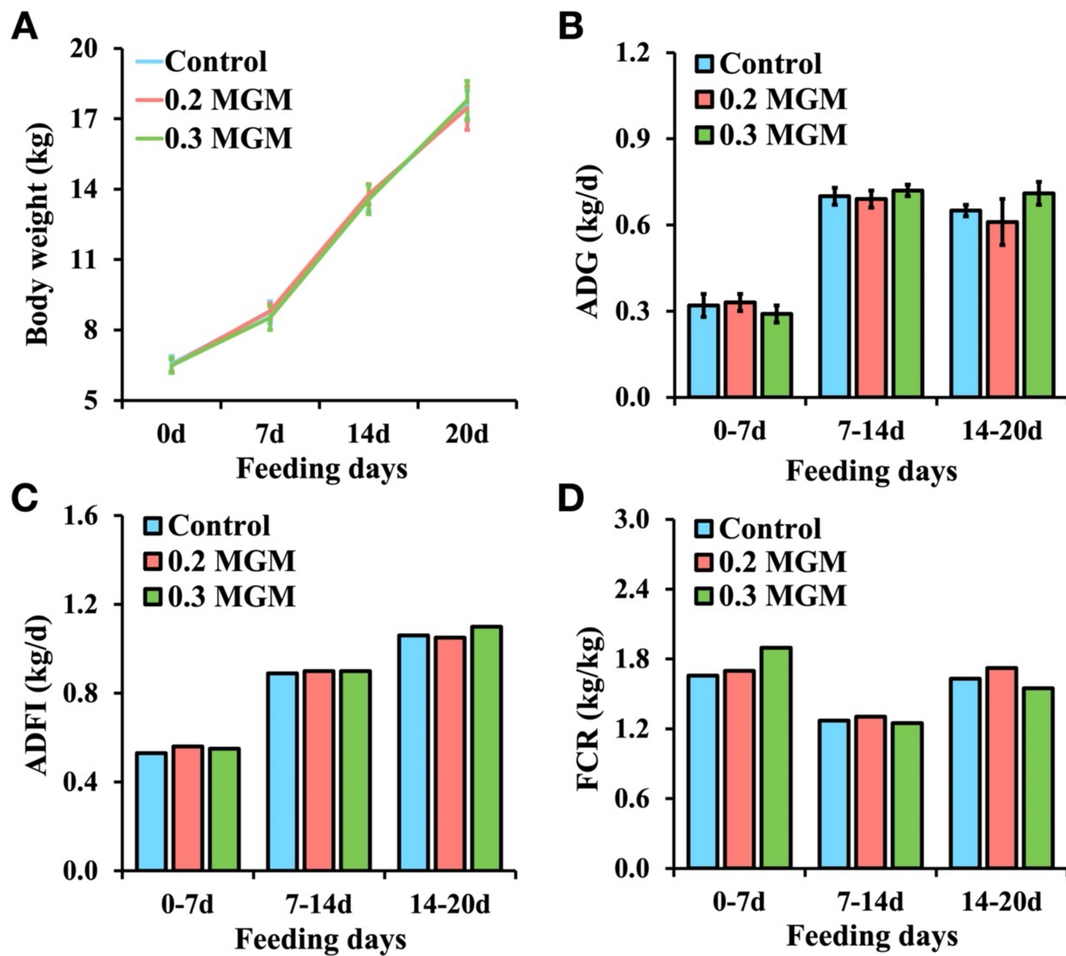


Figure 2.1. Effects of low-dosage MGM-P supplementation on growth performance in weaned piglets. Data in **A** and **B** are expressed as mean ± SEM (n = 8). There were no statistically significant differences among the three groups based on one-way analysis of variance.

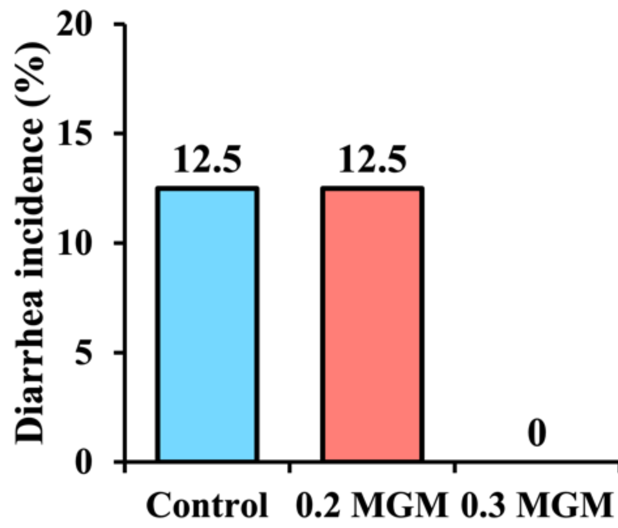


Figure 2.2. Effects of low-dosage MGM-P supplementation on diarrhea incidence in weaned piglets.

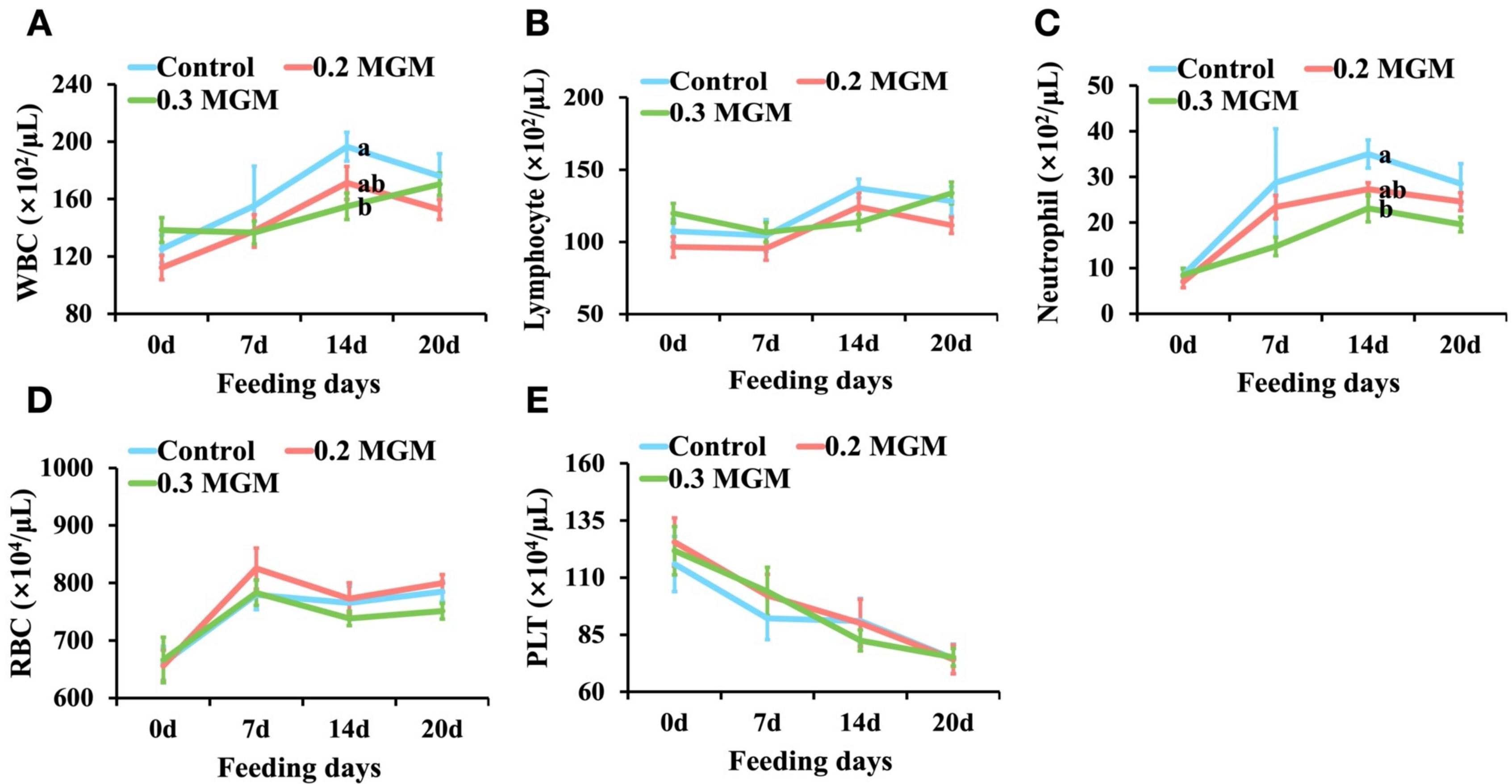


Figure 2.3. Effects of low-dosage MGM-P supplementation on blood hematology parameters in weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; *n* = 8. Different lowercase letters on the right side of the nodes indicate significant differences ($p < 0.05$). (**A**) the changes of the white blood cell count; (**B**) the changes of the lymphocyte count; (**C**) the changes of the neutrophil count; (**D**) the changes of the red blood cell count; (**E**), the changes of the platelet count.

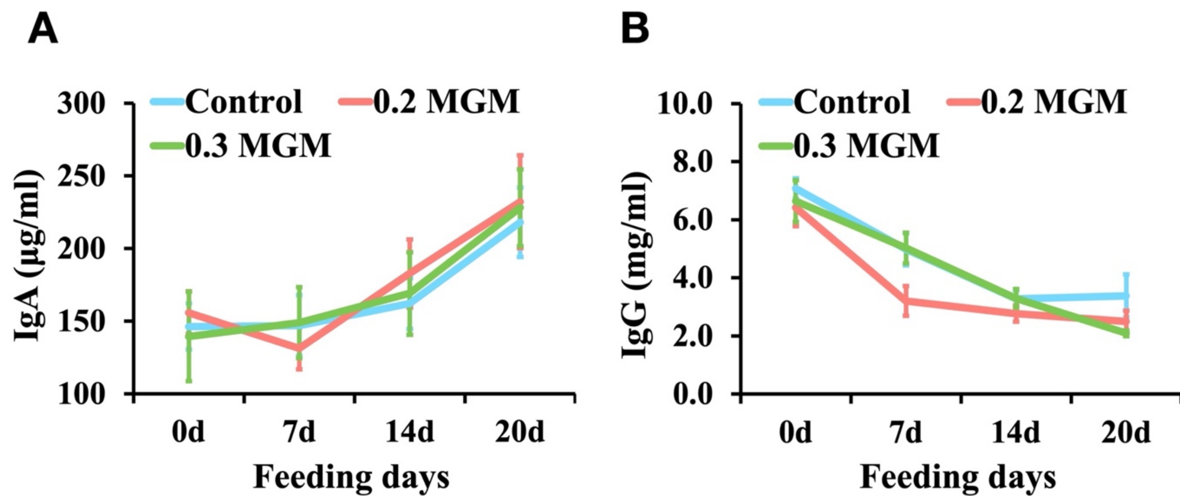


Figure 2.4. Effects of low-dosage MGM-P supplementation on blood immunoglobulin levels in weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; n = 8. There were no statistically significant differences among the three groups based on one-way analysis of variance. (**A**) the changes of the immunoglobulin A concentration; (**B**) the changes of the immunoglobulin G concentration.

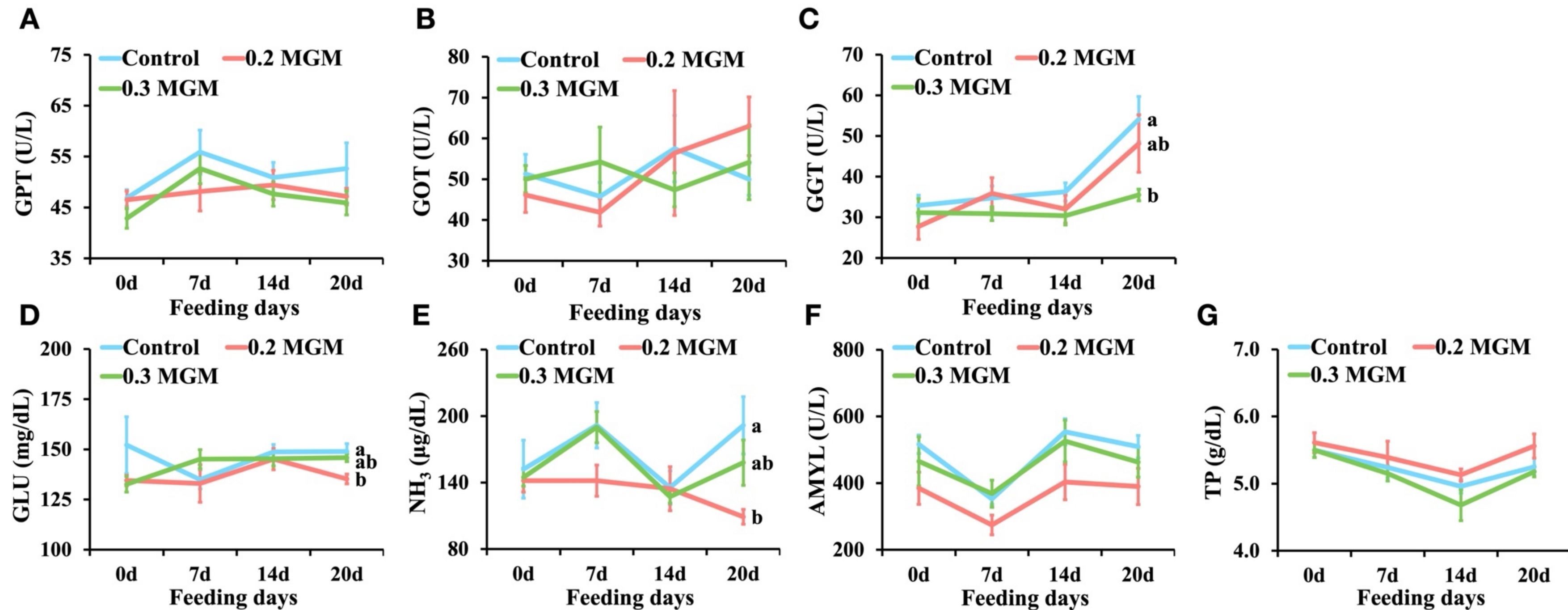


Figure 2.5. Effects of low-dosage MGM-P supplementation on blood biochemical parameters of weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; $n = 8$. Different lowercase letters on the right side of the nodes indicate significant differences ($p < 0.05$). (**A**) the changes of the glutamic pyruvic transaminase level; (**B**) the changes of the glutamic oxaloacetic transaminase level; (**C**) the changes of the gamma glutamyl transferase level; (**D**) the changes of the glucose concentration; (**E**) the changes of the ammonia concentration; (**F**) the changes of the amylase level; (**G**) the changes of the total protein concentration.

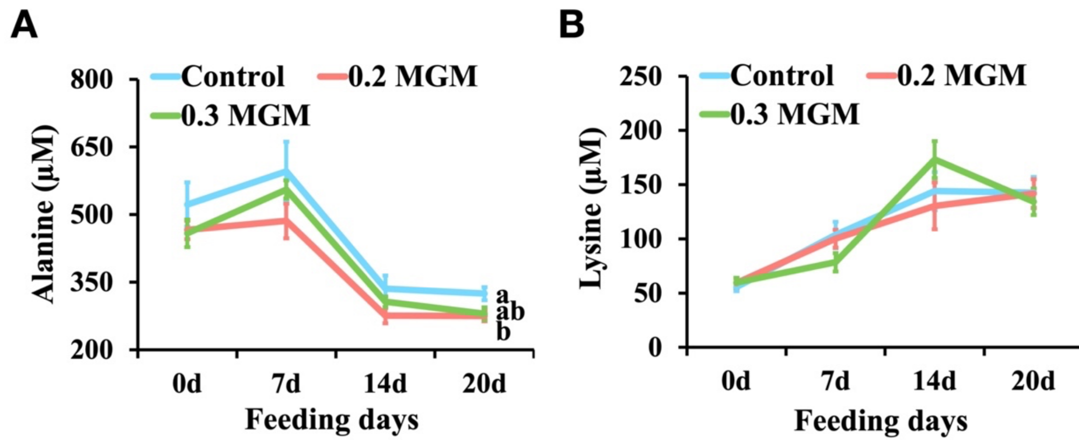


Figure 2.6. Effects of low-dosage MGM-P supplementation on plasma free amino acids of weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; $n = 8$. Different lowercase letters on the right side of the nodes indicate significant differences ($p < 0.05$). (**A**) the changes of the alanine concentration; (**B**) the changes of the lysine concentration.

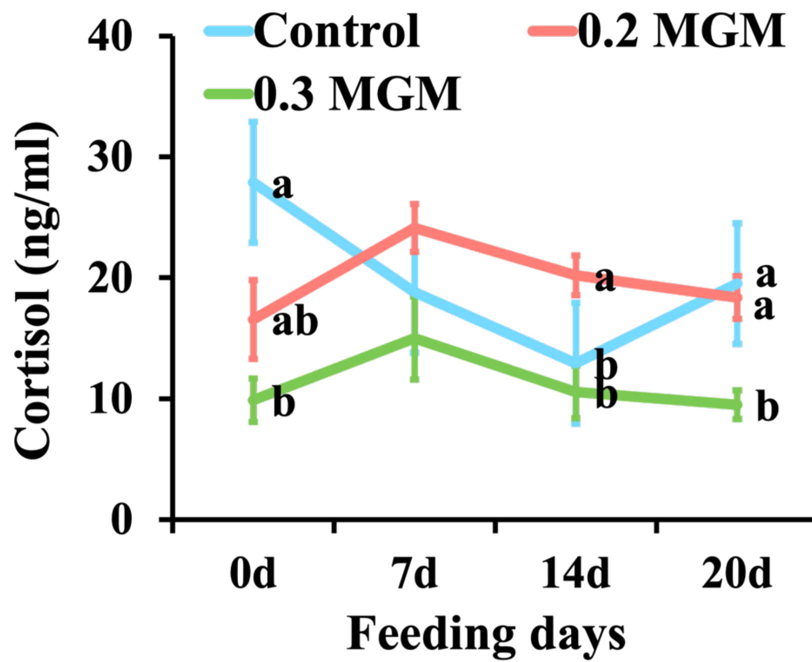


Figure 2.7. Effects of low-dosage MGM-P supplementation on the blood cortisol concentration of weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; n = 8. Different lowercase letters on the right side of the nodes indicate significant differences ($p < 0.05$).

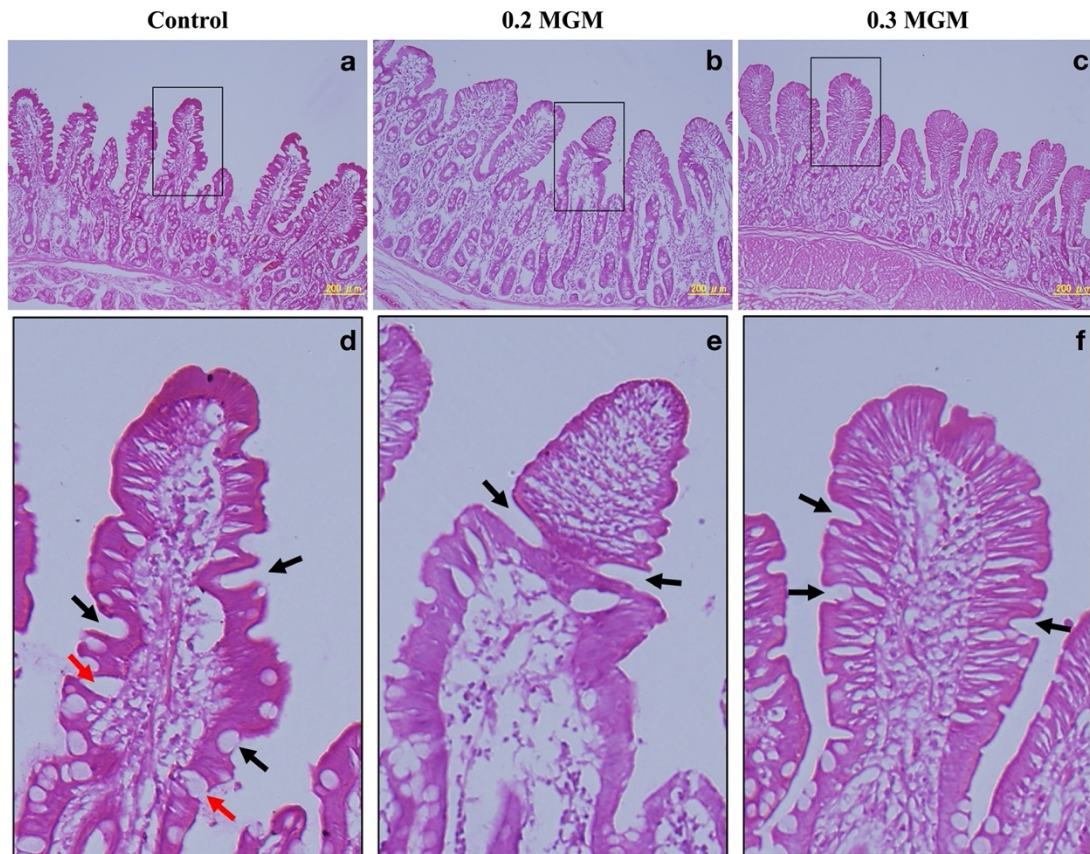


Figure 2.8. Effects of low-dosage MGM-P supplementation on morphology of the duodenum in piglets at 20 days post-weaning. (**a, b, c**) representative histological micrographs of the duodenum, produced by hematoxylin and eosin staining, scale bar, 200 μm; (**d, e, f**) villus architectures were examined by the representative square field of view in the **a, b, c** at 5× magnification. Pathological changes at the tips of intestinal villi are indicated by arrows. The exposed lamina propria are clearly visible in the control group (red arrow) (**d**).

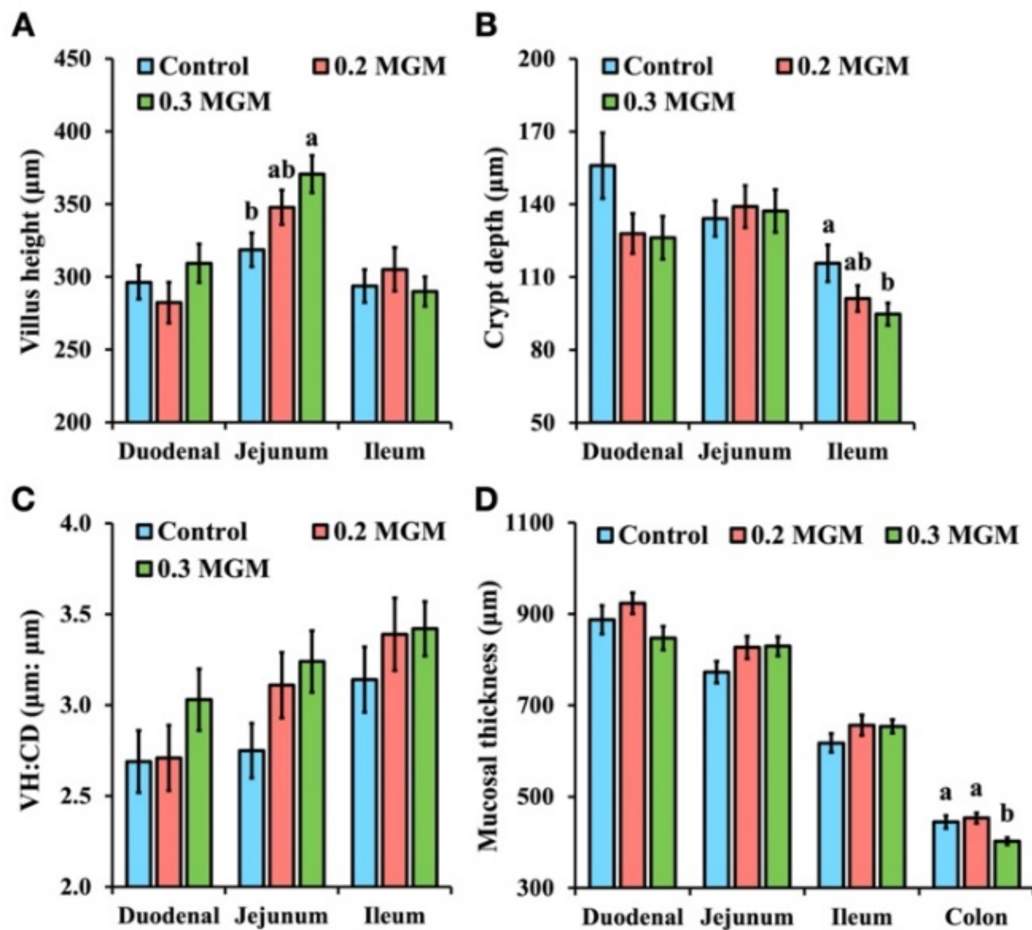


Figure 2.9. Effects of low-dosage MGM-P supplementation on the intestinal morphology of weaned piglets. Abbreviations: VH:CD, villus height/crypt depth. Values are expressed as mean ± SEM; n = 4. Different lowercase letters above the bars indicate significant differences ($p < 0.05$). (**A**) comparison of villus height in different groups (duodenum, jejunum and ileum); (**B**), comparison of crypt depth in different groups (duodenum, jejunum and ileum); (**C**) the ratio of villus height to crypt depth in different groups (duodenum, jejunum and ileum); (**D**) comparison of mucosal thickness in different groups (duodenum, jejunum, ileum and colon).

Chapter 3 Feasibility of replacing antibiotic additives with Quebracho tannin in early-weaned piglets

3.1. Introduction

Tannin is generally regarded as an anti-nutritional factor for monogastric animals and have the ability to reduce the digestive efficiency of dietary protein (Mariscal-Landín et al. 2004). This solidified perception affects the use of tannins in animal nutrition. The experimental results of Chapter 2 have shown that weaned piglets fed commercial feed containing 2 g/kg or 3 g/kg QT extract has not affect the performance of the piglet, and the 3 g/kg supplementation had helped to improve the villus morphology and alleviate the occurrence of diarrhea.

Since 3 g/kg QT supplementation has a better effect, it is worthwhile to further investigate whether the level of QT addition can be continued to increase within a reasonable range for maximum antimicrobial or other beneficial effects, without affecting piglet growth. Finding this delicate balance is the key to replacing antibiotic additives with QT. Once the ideal amount of QT is found, comparisons with the effects of antibiotic additives need to be made to demonstrate the potential for QT to replace antibiotics.

Flavomycin is considered a relatively safe antibiotic because of its non-absorbability in the gastro-intestinal tract of animals, and its widespread use as a growth promoter in pig feed in most countries (Ochetim and Odur 1979). It can hinder the formation of the murein polysaccharide strands and has a strong inhibitory effect on Gram-positive bacteria (Butaye et al. 2003). According to Dealy's study, the use of flavomycin supplemented feed for weaned piglets was effective in reducing the duration and prevalence of *Salmonella* shedding in them (Dealy and Moeller 1976 p. 1). In addition, one study reported that flavomycin also had an inhibitory effect on the colonization of multiresistant E. coli in pig intestine (van den Bogaard et al. 2002).

We selected and added appropriate doses of Flavomycin under the guidance of the antibiotic seller to simulate the actual production process in the pig farm, as a positive control to investigate the possibility of using QT as an alternative to antibiotics in the diet of early weaned piglets.

Therefore, higher doses of QT supplementation (0.5% and 1.0%) were attempted in the present study. The effects of QT on the growth and health of weaning piglets were assessed by analyzing blood parameters, diarrhea frequency, and organ weight, and then the appropriate dosage was determined and the feasibility of replacing antibiotics with QT to relieve weaning stress in piglets was assessed.

3.2. Materials and Methods

3.2.1. Materials

As in Chapter 2, MGM-P (provided by Kawamura Ltd. Tokyo, Japan) was employed as a source of QT.

3.2.2. Animals, Treatments, and Experimental Design

The experiment was conducted at the Animal Resource Science Center of the University of Tokyo (Kasama, Japan), and approved (P20-097) by the Animal Care and Use Committee of the Faculty of Agriculture, The University of Tokyo. Four pregnant specific-pathogen-free sows were purchased from Nakamura Chikusan (Ibaraki, Japan) at 1 week before delivery. Thirty-eight piglets (Duroc × Landrace × Yorkshire) were born within 2 days. The lactation period was 21 days. The male piglets were castrated at 2 weeks of age. Concurrently, all piglets were numbered; from 2 weeks of age, they were provided Antibacterial-substance-free (ASF) early-stage fodder (Marubeni Nisshin Feed, Tokyo, Japan) as creep feed, and during the experimental period. The piglets were weighed at 21 days of age. Using the Experimental Animal Allotment Program (version 1.1) in accordance with the method established by Kim and Lindemann (Kim and M.D. Lindemann. 2007), 36 piglets (5.84±0.21 kg) were selected and divided into four groups (*n* = 9 per group) according to weight and sex. The animals

in each group were divided into three identical pens containing three piglets each (**Table 3.1**).

The NC (negative control) group received ASF early-stage fodder without any added MGM-P; the LT (low-level treatment) and HT (high-level treatment) groups received ASF early-stage fodder with 5 g/kg and 10 g/kg MGM-P, respectively; the PC (positive control) group received ASF early-stage fodder with 0.1 g/kg Flavomycin[80] (Huvepharma Co., Ltd, Sofia, Bulgaria). The experimental period was 21 days after weaning.

3.2.3. Diet and Animal Management

Table 2.3 lists the ingredients in ASF early-stage fodder, which meets the National Research Council standards (**Table 2.4**) (NRC 2012). All piglets were raised in the same high-bed nursery house, equipped with an air conditioner and mechanical ventilation, molded plastic pen floors, a feed hopper, and a SUEVIA water cup. Throughout the experimental period, piglet health status was checked and recorded twice daily.

3.2.4. Growth Performance

The piglets were weighed at the same time on 0 (weaning day), 7, 14, and 21 d after weaning; the amounts of fodder consumed in each pen were recorded. The ADG, ADFI, and FCR were analyzed.

3.2.5. Diarrhea manifestations

To determine the incidence of diarrhea, piglet feces were observed twice per day (9:00 am and 3:00 pm) and classified into one of the following grades based on appearance; grade 1, hard cylinders; grade 2, soft cylinders; grade 3, thick and mushy feces; and grade 4, sloppy feces. In the present study, grade 4 was defined as diarrhea. The diarrhea incidence was the sum of piglets with diarrhea once or more throughout the experimental period, divided by the total number of piglets in each group. When

diarrhea does not occur, the fecal scores is calculated to predict the approach degree to diarrhea.

Fecal score = (sum of fecal score of diarrhea piglets)/(total number of piglets × number of days of experiment)

3.2.6. Blood Sampling

Blood was collected from the jugular vein immediately after weighing on days 1, 7, 14, and 20. A 21-gauge needle (VENOJECT II; Terumo, Tokyo, Japan) was used to harvest blood for storage in 5-mL collection tubes containing EDTA-Na.

3.2.7. Blood Hematology Analysis

Blood hematology analyses, including white blood cell (WBC) count, lymphocyte count, neutrophil count, red blood cell (RBC) count, and platelet (PLT) count, were performed using a pocH-100iV Diff hematology analyzer (Sysmex Corp., Kobe, Japan).

3.2.8. Plasma Collection and Biochemical Examination

After hematology analyses, the blood was centrifuged for 20 min (3000 rpm) at 4 °C to obtain plasma. The plasma was immediately subjected to biochemical analyses, and the remaining plasma was stored at −80 °C for subsequent use.

Blood biochemical examinations were performed using an automatic dry-chemistry analyzer (DRI-CHEM 3500s; Fujifilm, Tokyo, Japan). The analysis included glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), gamma glutamyl transferase (GGT), ammonia (NH_3), blood urea nitrogen (BUN), amylase (AMYL), glucose (GLU), total protein (TP), albumin (ALB) and triglyceride (TG).

3.2.9. Plasma free amino acids

In total, 20 amino acids were tested in this experiment, including 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), 3 semi-essential amino acids (cysteine, tyrosine, and

glutamine), and 7 non-essential amino acids (aspartic acid, serine, alanine, glycine, glutamic acid, proline, and asparagine). The analysis was carried out using LC/MS/MS Method Package for Primary Metabolites version 2.0 (Shimadzu, Kyoto, Japan) with a Shimadzu LCMS-8030 system. In brief, each plasma sample (50 µL) was deproteinized using a methanol-containing internal standard. The supernatant was filtered, dried, and dissolved; 20 µL of sample was then injected for liquid chromatography/mass spectrometry (LC/MS) analysis. The ultra-high-performance liquid chromatographic system in this study was equipped with a DGU-20 A_{3R} degasser, two LC-20 AD binary pumps, an SIL-20 AC_{HT} auto-sampler, a CBM-20A control module, an SPD-20A detector, and a CTO-20 AC column oven. It was then coupled with an LCMS-8030 triple quadrupole mass spectrometer (Shimadzu) with an electrospray ionization source. For liquid chromatography analysis, a Discovery HS F5-3 column (2.1 mm × 150 mm, 3 µm, Sigma-Aldrich, St. Louis, MO, USA) was used. The analysis was performed by means of a gradient system with two mobile phases: (A), a mixture of 0.1% formic acid (LC/MS grade; Wako Pure Industries, Osaka, Japan) and water (LC/MS grade; Wako Pure Industries); and (B), acetonitrile (LC/MS grade; Wako Pure Industries) containing 0.1% formic acid. The analysis began at 0% (B) for 2 min, with the concentration of (B) increasing to 25% at 5 min, 35% at 11 min, and 95% at 15 min, remaining at 95% until 20 min, and then decreasing to 0% at 20.1 min and remaining at this level until 25 min. The column temperature was 40°C, the flow rate was 0.25 mL/min, and the injection volume was 3 µL. For mass spectrometry analysis conditions, the nebulizer flow rate was 2 L/min and the drying gas flow rate was 15 L/min. The DL and heat block temperatures were 250°C and 400°C, respectively. 2-Morpholinoethanesulfonic acid (Dojindo, Kumamoto, Japan) and L-methionine sulfone (Wako Pure Industries) were used as internal standards in the analysis. Program monitoring and data acquisition were controlled using LabSolutions LCMS software (Shimadzu).

3.2.10. Actual and Relative Weights/Lengths of Organs and Intestines

After the feeding trial, one piglet near the average BW for each pen underwent induction of deep anesthesia via thiopental sodium (Ravonal 0.5 g; Mitsubishi Tanabe Pharma, Osaka, Japan) injection into the jugular vein; they were then sacrificed. Necropsies were performed and the organs (liver, pancreas, spleen, kidney, stomach, small intestine, and large intestine) were carefully removed. The weights of all organs, including individual intestinal tract sections, were measured. The relative organ weights were calculated as the organ weight divided by BW (%). The lengths of individual intestinal tract sections were measured and the relative lengths of intestinal tract sections to piglet BW were also calculated (cm/kg).

3.2.11. Statistical Analysis

Data analysis was performed using JMP Pro software (version 15.2.0, SAS Institute Inc., Cary, NC, USA). One-way analysis of variance was used to compare differences among experimental groups. When the p-value from analysis of variance was < 0.05, pairwise differences were assessed using the Tukey–Kramer honestly significant difference test. p-values < 0.05 were considered to indicate statistical significance. Results are presented as the means ± standard errors of the mean.

3.3. Results

3.3.1. Growth Performance

Table 3.2 and **Figure 3.1** show the result of growth performance and feed efficiency. During the feeding experiment, all piglets stayed healthy, and no animal losses occurred. In the first two weeks after weaning, we observed that the FCR of LT group was the lowest, on the contrary, FCR of HT group was the highest, and significantly higher ($p < 0.05$) than that of the LT and PC groups. A slight trend of better body weight was recognized after weaning in the LT group. However, at all stages, the four feeding treatments showed no difference in ADG and ADFI ($p > 0.05$).

3.3.2. Diarrhea manifestations

No signs of diarrhea were recorded during the experimental period. Therefore, the fecal score was used to predict the approach degree of diarrhea (**Figure 3.2**). Within 2 days after weaning, the fecal scores of the piglets in the NC and LT groups showed a rapid upward trend, and then the LT group quickly declined. Starting on the 7 th day after weaning, all groups showed elevated fecal scores, and the HT group was probably the closest to diarrhea. After that, the HT group remained high until 13 d and then descend. In contrast, piglets in LT group remained relatively low in most periods. Although the antibiotic supplement group was slightly higher than the LT group on the average, the overall change was more stable.

3.3.3. Blood Hematology Analysis

The changes of blood hematology in piglets after weaning can be observed in **Figure 3.3**. There were no significant differences in blood hematology counts, including WBC, lymphocytes, neutrophils, RBC and platelet counts.

3.3.4. Blood Biochemical Analysis

As shown in **Table 3.3** and **Figure 3.4**, no significant differences were observed in indicators related to liver metabolism, including GPT, GOT, and GGT ($p > 0.05$). The blood glucose and total protein concentration related to nutrient metabolism were also not affected by different treatments ($p > 0.05$). However, antibiotic supplementation significantly increased ($p < 0.05$) the TG concentration in piglet plasma at 21 d in comparison with that of the NC and LT groups.

3.3.5. Plasma free amino acids

Changes in the plasma free amino acids distribution of the weanling pigs are shown in **Table 3.4** and **Figure 3.5**. Dietary 1.0% MGM-P supplementation significantly reduced ($p < 0.05$) the concentration of arginine and cysteine in piglet blood at 21 d compared with PC group. Also, piglets in HT group had significantly reduced ($p < 0.05$) phenylalanine concentration at 21 d compared with piglets offered basal diet and diet

containing antibiotic. The inclusion of MGM-P or antibiotic in piglet diets did not affect the plasma concentrations of alanine and lysine, however, around the last week after weaning, the lysine concentration of piglets in the HT group tended to be lower, but the difference was not significant ($p > 0.05$).

3.3.6. Actual and Relative Weights/Lengths of Organs and Intestines

Autopsy at the end of the experiment found no abnormalities in the organs. The effect of dietary MGM-P supplementation on actual and relative weights or lengths of organs and intestines in piglets is presented in **Table 3.5** and **Table 3.6**. The dietary treatments had no influence on these relevant parameters of the organ in piglets ($p > 0.05$).

3.4. Discussion

Although the positive effect of QT supplementation on feed efficiency was only determined in the first two weeks, this is already an exciting result. Few studies have reported the positive results of tannins on pig growth performance. In fact, tannin has been considered for a long time in the past as an anti-nutritional factor of animals, and may negatively affect the growth performance of both monogastric and regurgitant animals (Mueller-Harvey 2006). The reason comes from two aspects: one is that tannins can combine with protein to form a precipitate, which will produce astringent taste to reduce feed intake and inhibit the activity of digestive enzymes in the animal's gastrointestinal tract when the animal eats (Martens et al. 2012); the other is the potential toxicity characteristics of tannins (Filippich et al. 1991). However, studies in recent decades have denied these views to a certain extent. The addition of 0.3% MGM-P to piglet feed was found in chapter 2 with no overall effect on piglet growth, but may improve intestinal villi morphology and inhibit diarrhea in piglets. Su et al. (2019) also reported that adding 1 g/kg MGM-P can significantly improve the ADG and ADFI of finishing pigs, the feed utilization rate, and decrease the number of E. coli in feces. Similar results were obtained in this study. The addition of 0.5% MGM-P effectively

improved feed efficiency and made the body weight change of piglets slightly superior. Even this positive effect is equal to or slightly stronger than antibiotic additives. Experience has been gained in using antibiotics for years, that is, antibiotics can only play their best role when animals are in poor health and living conditions are unhygienic (Prescott J.F. and Baggot J.D. 1993). In addition, it is worth highlighting that the higher level of addition (1.0%) did not play a positive role in the current experiment. From previous studies, it is not difficult to find that both the growth failure and poisoning caused by tannins are related to the intake of very high levels by animals (Garg et al. 1992; Frutos et al. 2020). However, the high level refers to how many doses are not easy to describe. Because most of the raw ingredient in feed contain tannins, its concentration will also be affected by many factors such as the feed type, ingredients proportion, processing method, and the storage time (Shahidi and Ambigaipalan 2015). Although the 1.0% increase in MGM-P does not affect piglet health, it is not as beneficial as 0.5% in terms of growth performance. Combining the results of Chapter 2, we can speculate that the 0.5% addition amount seems to be closer to the appropriate addition amount.

In contrast to the experimental results in Chapter 2, no diarrhea occurred in any of the piglets treated in this experiment. This may be related to the low rearing density in this experiment, as the piglets were subjected to less weaning stress at a smaller rearing density. The trend of diarrhea occurrence was predicted by fecal scoring, and the results showed that the 1.0% addition level not only failed to promote the growth of piglets, but also had a negative impact on the fecal scores. The higher fecal score indicates changes in the health status of the piglets, which is also consistent with growth performance. Although it is difficult to determine why high levels of QT did not reduce fecal scores, the slightly higher number of neutrophils in the HT group at 7-21 days post-weaning based on blood hematology implies that mild inflammation may have occurred, as neutrophil is a key component of the innate response to the inflammatory process (Fournier and Parkos 2012). In terms of WBC counts, piglets had lower WBC

counts at weaning, either compared to those in Chapter 2 or to the normal range of WBC counts given by Czech et al. (2018). This possibly be due to the lower initial body weight of the piglets, which usually have weaker immunity. Then during weaning, the increase in WBC count was also smaller compared to Chapter 2, which was also largely related to the low rearing density, and the slowdown in WBC count increase after weaning was consistent with the 0 incidence of diarrhea.

Hydrolysable tannins by themselves or degradation products (e.g. gallic acid) can cause toxic effects after being absorbed by the small intestine and may lead to liver damage (Jansman 1993). However, it has not been reported whether QT is poisonous. After liver cell injury or permeability change of liver membrane, GOT, GPT and GGT will leak into peripheral blood through membrane, and the concentration change of these enzymes can be observed (Zhang et al. 2016). Our results in this regard did not show anomalies, it indicates that 1.0% supplement level is acceptable for animals. As monitoring indicators of nutritional status, blood glucose and total protein content were not affected by QT supplementation. The triglyceride in blood comes from food as well as being produced by the body, and is secreted from liver into blood through triglyceride-rich lipoproteins. The significantly increased TG concentration of piglets in PC group at 21 d indicates the strengthening of this process or the increase of lipoprotein content (Ao et al. 2011).

The intake of tannins by monogastric animals may affect the metabolism of protein and amino acids, so we analyzed the changes in blood free amino acids at different periods after weaning. Arginine is mainly metabolized and absorbed in the small intestine, and its importance in intestinal immune response was confirmed by a large number of studies (Wang et al. 2009). According to reports, enteral administration of arginine to mice can alleviate mucosal injury, improve intestinal morphology and increase cell proliferation index of jejunum and ileum (Sukhotnik et al. 2004). Our study shows: at 21 days after weaning, the HT group had significantly lower arginine concentration than the other three groups, suggesting a reduction in sources or an

increase in use. Due to the higher level of tannin supplementation in the HT group, which allowed more tannins to bind to proteins in the feed and prevent their metabolism in the gut, so the amount of amino acids that can be absorbed by the gut becomes less, which may lead to insufficient arginine content in the intestinal mucosa and affect the development of the intestinal mucosa, and correspondingly, the fecal score is also higher. Phenylalanine acts as a competitive inhibitor in regulating intestinal amino acid absorption (Liu et al. 2017). Therefore, the concentration of phenylalanine in QT supplementation groups were lower on 21 d, indicating that QT helps to improve the utilization efficiency of amino acids in the intestine. Cysteine is synthesized insufficiently by piglets under some feeding conditions (e.g., corn and soybean meal), so they are conditionally essential amino acids for piglets (Rezaei et al. 2013). The cysteine concentration of piglets in the antibiotic supplementation group and LT group gradually increased, and at 21 days post-weaning, cysteine concentrations in the HT group remained higher than in the negative control group, indicating that cysteine metabolism was less affected by QT supplementation. In contrast to the results in Chapter 2, neither 0.5% nor 1.0% MGM-P supplementation affected alanine metabolism. Again, no significant differences in lysine concentrations were observed. But it should be noted that lysine and arginine are factors linked to growth hormone release in young children through the somatotropic axis, and their intake is effective for good early growth (Soliman et al. 2021). The lower concentrations of these two amino acids in piglets of HT group during the last week of feeding may be associated with poor growth performance.

Wang et al. (2020b) added 0, 0.05, 0.1 and 0.15% tannic acid and antibiotic to the diets of weaned piglets on 21-day-old weaned piglets, respectively, and found that none of them had an effect on the relative organ weight. These results are consistent with the observations of our work in Chapter 2. So far, there are not many studies on the effect of high-level condensed tannin addition on the relative organ weight of piglets. However, the current research results show that the addition of 1.0% MGM-P still has

no effect on the development of organs, and the pathological features of organs are not observed during dissection.

3.5. Conclusions

In conclusion, although adding a high level of 1.0% MGM-P to the diet does not affect health of the piglets, it has no advantage in growth performance. On the contrary, the addition of 0.5% MGM-P is helpful to improve the feed efficiency of piglets within two weeks after weaning, and the body weight is also slightly superior compared to antibiotic addition group. It has no adverse effects on piglet health, reduces fecal scores to a certain extent. Combined with our previous research, it can be roughly determined that about 0.5% is closer to the optimal additive amount.

Table 3.1. Experimental animal allotment.

Group	n			BW			
	Male	Female	Total	Mean	SEM	*p*-value	CV (%)
NC	5	4	9	5.86	0.42	1.00	0.31
LT	5	4	9	5.84	0.44		
HT	5	4	9	5.85	0.49		
PC	5	4	9	5.82	0.42		

Abbreviations: n, number of piglets; BW, body weight; SEM, standard error of the mean; CV, coefficient of variation.

Table 3.2. Effects of high-dosage MGM-P supplementation on growth performance in weaned piglets.

Item	Initial BW (kg)	Final BW (kg)	ADG (kg)	ADFI (kg)	FCR (kg/kg)
0-14 d					
NC	5.86±0.42	12.50±0.97	0.47±0.05	0.60±0.03	1.27±0.03[ab]
LT	5.84±0.44	12.87±0.86	0.50±0.03	0.60±0.02	1.20±0.02[b]
HT	5.85±0.49	12.19±0.91	0.45±0.04	0.61±0.06	1.34±0.02[a]
PC	5.82±0.42	12.50±0.67	0.48±0.02	0.59±0.03	1.23±0.01[b]
0-21 d					
NC	5.86±0.42	17.68±1.24	0.56±0.04	0.74±0.05	1.31±0.01
LT	5.84±0.44	18.25±1.10	0.59±0.03	0.74±0.04	1.26±0.02
HT	5.85±0.49	17.35±1.11	0.55±0.03	0.73±0.06	1.33±0.04
PC	5.82±0.42	17.77±0.72	0.57±0.02	0.74±0.05	1.29±0.03

Values of BW and ADG are expressed as mean ± SEM (n = 9); Values of ADFI and FCR are expressed as mean ± SEM (n = 3). [a,b] Mean values within a row with dissimilar superscript letters are significantly different ($p < 0.05$).

Table 3.3. Effects of high-dosage MGM-P supplementation on blood biochemical parameters in weaned piglets.

Item	GPT (U/L)	GOT (U/L)	GGT (U/L)	NH_3 (μg/dL)	BUN (mg/dL)	AMYL (U/L)	GLU (mg/dL)	TP (g/dL)	ALB (g/dL)	TG (mg/dL)
0d[1]										
NC	35.56±1.11	31.89±2.39	24.56±4.63	121.00±14.65	6.82±0.75	868.22±102.16	123.56±2.56	4.53±0.15	2.92±0.13	53.44±6.02
LT	31.44±1.57	32.33±2.07	22.67±2.19	113.78±4.72	5.64±0.31	971.22±132.76	123.22±3.76	4.77±0.13	2.97±0.1	63.11±13.68
HT	34.56±1.08	39.78±7.44	20.67±1.55	150.22±26.81	5.96±0.71	1060.22±159.59	121.00±3.99	4.54±0.06	2.96±0.09	47.78±7.83
PC	33.44±1.25	32.56±2.02	21.67±2.10	168.56±40.33	6.68±0.57	1040.67±66.55	132.67±4.95	4.94±0.14	3.28±0.12	77.67±15.42
7d										
NC	39.33±1.31	38.11±3.26	26.56±1.34	103.33±7.14	9.41±0.70	971.22±84.76	142.67±6.55	4.82±0.16	3.30±0.12	28.78±7.69
LT	37.78±2.10	37.22±4.02	28.56±1.77	124.00±12.55	7.96±0.52	1047.11±115.91	135.67±3.55	5.10±0.11	3.49±0.1	18.00±2.90
HT	38.56±1.19	47.78±7.05	28.11±1.42	115.11±8.96	7.44±0.34	1134.78±144.79	134.00±6.46	4.93±0.10	3.58±0.09	19.44±2.37
PC	36.33±1.25	31.33±1.64	24.67±1.99	102.22±3.15	8.47±0.40	1109.89±61.50	133.78±4.28	5.08±0.04	3.66±0.04	14.78±1.82
14d										
NC	39.67±1.31	45.89±4.69	36.00±3.70	122.33±15.85	12.62±1.40	891.00±87.59	140.56±5.69	5.18±0.14	3.89±0.14	17.56±2.43
LT	36.56±0.65	46.89±3.73	43.56±4.87	209.44±48.76	13.52±1.25	951.11±117.17	162.22±11.37	5.37±0.08	4.10±0.10	30.00±6.84
HT	37.78±1.04	44.22±5.28	36.22±3.05	148.00±13.23	9.83±0.93	1126.44±129.96	157.11±10.82	5.20±0.11	4.02±0.10	31.00±5.37
PC	36.67±1.66	44.78±3.57	33.67±3.11	153.78±8.15	13.49±0.43	881.56±56.87	147.67±6.66	5.24±0.13	4.07±0.08	29.00±4.40
21d										
NC	39.56±1.29	39.44±3.66	30.56±3.04	110.44±10.39	16.36±1.36	744.22±82.69	141.33±3.15	5.00±0.07	4.08±0.08	16.00±1.25[b]
LT	36.00±1.30	38.56±4.33	32.33±1.72	131.56±11.82	16.81±1.22	868.00±116.01	153.67±8.83	5.08±0.11	4.03±0.09	16.22±1.67[b]
HT	38.44±2.18	50.78±13.6	30.22±1.53	151.22±32.61	14.72±1.13	1013.89±124.27	148.22±11.18	4.97±0.08	4.00±0.09	21.78±3.60[ab]
PC	38.33±1.94	51.11±4.44	30.44±2.57	153.44±14.69	17.09±0.81	824.67±42.78	143.78±4.09	5.14±0.11	4.16±0.11	35.22±8.81[a]

Abbreviations: GPT, glutamate-pyruvate transaminase; GOT, glutamate-oxaloacetate transaminase; GGT, gamma-glutamyl transpeptidase; NH_3, ammonia; BUN, blood urea nitrogen; AMYL, amylase; GLU, glucose; TP, total protein; ALB, albumin; TG, triglyceride. All data are expressed as mean ± SEM; n = 9. [1] Blood was collected before the provision of feed with MGM-P on the day of weaning. [a,b] Mean values within a row with dissimilar superscript letters are significantly different ($p < 0.05$).

Table 3.4. Effects of high-dosage MGM-P supplementation on plasma free amino acids in weaned piglets (uM).

Item	Asparagine	Aspartic acid	Serine	Alanine	Glycine	Glutamine	Threonine	Cysteine	Glutamic acid	Proline	Lysine	Histidine	Arginine	Valine	Methionine	Tyrosine	Isoleucine	Leucine	Phenylalanine	Tryptophan
0d[1]																				
NC	99.11±7.28	82.84±2.46	198.10±7.71	542.44±24.43	854.17±47.33	382.91±20.71	241.79±17.58	3.48±0.43	102.31±6.80	264.39±17.70	146.24±11.03	35.33±1.39^{ab}	117.02±8.48	205.89±13.28	67.48±7.24	199.30±16.57	108.72±7.30	142.54±9.84	93.90±7.20	58.77±4.38
LT	85.05±7.06	80.87±3.25	176.65±11.67	478.86±39.12	804.03±47.92	336.25±26.34	212.65±18.31	2.47±0.46	98.96±9.45	225.74±21.15	117.26±13.90	29.02±2.91^{b}	91.59±7.38	171.33±17.28	58.66±6.80	151.67±17.42	93.07±11.20	119.52±15.11	77.64±9.44	51.18±4.91
HT	93.25±5.26	89.72±2.55	195.43±10.64	502.45±19.45	787.56±35.30	378.32±13.02	234.27±14.70	3.61±0.55	106.24±7.59	233.97±11.25	130.70±8.56	32.66±1.54^{ab}	106.73±7.04	185.57±13.06	65.97±6.26	167.08±7.81	103.01±6.55	128.85±6.23	75.34±5.69	54.17±2.30
PC	99.94±6.31	83.42±2.48	215.21±12.63	550.5±32.11	854.36±46.98	363.79±18.53	252.83±18.03	3.04±0.57	94.88±6.15	259.25±21.84	144.92±10.04	37.46±1.94^{a}	111.20±10.19	208.68±17.25	64.23±5.05	180.20±16.31	105.68±9.73	144.06±14.91	94.69±10.15	58.37±4.78
7d																				
NC	77.40±4.13	66.15±2.69	138.44±11.84	362.49±26.56	687.64±43.69	370.65±12.00	268.39±12.91	2.48±0.34	100.58±9.96	177.93±10.29	90.01±12.15	25.84±1.78	91.50±9.28	223.25±9.91	180.47±29.71	136.55±12.89	123.21±10.66	123.38±8.47	77.79±5.82	45.42±4.12
LT	71.96±3.48	68.120±1.40	142.23±7.59	329.68±21.09	778.78±40.02	355.13±17.67	280.89±17.60	3.02±0.45	101.05±8.73	163.01±6.51	56.35±7.33	21.57±1.30	83.16±7.45	205.25±11.23	169.04±23.69	126.86±8.68	105.81±6.81	104.57±6.82	61.15±4.79	41.47±3.26
HT	75.62±5.32	69.20±2.56	147.15±16.38	382.81±23.05	795.06±34.43	388.97±22.18	285.63±25.65	2.64±0.50	104.79±6.90	173.10±11.76	69.17±17.09	25.70±3.74	92.48±15.19	221.45±17.51	156.32±22.62	135.01±13.55	115.80±7.98	121.14±14.03	70.86±8.23	46.92±5.03
PC	75.32±3.68	70.66±1.42	145.22±6.66	366.31±21.18	742.91±25.91	374.58±12.29	297.52±10.19	3.79±0.48	111.78±5.65	173.34±8.43	62.34±8.80	23.50±1.40	83.55±7.08	231.86±13.28	161.52±18.20	150.16±6.60	126.95±6.84	117.95±9.35	68.18±4.60	48.99±2.55
14d																				
NC	80.15±5.41	66.48±2.30	161.20±13.29	322.29±26.33	1036.56±46.09	403.09±24.94	315.86±19.09	3.60±0.53	103.74±9.78	196.28±13.44	103.44±12.17	32.71±3.42	88.31±9.44	244.93±14.18	157.73±19.64	150.64±12.54	119.81±9.02	138.89±14.97	71.88±5.40	58.97±4.09
LT	89.08±5.97	73.43±3.31	188.29±12.60	342.76±28.71	1115.89±62.15	431.36±20.47	353.60±18.33	3.67±0.66	122.31±16.26	207.59±12.17	119.63±10.04	34.31±2.45	105.01±8.26	263.76±11.33	189.87±27.39	162.21±11.47	125.34±7.45	146.45±9.42	77.88±6.06	64.04±6.37
HT	83.82±3.45	72.00±1.77	173.09±10.78	369.20±20.08	1166.68±37.43	430.94±18.39	322.15±12.04	2.54±0.38	116.16±10.07	201.65±7.86	105.11±9.59	31.32±2.89	90.49±6.72	244.50±10.80	144.84±17.70	144.18±7.26	122.38±6.61	144.88±10.81	69.08±4.81	60.68±3.11
PC	84.12±8.02	65.01±1.99	169.67±15.24	303.98±28.8	1001.68±34.42	370.85±23.13	328.72±25.89	4.15±0.51	105.99±7.10	185.69±17.61	113.65±16.55	33.00±3.39	92.63±11.35	252.05±19.60	156.64±18.94	149.00±11.17	126.58±11.14	145.28±18.50	71.45±5.73	59.06±3.81
21d																				
NC	121.30±9.60	90.16±2.57	246.08±11.85	412.72±36.74	1332.01±97.85	584.51±31.74	534.64±38.02	4.25±0.46^{b}	117.39±9.27	312.39±21.41	181.57±16.38	56.21±3.44	140.17±9.93^{ab}	428.87±23.22	191.72±27.29	208.43±16.51	209.46±15.51	253.48±21.9	123.77±9.17^{a}	99.91±6.61
LT	107.68±7.56	87.10±2.25	247.16±20.01	382.81±29.88	1197.38±34.30	559.88±39.28	506.29±27.20	5.08±0.39^{ab}	143.44±10.75	296.31±26.01	162.47±13.22	52.34±3.49	141.28±9.94^{ab}	411.25±30.88	192.54±18.37	201.56±14.64	191.14±15.81	228.61±21.91	102.29±10.07^{ab}	89.6±7.44
HT	107.61±9.77	89.42±2.85	228.38±25.14	421.26±39.34	1157.93±33.96	571.06±26.15	460.32±37.97	4.83±0.69^{b}	147.48±12.62	286.16±29.25	151.33±12.50	51.52±5.92	118.64±8.58^{b}	384.42±24.24	187.56±31.36	183.84±13.76	174.86±11.53	213.69±16.29	88.40±7.43^{b}	80.74±5.57
PC	122.18±7.08	88.04±2.30	278.48±18.38	447.46±17.18	1253.05±53.16	570.69±16.80	565.84±33.80	7.05±0.69^{a}	135.80±10.59	300.33±14.71	196.23±12.94	59.73±4.59	164.42±13.76^{a}	436.73±14.28	164.57±12.16	206.65±10.13	218.51±9.58	271.63±12.21	121.46±7.40^{a}	96.83±5.38

All data are expressed as mean ± SEM; n = 9. [1] Blood was collected before the provision of feed with MGM-P on the day of weaning. ^{a,b} Mean values within a row with dissimilar superscript letters are significantly different ($p < 0.05$).

Table 3.5. Effects of high-dosage MGM-P supplementation on the actual organ weight/length of weaned piglets.

Item	Liver (g)	Pancreas (g)	Spleen (g)	Kidney (g)	Stomach (g)	Small intestine weight (g)	Small intestine length (m)	Large intestine weight (g)	Large intestine length (m)
NC	597.27±86.60	38.47±5.43	41.47±2.25	135.73±25.50	104.10±18.65	595.97±73.98	1188.00±27.26	202.07±20.78	219.17±10.14
LT	531.93±72.17	35.67±6.24	47.87±7.60	121.87±22.44	86.87±12.66	603.53±99.37	1140.17±62.98	185.23±28.52	220.00±17.24
HT	514.50±87.61	35.53±6.16	37.63±2.19	119.10±25.52	87.90±9.40	582.07±36.16	1123.00±31.47	182.50±16.20	215.00±12.58
PC	536.10±45.63	39.07±5.32	40.67±3.41	128.30±6.33	96.27±10.26	545.03±11.39	1105.00±57.36	177.07±12.16	200.33±10.27

All data are expressed as mean ± SEM; $n = 3$. There were no statistically significant differences among the four groups based on one-way analysis of variance.

Table 3.6. Effects of high-dosage MGM-P supplementation on the relative organ weight/length of weaned piglets.

Item	Liver (%)	Pancreas (%)	Spleen (%)	Kidney (%)	Stomach (%)	Small intestine weight (%)	Small intestine length (cm/kg)	Large intestine weight (%)	Large intestine length (cm/kg)
NC	2.88±0.15	0.19±0.01	0.21±0.02	0.65±0.03	0.49±0.03	3.10±0.84	59.32±7.93	0.99±0.08	10.86±1.19
LT	2.65±0.12	0.18±0.01	0.24±0.00	0.60±0.03	0.43±0.02	3.07±0.48	58.61±7.72	0.92±0.02	11.19±1.10
HT	2.49±0.06	0.17±0.02	0.19±0.02	0.57±0.03	0.43±0.03	2.90±0.24	56.61±7.05	0.91±0.08	10.72±0.94
PC	2.59±0.10	0.19±0.01	0.20±0.03	0.62±0.03	0.46±0.02	2.66±0.17	53.58±1.60	0.86±0.01	9.86±1.22

All data are expressed as mean ± SEM; $n = 3$. There were no statistically significant differences among the four groups based on one-way analysis of variance.

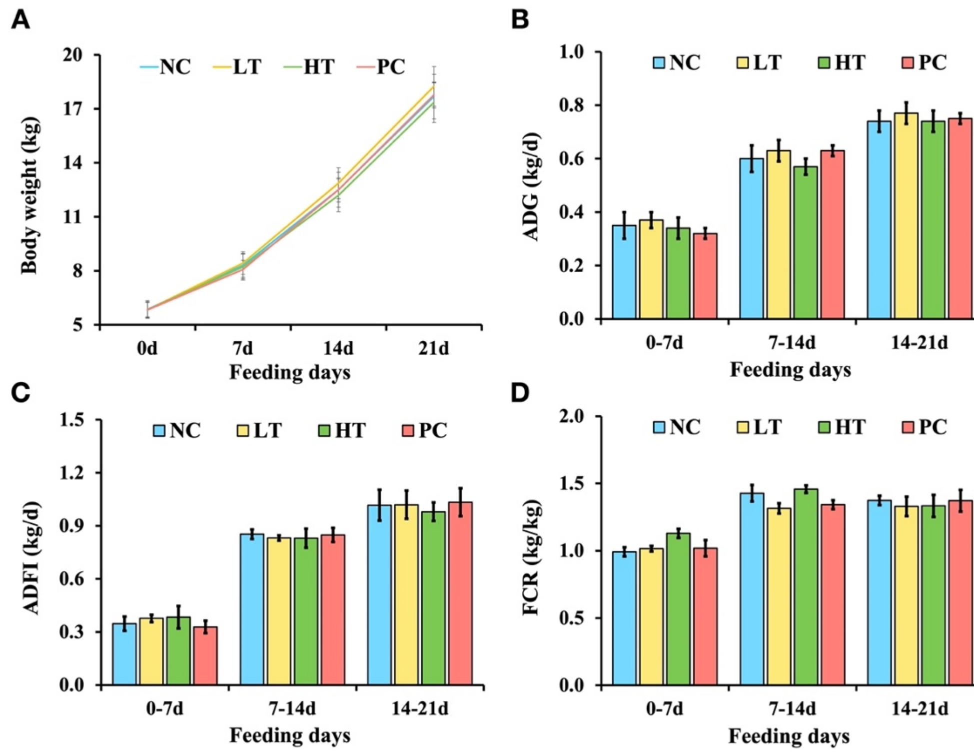


Figure 3.1. Effects of high-dosage MGM-P supplementation on growth performance in weaned piglets. Data in **A** and **B** are expressed as mean ± SEM (n = 9); Data in **C** and **D** are expressed as mean ± SEM (n = 3). There were no statistically significant differences among the four groups based on one-way analysis of variance.

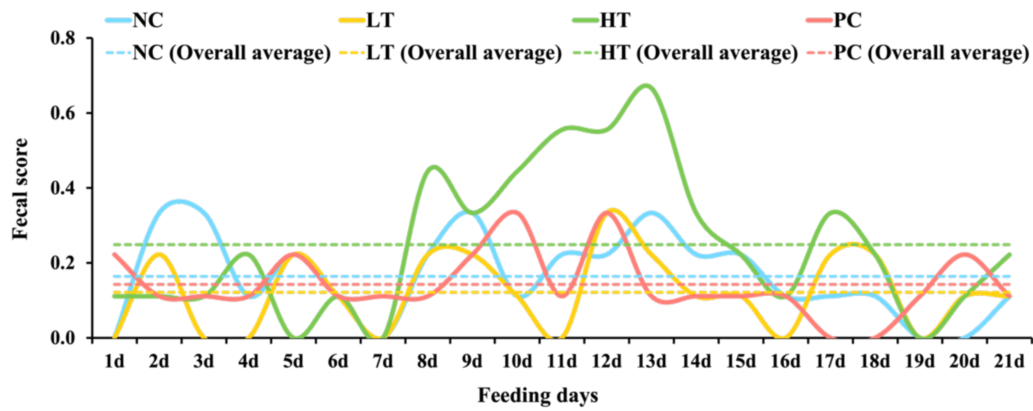


Figure 3.2. Effects of high-dosage MGM-P supplementation on fecal scores in weaned piglets. Data are expressed as mean (n = 3). There were no statistically significant differences among the four groups based on one-way analysis of variance.

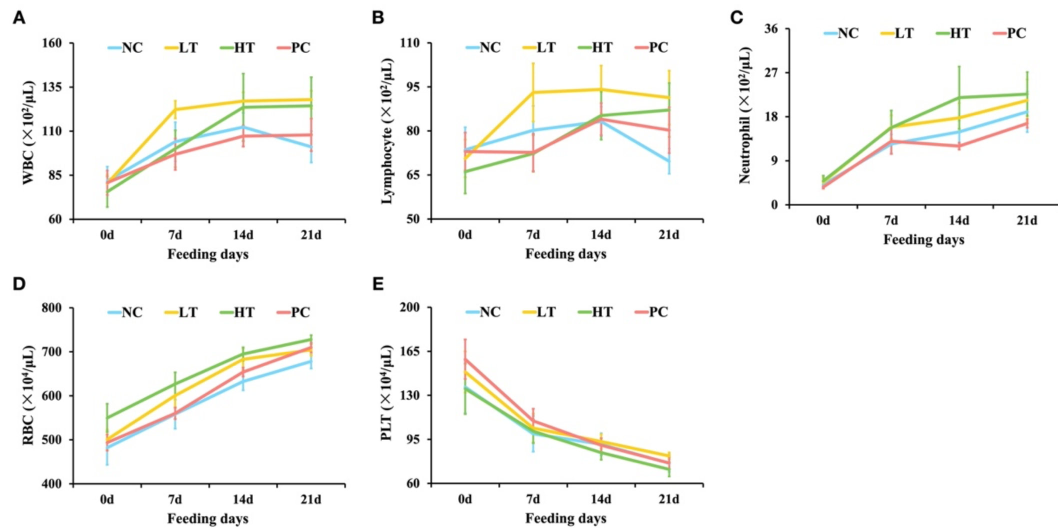


Figure 3.3. Effects of high-dosage MGM-P supplementation on blood hematology parameters in weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; $n = 9$. There were no statistically significant differences among the four groups based on one-way analysis of variance. (**A**) the changes of the white blood cell count; (**B**) the changes of the lymphocyte count; (**C**) the changes of the neutrophil count; (**D**) the changes of the red blood cell count; (**E**), the changes of the platelet count.

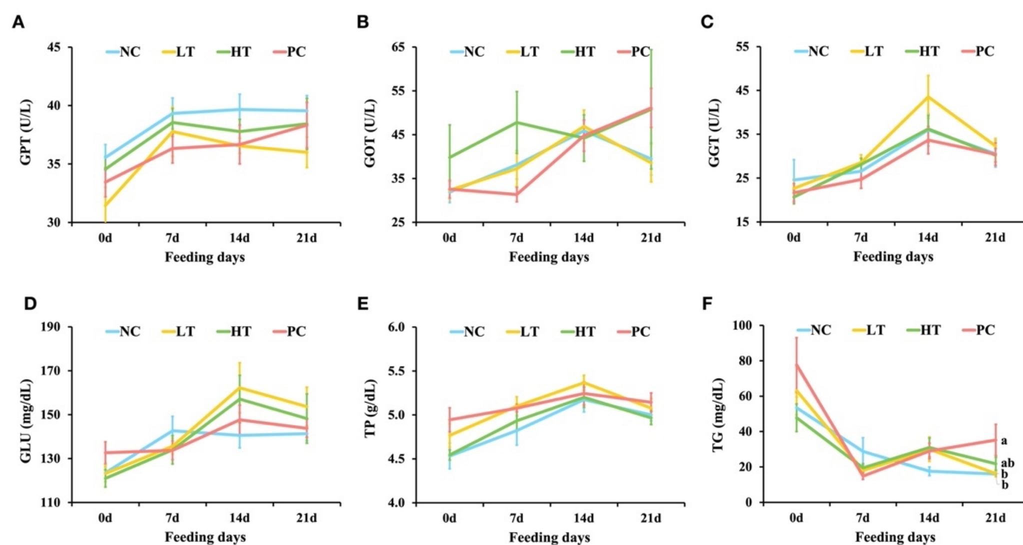


Figure 3.4. Effects of high-dosage MGM-P supplementation on blood biochemical parameters of weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; n = 9. Different lowercase letters on the right side of the nodes indicate significant differences ($p < 0.05$). (**A**) the changes of the glutamic pyruvic transaminase level; (**B**) the changes of the glutamic oxaloacetic transaminase level; (**C**) the changes of the gamma glutamyl transferase level; (**D**) the changes of the glucose concentration; (**E**) the changes of the total protein concentration; (**F**) the changes of the triglycerides level.

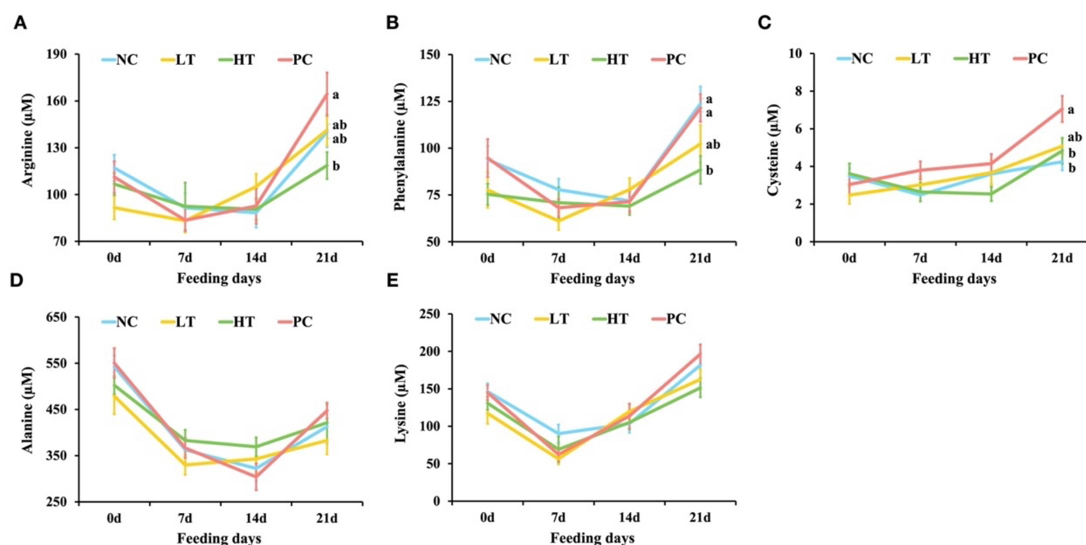


Figure 3.5. Effects of high-dosage MGM-P supplementation on the blood free amino acid concentration of weaned piglets. Blood for 0 d was collected before MGM-P-supplemented feed was provided on the day of weaning. Values are expressed as mean ± SEM; $n = 9$. Different lowercase letters on the right side of the nodes indicate significant differences ($p < 0.05$). (**A**) the changes of the arginine concentration; (**B**) the changes of the phenylalanine concentration; (**C**) the changes of the cysteine concentration; (**D**) the changes of the alanine concentration; (**E**) the changes of the lysine concentration.

Chapter 4 Effect of long-term dietary Quebracho tannin supplementation on growth performance and overall health of grow-finish pigs

4.1. Introduction

Although the addition of antibiotics is the most effective for improving the growth and efficiency of piglets (Li 2017), however, in order to achieve higher productivity and economic benefits, farmers have gradually expanded the use of antibiotic additives from the weaning stage to every stage of pig growth.

It has been found that the use of antibiotics in diet can improve the sow's reproductive performance, pregnancy rate, litter size and farrowing rate. Supplementation of antibiotics can also improve the growth performance and feed efficiency of growing pigs (Li 2017). It is pointed out in a literature that feeding swine with subtherapeutic levels of antibiotics can increase body weight gain by 3.3-8.8% and increase feed efficiency by 2.5-7.0% (M. Ellin Doyle 2001).

Since during the fattening stage, pig health and growth performance are directly linked to economic performance, it is necessary to evaluate the effectiveness of QT as an additive during this stage. Our previous studies on the application of QT in piglets at the weaning stage have given encouraging results, showing that some improvement in feed efficiency already after 0.5% MGM-P supplementation. However, since piglets are exposed to QT for only 3 weeks, it is worth exploring further whether continued supplementation at this level during the longer grow-finish phase still has positive effects and whether it is safe. Therefore, in this experiment, the effects of 0.5% MGM-P on performance, hematology, blood biochemistry and free amino acids of fattening pigs were further investigated. At the best of our knowledge, few studies have evaluated the potential of QT as an antibiotic alternative in growing and finishing pigs. Therefore, this study will create a more comprehensive theoretical basis for the practical application of QT in the pig industry.

4.2. Materials and Methods

4.2.1. Materials

As in Chapter 2, MGM-P (provided by Kawamura Ltd. Tokyo, Japan) was employed as a source of QT.

4.2.2. Animals, Treatments, and Experimental Design

The experiment was conducted at the Animal Resource Science Center of the University of Tokyo (Kasama, Japan), and approved (P20-097) by the Animal Care and Use Committee of the Faculty of Agriculture, The University of Tokyo. Twenty-four pigs (Duroc × Landrace × Yorkshire), from 68 to 69 days of age with an initial average body weight of 37.60 ± 0.86 kg, were allocated into two dietary treatments with the Experimental Animal Allotment Program (Kim and M.D. Lindemann. 2007) according to initial BW, sex and litter. The animals in each group were divided into three identical pens containing four pigs each (**Table 4.1**). The two dietary treatments consisted of the basal diet treatment (C) and the 0.5% MGM-P supplementation group (T). During the 16 weeks grow-finish feeding trial, BW and feed intake were recorded every 4 weeks. All pigs were checked daily for general health and eating status during the experimental period.

4.2.3. Diet and Animal Management

Three dietary phases were formulated in order to meet or exceed the National Research Council standards (NRC 2012). The ingredients composition and nutritional levels for starter phase (1–2 weeks), grower phase (3–6 weeks), and finisher phase (7-16 weeks) are summarized in **Table 4.2**. The pigs were housed in a same semi-enclosed pig house without temperature control, and the houses were kept clean daily. All animals were given free access to feed and water using a 4-hole stainless-steel feeder, and a stainless-steel nipple waterer. A standard immune procedure was implemented throughout the trial.

4.2.4. Growth Performance

The pigs were weighed at the same time on 0, 4, 8, 12, and 16 weeks after administration; the amounts of fodder consumed in each pen were recorded. The ADG, ADFI, and FCR were analyzed.

4.2.5. Blood Sampling

Blood was collected from the jugular vein immediately after weighing on 0, 4, 8, 12, and 16 weeks. A 21-gauge needle (VENOJECT II; Terumo, Tokyo, Japan) was used to harvest blood for storage in 5-mL collection tubes containing EDTA-Na.

4.2.6. Blood Hematology Analysis

Blood hematology analyses, including white blood cell (WBC) count, lymphocyte count, neutrophil count, red blood cell (RBC) count, and platelet (PLT) count, were performed using a pocH-100iV Diff hematology analyzer (Sysmex Corp., Kobe, Japan).

4.2.7. Plasma Collection and Biochemical Examination

After hematology analyses, the blood was centrifuged for 20 min (3000 rpm) at 4 °C to obtain plasma. The plasma was immediately subjected to biochemical analyses, and the remaining plasma was stored at −80 °C for subsequent use.

Blood biochemical examinations were performed using an automatic dry-chemistry analyzer (DRI-CHEM 3500s; Fujifilm, Tokyo, Japan). The analysis included glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), gamma glutamyl transferase (GGT), ammonia (NH_3), blood urea nitrogen (BUN), amylase (AMYL), glucose (GLU), total protein (TP), albumin (ALB) and triglyceride (TG).

4.2.8. Plasma free amino acids

In total, 20 amino acids were tested in this experiment, including 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine,

threonine, tryptophan, and valine), 3 semi-essential amino acids (cysteine, tyrosine, and glutamine), and 7 non-essential amino acids (aspartic acid, serine, alanine, glycine, glutamic acid, proline, and asparagine). The analysis was carried out using LC/MS/MS Method Package for Primary Metabolites version 2.0 (Shimadzu, Kyoto, Japan) with a Shimadzu LCMS-8030 system. In brief, each plasma sample (50 µL) was deproteinized using a methanol-containing internal standard. The supernatant was filtered, dried, and dissolved; 20 µL of sample was then injected for liquid chromatography/mass spectrometry (LC/MS) analysis. The ultra-high-performance liquid chromatographic system in this study was equipped with a DGU-20 A_{3R} degasser, two LC-20 AD binary pumps, an SIL-20 AC_{HT} auto-sampler, a CBM-20A control module, an SPD-20A detector, and a CTO-20 AC column oven. It was then coupled with an LCMS-8030 triple quadrupole mass spectrometer (Shimadzu) with an electrospray ionization source. For liquid chromatography analysis, a Discovery HS F5-3 column (2.1 mm × 150 mm, 3 µm, Sigma-Aldrich, St. Louis, MO, USA) was used. The analysis was performed by means of a gradient system with two mobile phases: (A), a mixture of 0.1% formic acid (LC/MS grade; Wako Pure Industries, Osaka, Japan) and water (LC/MS grade; Wako Pure Industries); and (B), acetonitrile (LC/MS grade; Wako Pure Industries) containing 0.1% formic acid. The analysis began at 0% (B) for 2 min, with the concentration of (B) increasing to 25% at 5 min, 35% at 11 min, and 95% at 15 min, remaining at 95% until 20 min, and then decreasing to 0% at 20.1 min and remaining at this level until 25 min. The column temperature was 40°C, the flow rate was 0.25 mL/min, and the injection volume was 3 µL. For mass spectrometry analysis conditions, the nebulizer flow rate was 2 L/min, and the drying gas flow rate was 15 L/min. The DL and heat block temperatures were 250°C and 400°C, respectively. 2-Morpholinoethanesulfonic acid (Dojindo, Kumamoto, Japan) and L-methionine sulfone (Wako Pure Industries) were used as internal standards in the analysis. Program monitoring and data acquisition were controlled using LabSolutions LCMS software (Shimadzu).

4.2.9. Actual and Relative Weights/Lengths of Organs and Intestines

After the feeding trial, two pigs near the average BW for each pen underwent induction of deep anesthesia via thiopental sodium (Ravonal 0.5 g; Mitsubishi Tanabe Pharma, Osaka, Japan) injection into the jugular vein; they were then sacrificed. Necropsies were performed and the organs (liver, pancreas, spleen, kidney, stomach, small intestine, and large intestine) were carefully removed. The weights of all organs, including individual intestinal tract sections, were measured. The relative organ weights were calculated as the organ weight divided by BW (%). The lengths of individual intestinal tract sections were measured and the relative lengths of intestinal tract sections to piglet BW were also calculated (cm/kg).

4.2.10. Statistical Analysis

Data analysis was performed using JMP Pro software (version 15.2.0, SAS Institute Inc., Cary, NC, USA). The statistical significance between averages of two groups at each stage was determined using the independent sample t test, with the results presented as means ± SEM. *($p < 0.05$) and **($P < 0.01$) respectively indicate statistical significance and extreme significance.

4.3. Results

4.3.1. Growth Performance

As far as the whole experiment period is concerned, MGM-P had no effect ($p > 0.05$) on animal performance (**Table 4.3**). The weight change, ADG, ADFI, and FCR in each trial phase are listed in **Figure 4.1**. During 0 to 8 w after administration, there were no differences in BW, gain, and feed efficiency between the two groups. During 8 to 12 w, MGM-P group had a higher ADG compared with control group ($p < 0.01$). At the same time, lower FCR can be observed in this group, but the difference is not significant ($p > 0.05$). However, there has been no difference in performance between two groups since 12 w.

4.3.2. Blood Hematology Analysis

The blood hematology of grow-finish pigs in MGM-P supplementation from 0 to 16 w is presented in **Figure 4.2**. MGM-P had no effect on blood hematology measured in this study ($p > 0.05$).

4.3.3. Blood Biochemical Analysis

As shown in **Table 4.4** and **Figure 4.3**, diet supplemented with 0.5% MGM-P decreased ($p < 0.05$) the GOT level of plasma in pigs at the end of the feeding compared to control, however, other indicators related to liver metabolism, including GPT, GGT, blood ammonia and urea nitrogen, were not affected ($p > 0.05$).

4.3.4. Plasma free amino acids

As shown in **Table 4.5** and **Figure 4.4**, significant differences between dietary supplement and non-supplement of MGM-P were detected for several plasma free amino acids. In the blood collected initially of the experiment, pigs in the T group had higher ($p < 0.05$) lysine and tyrosine concentration relative to the control group. Then they dropped rapidly, and the difference disappeared. Compared with pigs in the control group at 12 w, pigs in the MGM-P supplementary group had higher ($p < 0.05$) concentrations of proline and tyrosine in their blood. During to entire experimental period, both tyrosine and phenylalanine concentrations were characterized by consistent changes.

4.3.5. Actual and Relative Weights/Lengths of Organs and Intestines

The actual and relative weights or lengths of organs and intestines were measured and reported in **Table 4.6** and **Table 4.7**. Compared with the control group, feed MGM-P addition markedly increased ($p < 0.05$) the weight of kidneys. However, it did not have an effect on relative kidney weight ($p > 0.05$). Except for the kidneys, neither the weight of other organs nor the length of the intestine were affected by MGM-P supplementation ($p > 0.05$).

4.4. Discussion

As a symbol of modern animal husbandry, antibiotics have been widely used since 1950s to improve the growth efficiency of animals, but the specific mechanisms of action are still not fully elucidated. Antibiotics are said to reduce the population of intestinal microbes and systemic inflammation, thereby reducing the non-growth energy consumption to promote production efficiency, which is considered to be responsible for promoting production efficiency (Callaway et al. 2021). Supplementing tannins in pig diets also has been demonstrated in the affection of gut microbes and reduction of microflora diversity (Tretola et al. 2019). Moreover, either hydrolysable tannins or condensed tannins, in previous studies, have been proved to have anti-inflammatory activity (M. L. R. Mota et al. 1985). Therefore, the use of tannins as a feed supplement is likely to play a role similar as antibiotics to promote pig production performance. The current study confirmed that the ADG of pigs was significantly improved by supplementing tannin. Best of our knowledge, at present, there are few studies that have reported about the positive influence of tannins in the growth of monogastric animals. This is mainly due to the anti-nutritional properties of tannins by forming complexes with proteins to inhibit the activity of proteolytic enzymes in digestive secretions (Etuk et al. 2012). In contrast, benefits of tannins for ruminants including growth, development and health has been proven by a lot of trials since over 20 years ago (Waghorn 2008).

Hematological parameters are important clinical indicators indicative of the pathophysiological status of animals. Czech et al. (2018) determined the range of WBC counts in growing (31-60 kg) and fattening (61-110 kg) pigs, ranging from 5.92 ~ 36.2 (10^3/μL) and 9.02 ~ 27.04 (10^3/μL). The WBC counts in the blood of pigs at all stages in this experiment were within this range. None of the hematological parameters, including WBC, were affected by MGM-P supplementation. The effect of QT on WBC counts may be influenced by several factors such as the rearing environment and the growth stage of the pigs.

Hydrolysable tannins can be hydrolyzed in the gastrointestinal tract to smaller molecules which are absorbed and may cause toxicity to liver in the metabolism, which may be another reason why tannins are restricted to be used in livestock feed (Smeriglio et al. 2017). Bone et al. (2013) therefore concluded that animals should avoid long-term intake of tannins, especially hydrolysable tannins. But the toxicity of QT has not yet been reported, and the negative affection of 0.5% MGM-P on liver has not been observed in our previous studies on piglets. Also, the present study found that long-term supplementation of 0.5% MGM-P may be harmless to the liver of grow-finish pigs, even the level of GOT in the blood is reduced, implying a protective effect of QT on liver function as the transaminase activities have always been considered as sensitive indicators of hepatic injury (L et al. 2015). It have been reported in the literatures that tannins can effectively repair liver damage by inhibiting lipid peroxidation and inflammation (Chu et al. 2016). Additionally, the presence of mycotoxins in the feed may also be one of the causes of liver lesions, but vegetable tannins have been given to the evidence of antimycotic and antimycotoxin properties (Anjorin et al. 2013).

Amino acids are regulators of the growth performance, gut microbiota and intestinal immunity, imply that the promotion of growth performance by feed additives might rely on their influence on the amino acid profiles (Chen et al. 2019b). As the first-limiting amino acid in pig diets, the plasma lysine concentration of pigs in the MGM-P supplemented group was significantly higher than that of the control group at the beginning of the experiment and decreased significantly after the addition of MGM-P, probably related to the effect of tannins, but remained consistent with the control group thereafter, thus not causing adverse effects on growth and development. Proline proteins tend to produces complexes with tannins since their small size and open loose structure, therefore the addition of tannins to the diet may increase the excretion of proline and decrease its digestibility (Galassi et al. 2019). However, at 12 w, we observed the pigs in the MGM-P group had higher proline levels, and remained higher after tannin supplementation, implying increased proline utilization in the control group.

Since proline is a major component of collagen can make up fibrotic extracellular matrix, therefore the lower proline concentration in the control group may also be associated with pathological fibrosis of the structure in vivo (Wu et al. 2009). It needs further investigation. The changing trend of tyrosine was similar to that of proline, being higher in the treated group than in the control group before the beginning of the experiment and still significantly higher than in the control group at 12 w, implying that the addition of 0.5% MGM-P had little effect on these amino acids' metabolism. Phenylalanine is an essential amino acid and participates in a lot of metabolic pathways (Zhou et al. 2016). Since it can be used for tyrosine synthesis, the changing trends of the two amino acids in the blood were consistent. The higher tyrosine concentration of pigs in MGM-P group indicates that this essential amino acid is sufficient or the reaction of transforming it into tyrosine is active. In addition, the conversion of phenylalanine to tyrosine is related to renal function, and this process will be blocked when renal failure is severe, indicating that the renal function is intact.

Few studies have examined the effects of tannins on the organ and relative organ weights of pigs. Khanyile et al. (2017) found that as the amount of Acacia tortilis leaf meal, which is rich in tannins, increased in the diet, the hepatosomatic index and scaled kidney weight of pigs showed clear upward trends. This is consistent with our finding that dietary 0.5% MGM-P supplementation increases the weight of kidney. No abnormal changes were found in the measurements of blood ammonia and urea nitrogen, so enlargement and hyperplasia due to lesions can be excluded. Since the kidney is the main site of nutrient reabsorption as well as endocrine, the increase in kidney weight may be associated with the increased reabsorption of nutrients from this organ, and implies an enhanced ability of the kidneys to excrete waste products (Yu et al. 2017).

4.5. Conclusions

In conclusion, this study confirmed that long-term dietary supplementation with 0.5% MGM-P has a positive effect on the growth performance in grow-finish pigs. The

addition of MGM-P to the diet resulted in an increase in ADG, although this significant difference occurred only at 8-12 w. As for the blood parameters though, there was no significant effect on hematology, the blood GOT levels was improved, which indicate that the liver maintained a good functional level. These results indicate that long-term supplementation with 0.5% MGM-P during the grow-finish stage is not harmful to the health of pigs and has a positive effect on production.

Table 4.1. Experimental animal allotment.

Group	n			BW			
	Male	Female	Total	Mean	SEM	*p*-value	CV (%)
C	8	4	12	37.65	1.20	0.96	0.19
T	8	4	12	37.55	1.28		

Abbreviations: n, number of pigs; BW, body weight; SEM, standard error of the mean; CV, coefficient of variation.

Table 4.2. Ingredients and chemical composition of basal diets (as-fed basis).[1]

Items	Starter	Grower	Finisher
Ingredients [2] (%)			
Cereals [3]	52	56	70
Animal feed [4]	21	1	0
Oil seed meal [5]	17	21	13
Bran [6]	0	7	14
Others [7]	10	15	3
Chemical composition [8]			
DM (%)	89.0	87.5	87.9
CP (%)	21.8	16.4	13.6
EE (%)	7.4	5.60	3.3
CF (%)	2.1	3.00	3.3
Ash (%)	5.5	4.20	4.2
NFE (%)	52.2	58.3	63.5
GE [9] (Mcal/Kg)	4.27	4.10	3.95

[1] Experimental group diet was based on this diet with the addition of MGM-P at 0.5%.
[2] Ingredients are provided by Marubeni Nisshin Feed Co., Ltd., Tokyo.
[3] Composition: corn, wheat, wheat flour, bread flour, corn starch, potato starch, dextrin, climacteric corn, milo, rice, low quality wheat flour, cassava, barley.
[4] Composition: dried whey, mixed meal of pork and chicken ingredients, fish meal, plasma protein, concentrated whey protein.
[5] Composition: soybean oil residue, flaxseed oil residue, rape seed oil residue, corn jam meal.
[6] Composition: corn gluten feed, rice bran, wheat bran, Distiller's Dried Grains with Solubles.
[7] Composition: confectionery waste, specific animal fats, dextrose, lignocellulosic, calcium phosphate, salt, beet bulb, calcium carbonate, citric acid, dried yeast cell wall, whole egg powder, lactic acid bacteria, baker's yeast, silicic acid, oligosaccharides, ginkgo biloba extract, garlic extract, Chenopodiaceous extract, licorice extract, pine needle extract, hawthorn extract, butyric acid bacteria, saccharification bacteria, brewer's yeast, aluminum silicate, galactooligosaccharide, molasses, yucca extract.
[8] DM, dry matter; CP, crude protein; EE, ether extract (crude fat); CF, crude fiber; Ash, crude ash; NFE, nitrogen free extract; GE, gross energy.
[9] The GE was calculated with the following equation:

$$GE\ (Mcal/kg)=(5.67\times CP(\%)+9.68\times EE(\%)+4.25\times NFE(\%)+4.90\times CF(\%)/100$$

Table 4.3. Effects of long-term dietary MGM-P supplementation on growth performance in grow-finish pigs (throughout the experimental period).

Item	Initial BW (kg)	Final BW (kg)	ADG (kg)	ADFI (kg)	FCR (kg/kg)
C	37.65±1.20	144.60±3.54	0.97±0.03	3.21±0.10	3.31±0.05
T	37.55±1.28	148.35±2.43	1.01±0.02	3.30±0.10	3.26±0.04

All data are expressed as mean ± SEM (n = 12). There were no statistically significant differences between two groups according to the Student's t-test.

Table 4.4. Effects of long-term dietary MGM-P supplementation on blood biochemical parameters in grow-finish pigs.

Item	GPT (U/L)	GOT (U/L)	GGT (U/L)	NH_3 (μg/dL)	BUN (mg/dL)	AMYL (U/L)	GLU (mg/dL)	TP (g/dL)	ALB (g/dL)	TG (mg/dL)
0w										
C	41.33±1.28	53.42±7.99	52.08±6.23	119.00±4.86	17.78±1.62	669.17±64.79	126.5±3.84	5.87±0.06	4.50±0.06	27.00±3.77
T	43.17±2.36	53.67±5.88	58.83±8.54	121.67±11.10	19.76±1.78	672.08±67.21	137.83±4.45	5.98±0.06	4.57±0.03	31.17±3.79
4w										
C	51.25±4.21	34.42±2.55	33.17±1.76	94.17±3.80	11.58±0.58	549.00±65.44	114±24.26	5.68±0.10	4.10±0.09	27.08±2.45
T	45.25±3.20	38.67±4.80	31.67±1.61	99.08±4.68	11.44±0.91	570.33±47.58	94.42±2.82	5.73±0.11	4.02±0.07	28.67±2.87
8w										
C	45.83±1.82	31.33±1.37	42.50±1.48	106.00±6.24	12.96±0.95	556.08±68.48	100.33±3.64	6.52±0.10	4.43±0.07	33.42±5.20
T	43.91±2.27	37.18±4.37	39.82±1.95	119.27±5.41	12.79±1.17	455.64±72.32	104.45±3.88	6.45±0.17	4.45±0.11	47.00±16.13
12w										
C	49.58±1.84	35.67±3.37	42.67±0.99	135.58±9.27	11.67±0.89	288.33±26.97	84.08±3.08	6.75±0.09	4.58±0.11	23.92±2.15
T	47.45±2.09	34.27±2.33	43.00±1.78	120.00±5.71	13.91±1.13	304.82±33.71	86.82±2.99	6.70±0.06	4.57±0.05	29.91±2.73
16w										
C	51.75±2.03	42.42±3.53	45.92±1.87	228.00±10.7	16.97±0.67	387.25±68.86	70.17±5.62	12.24±5.25	4.86±0.11	27.25±2.47
T	49.45±2.26	31.45±1.38*	44.00±1.91	208.82±6.79	16.43±0.85	276.64±21.54	74.82±1.89	7.08±0.16	4.91±0.08	28.27±3.36

Abbreviations: GPT, glutamate-pyruvate transaminase; GOT, glutamate-oxaloacetate transaminase; GGT, gamma-glutamyl transpeptidase; NH_3, ammonia; BUN, blood urea nitrogen; AMYL, amylase; GLU, glucose; TP, total protein; ALB, albumin; TG, triglyceride. All data are expressed as mean ± SEM (n = 12). For a given row, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 4.5. Effects of long-term dietary MGM-P supplementation on plasma free amino acids in grow-finish pigs (uM).

Item	Asparagine	Aspartic acid	Serine	Alanine	Glycine	Glutamine	Threonine	Cysteine	Glutamic acid	Proline	Lysine	Histidine	Arginine	Valine	Methionine	Tyrosine	Isoleucine	Leucine	Phenylalanine	Tryptophan
0w																				
C	65.93±2.65	36.99±0.92	157.36±9.64	310.26±20.48	991.08±39.74	433.80±13.58	213.11±12.78	1.87±0.17	98.50±7.71	234.72±14.72	202.67±11.46	62.33±3.83	113.18±10.21	219.28±14.4	73.12±7.06	71.52±3.24	115.98±6.13	155.28±7.97	71.58±3.24	59.69±2.62
T	77.50±3.42*	35.87±1.53	172.78±9.08	351.55±23.87	988.98±28.13	436.11±13.33	270.65±14.69*	2.22±0.14	101.24±8.57	274.88±15.30	256.91±15.49*	70.12±3.43	134.14±12.74	248.23±16.05	94.12±12.25	95.72±8.87*	125.77±6.84	180.18±11.05	78.23±5.63	68.24±3.47
4w																				
C	69.00±2.02	38.28±1.50	168.15±5.74	276.84±11.76	874.96±29.64	443.12±9.59	141.63±6.75	1.36±0.11	81.52±3.58	235.37±4.91	183.70±9.95	66.89±1.64	144.11±8.65	206.06±8.13	45.65±1.31	82.85±2.03	99.25±3.7	184.13±4.76	71.76±2.42	58.29±2.18
T	70.52±2.77	38.95±1.71	160.45±8.93	267.83±9.90	901.53±35.51	472.29±15.31	153.32±7.25	1.70±0.19	87.37±4.07	245.46±8.47	184.44±8.68	62.88±3.58	122.05±6.83	204±9.7	49.36±2.62	82.65±3.61	102.6±3.95	184.23±6.46	68.34±3.35	62.36±3.4
8w																				
C	49.57±2.34	35.05±1.06	155.79±10.12	256.54±19.17	933.82±34.14	384.82±12.60	138.23±10.30	1.40±0.23	72.89±5.36	202.89±10.52	215.93±16.52	64.47±2.58	135.34±11.95	196.82±9.21	47.64±2.46	76.65±3.76	70.3±4.05	166.89±7.42	67.01±2.73	52.38±3.12
T	50.05±1.56	33.31±0.99	141.78±8.40	249.36±11.32	860.58±27.46	408.59±13.38	139.76±10.91	1.38±0.19	73.31±4.02	195.89±10.32	211.41±8.22	60.23±2.7	121.64±10.35	199.57±13.67	44.77±3.65	79.85±4.6	73.69±5.69	167.18±8.44	63.63±4.39	54.47±3.87
12w																				
C	37.96±0.96	27.48±0.41	109.65±4.32	225.25±12.46	812.26±43.64	333.77±10.90	111.22±5.06	1.19±0.08	60.28±3.10	134.23±4.54	167.20±6.19	48.33±2.1	97.19±5.43	180.03±6.99	35.52±1.24	58.17±1.8	73.06±3.82	146.7±4.69	58.23±2.23	49.81±2.47
T	38.51±1.97	28.76±0.82	124.44±7.19	224.46±14.88	772.51±29.05	342.89±9.94	127.60±8.05	1.01±0.15	66.87±5.61	166.61±9.24*	168.36±9.87	53.91±2.48	103.03±7.61	186±8.24	38.63±2.57	64.65±2.45*	69.59±3.81	152.47±5.45	58.42±2.85	51.77±2.11
16w																				
C	49.03±2.36	34.16±1.07	176.37±7.53	299.56±12.95	853.15±19.83	371.79±12.47	188.48±6.45	1.42±0.15	77.43±5.01	225.23±7.85	224.96±13.46	67.58±2.29	148.39±10.24	253.07±9.68	57.88±1.84	92.14±2.94	101.6±5.69	212.25±7.3	86.77±3.22	67.88±2.92
T	50.36±2.41	32.66±1.01	166.26±8.82	277.47±10.75	811.11±22.21	367.77±16.61	187.32±9.17	1.45±0.13	75.91±4.87	225.01±9.91	220.66±10.54	68.42±4.20	132.10±8.74	239.33±12.38	56.50±3.76	91.33±4.09	97.36±6.54	205.51±9.57	81.65±3.87	71.13±2.64

All data are expressed as mean ± SEM (n = 12). For a given row, mean values with asterisk indicate significant differences according to the Student’s t-test (*$p < 0.05$, ** $p < 0.01$).

Table 4.6. Effects of long-term dietary MGM-P supplementation on the actual weights/lengths of organs and intestines of grow-finish pigs.

Item	Liver (kg)	Pancreas (g)	Spleen (g)	Kidney (g)	Stomach (g)	Small intestine weight (kg)	Small intestine length (m)	Large intestine weight (kg)	Large intestine length (m)
C	2.65±0.05	151.57±9.85	175.08±12.76	404.55±21.95	543.92±27.00	2.02±0.08	18.90±0.97	1.80±0.07	5.41±0.15
T	2.55±0.05	142.16±11.70	208.04±11.78	461.84±8.58*	560.62±18.96	2.02±0.08	17.54±0.46	1.72±0.05	5.07±0.14

All data are expressed as mean ± SEM (n = 6). For a given row, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 4.7. Effects of long-term dietary MGM-P supplementation on the relative weights/lengths of organs and intestines of grow-finish pigs.

Item	Liver (%)	Pancreas (%)	Spleen (%)	Kidney (%)	Stomach (%)	Small intestine weight (%)	Small intestine length (cm/kg)	Large intestine weight (%)	Large intestine length (cm/kg)
C	1.78±0.02	0.10±0.01	0.12±0.01	0.27±0.01	0.37±0.02	1.35±0.06	12.63±0.55	1.18±0.04	3.62±0.12
T	1.65±0.05	0.09±0.01	0.13±0.01	0.30±0.01	0.36±0.02	1.31±0.05	11.37±0.36	1.11±0.03	3.29±0.13

All data are expressed as mean ± SEM (n = 6). There were no statistically significant differences between two groups according to the Student's t-test.

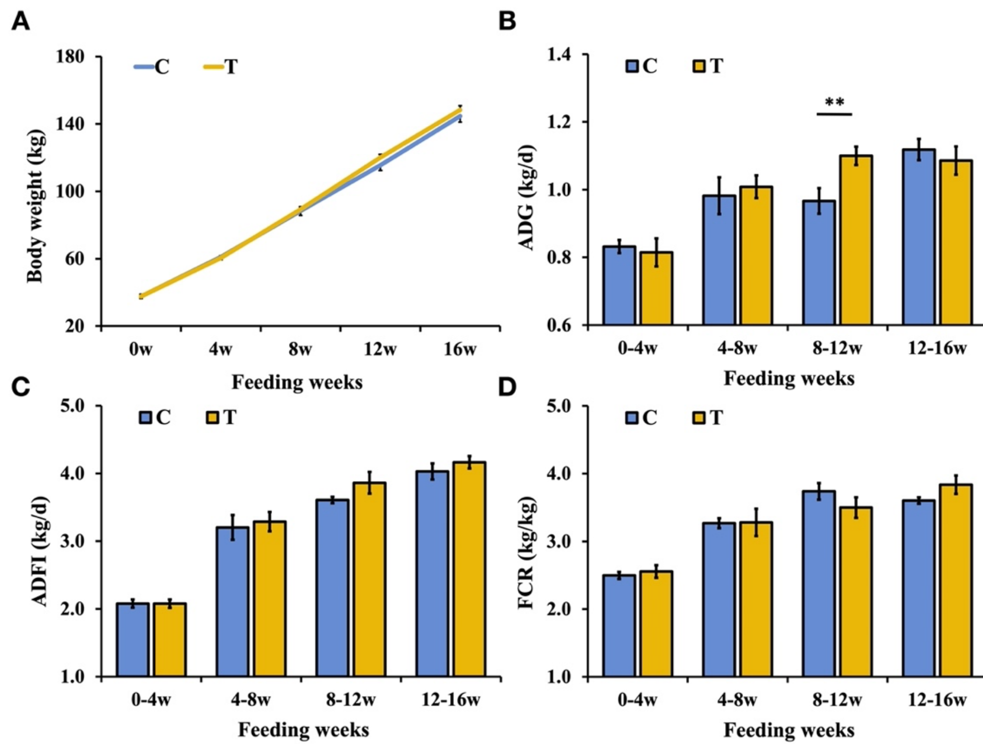


Figure 4.1. Effects of long-term dietary MGM-P supplementation on growth performance in grow-finish pigs. Data in **A** and **B** are expressed as mean ± SEM (n = 12); Data in **C** and **D** are expressed as mean ± SEM (n = 3). Asterisk above bars indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

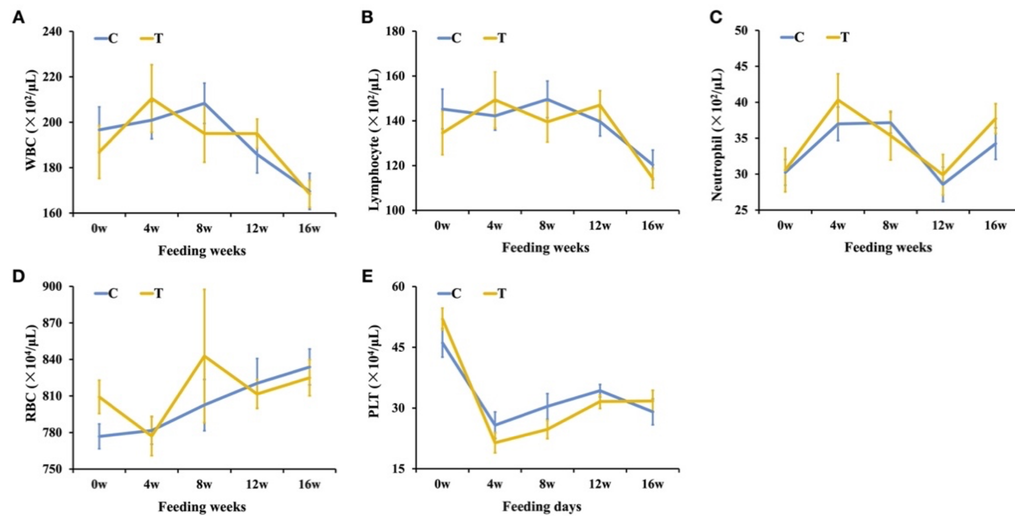


Figure 4.2. Effects of long-term dietary MGM-P supplementation on blood hematology parameters in grow-finish pigs. All data are expressed as mean ± SEM (n = 12). There were no statistically significant differences between two groups according to the Student's t-test. (**A**) the changes of the white blood cell count; (**B**) the changes of the lymphocyte count; (**C**) the changes of the neutrophil count; (**D**) the changes of the red blood cell count; (**E**), the changes of the platelet count.

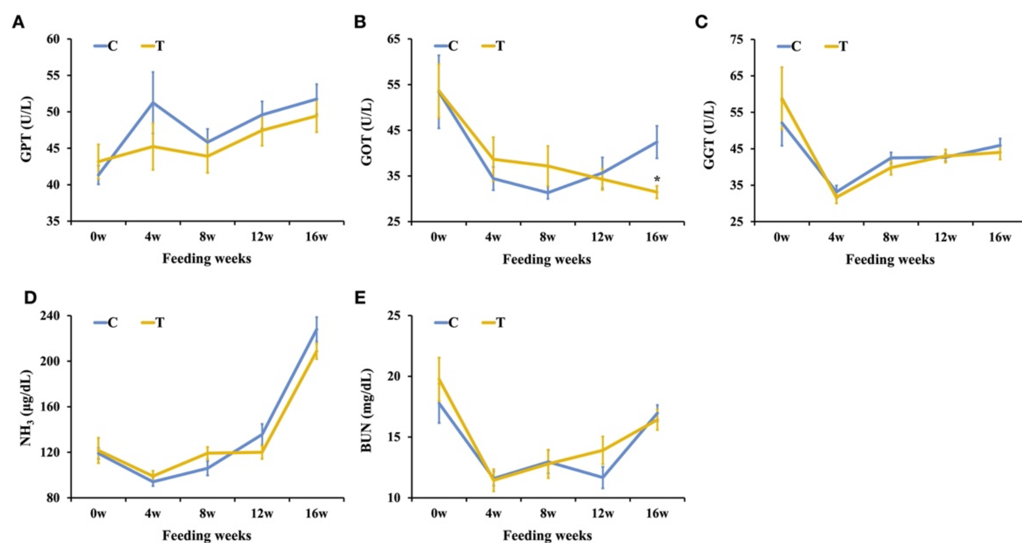


Figure 4.3. Effects of long-term dietary MGM-P supplementation on blood biochemical parameters of grow-finish pigs. All data are expressed as mean ± SEM (n = 12). Asterisk near the node indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$). (**A**) the changes of the glutamic pyruvic transaminase level; (**B**) the changes of the glutamic oxaloacetic transaminase level; (**C**) the changes of the gamma glutamyl transferase level; (**D**) the changes of the blood ammonia concentration; (**E**) the changes of the urea nitrogen concentration.

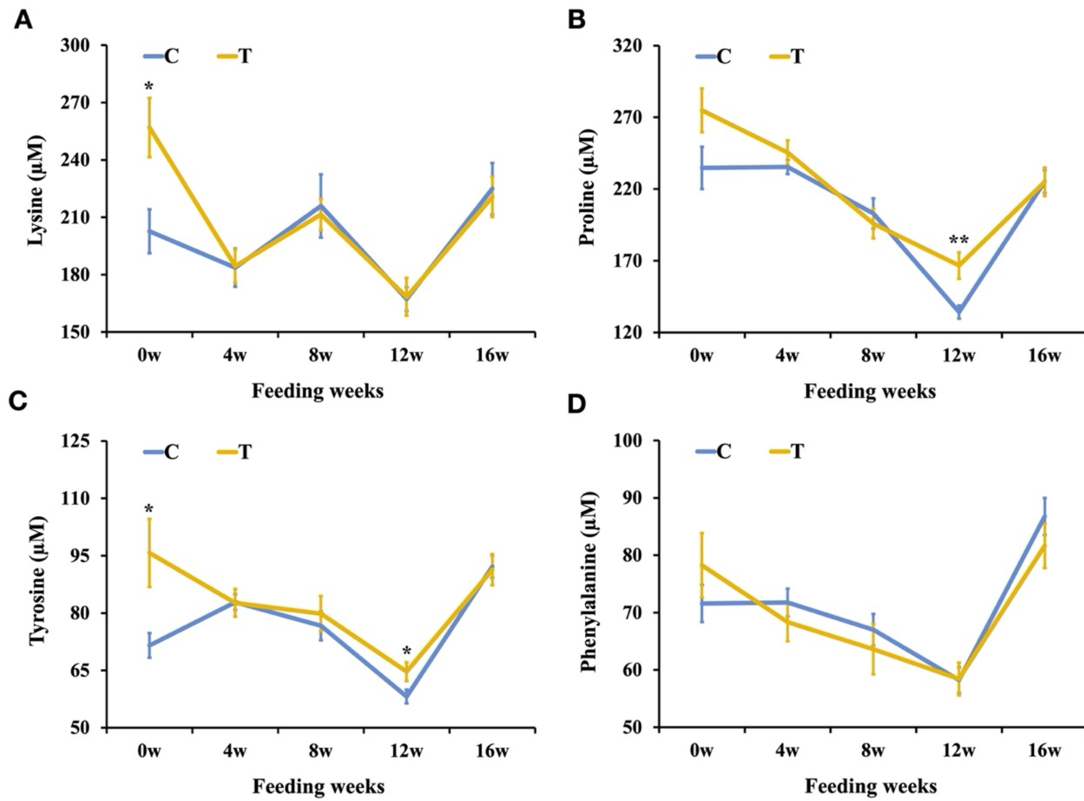


Figure 4.4. Effects of long-term dietary MGM-P supplementation on the blood free amino acids concentration of grow-finish pigs. All data are expressed as mean ± SEM (n = 12). Asterisk near the node indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$). (**A**) the changes of the lysine concentration; (**B**) the changes of the proline concentration; (**C**) the changes of the tyrosine concentration; (**D**) the changes of the phenylalanine concentration.

Chapter 5 Effects of supplementation with tannin-rich chestnut by-products on growth performance, blood parameter, and meat quality in finishing pigs

5.1. Introduction

In order to achieve carbon neutrality by 2050, the Green Deal issued by Europe has made a good guide for our work, creating closed loops of resources, to minimize waste and relieve environmental pressure, and converting food and its by-products into animal feed is an attractive option (Rao et al. 2021).

Chestnut is a very popular food and grown in the temperate regions of the Northern Hemisphere. It is reported that chestnut is distributed in 340,000 hectares of land around the world, with an annual output of about 1.1 million tons (Fernandes et al. 2011). Traditionally, chestnut in some areas of Europe have been opportunely used for swine feeding because their production exceeded human demand. Local people usually raise autochthonous pigs (*Cinta Senese*) in chestnut forests, and the pork products obtained are appreciated by consumers (Pugliese et al. 2009).

Iberian pig is a typical example, in the south-west of the Iberian Peninsula, the local Iberian pig also feed acorns and chestnuts through extensive or semi-extensive feeding mode during the late fattening phase (Rey et al. 1997; Bělková et al. 2017). The meat product thus obtained has a higher sensory score and consumers appeared to be prepared to pay more for such meat and its products (Dransfield et al. 2005). Unfortunately, with the decrease in available natural resources and the increase in demand for meat products. In today's intensive production model, a large number of commercially mixed diets are chosen as pig feed to meet market demand, which has directly contributed to the deterioration of pork quality (Tejeda et al. 2020).

Study on supplementary feeding of chestnuts has been of great interest in recent years in order to obtain high-quality pork similar to those from Iberia and Parma under intensive production mode. Pugliese (2013) in a study in pigs (*Cinta Senese*), pointed out feeding with chestnuts (90%) and bran during the last month of the fattening period

can help increase content of intermuscular fat and UFA. Carmen et al. (2016) supplemented the commercial feed with 25% dried chestnuts and found that the slaughter rate of Celta pig and the monounsaturated fatty acids content in the muscles increased. Moreover, as reported by Díaz et al (2009), adding 15% dried chestnuts in diets for pigs during the fattening period not only increased the content of polyunsaturated fatty acid C18:2 (ω6) and C18:3 (ω3) in the pork, but also reduced lipid oxidation and increased the water-holding capacity of meat. Although it is not conclusive so far exactly which components of chestnuts play a crucial role. However, it has been suggested that the tannins contained in chestnuts help stabilize fatty acids, thus maintaining the degree of unsaturation of fat in meat (Echegaray et al. 2020a).

The main component of chestnut is starch (up to 70%), while the contents of protein and fat are relatively low, which are 2-4% and 2-5% respectively (Barreira et al. 2009). Although chestnut fruits is rich in tannins, interestingly, Vekiari (2008) determined the concentrations of tannins in different chestnut fruit tissues, and found that tannins were mainly concentrated in the shells and mainly in the form of ellagitannins. According to their report, the contents of ellagitannins in the pericarp and integument of chestnut were found to vary between 0.04-0.19 $mg \cdot g^{-1}$ and 0.03-0.091 $mg \cdot g^{-1}$, respectively. Pinto et al. (2021) also determined the tannin content in chestnut shells, and the result was 10.6 mg/g of dry matter (in which hydrolysable tannins was 10.3 mg/g). The tannin content in the chestnut shells is even higher than in other parts, such as flowers and leaves (Barreira et al. 2008).

It is estimated that the shells account for about 10-15% of the weight of chestnuts (Gullon Patricia et al. 2018). And in the process of making chestnuts into food, a large number of chestnut shells will be discarded, thus result in a serious waste of resources. If these chestnut shells could be used as pig feed, it would not only contribute to sustainable production and environmental protection, but also have the potential to produce high quality pork, while reducing feed costs in the pig breeding process. However, as far as we know, no relevant studies have been reported so far.

Also, the current studies on the effects of feeding chestnuts on pork quality focus on the fatty acid while ignoring the changes in amino acids (Díaz et al. 2009; Domínguez et al. 2015; Jesús et al. 2017; Joo et al. 2018; Echegaray et al. 2020b). However, meat serves as a good source of dietary amino acids for humans, so the profile of amino acids in meat is also an important indicator to determine the quality of meat. Although the use of chestnuts has been shown to improve meat quality, to our knowledge, few studies have demonstrated the effect of chestnuts or their by-products on the profile of amino acids in meat (Bahelka et al. 2021).

Therefore, in the present study, chestnut shells were added to the finishing pigs' diet. The feasibility of chestnut shells supplementation was preliminarily explored by evaluating the chemical components (including tannins) of diet, and the growth performance and the blood parameters of pigs. Then, the application potential of chestnut shells in pork production was evaluated by investigating its effect on the sensory quality, chemical composition, fatty acid, and amino acid profiles of meat.

5.2. Materials and Methods

5.2.1. Animal ethics

All animal care and handling procedures conformed to the Guidelines for Animal Experiments of the University of Tokyo and were approved (P20-098) by the Animal Care and Use Committee of Life Science, Faculty of Agriculture, University of Tokyo.

5.2.2. Materials

The experiment was conducted at the Animal Resource Science Center of the University of Tokyo (Japan). The chestnut shells were provided by local corporations (Aiki Maron Co., Ltd, Kasama, Japan; Odaki Shoten Co., Ltd., Kasama, Japan), comprise outer shells and inner shells with a little pulp. The collected chestnut shells were shattered using an electric minced machine (PMM-12F, Minato, Fukuoka, Japan), and passed through a 10-mm screen. Every 10 kg of chestnut shell meal was packed

into a plastic bag, compacted, and sealed. Then they were stored in a refrigerator at -20°C until use.

5.2.3. Animals and experimental design

A total of 6 Duroc finishing pigs (165 d of age, 103.37±2.03 kg, from the same mother), including castrated males and females, bred by the Animal Resource Science Center of the University of Tokyo were selected as experimental animals and divided into control group and treatment group (3 pigs each, kept in a pen). The commercial diet purchased from Marubeni Nisshin Feed Co., Ltd (Tokyo, Japan) was used as the basal diet (**Table 5.1**), and it meets the National Research Council standards (NRC 2012). The control group fed the basal diet (C), and the treated group received the basal diet containing 60% chestnut shell meal (T). The pigs were housed in a same semi-enclosed pig house without temperature control, and the houses were kept clean daily. All animals were given feed and water using a 4-hole stainless-steel feeder, and a stainless-steel nipple waterer. The raising period lasted for 110 days. Taking account of the energy deficiencies of the alternative diet of chestnut shell meal, all pigs were allowed ad libitum access to food and water, and the hopper and drinker were constantly inspected to ensure adequate supplies throughout the trial period.

5.2.4. Sample collection

Feed: Chestnut shell meal were mixed with basal feed well, sampled while feeding, and then quickly placed in a −80°C refrigerator for storage.

Blood: The sampling procedures and methods are the same as we mentioned before (Piao et al. 2020). Blood samples were collected at the same time on days 0 (before treatment), 30, 60, 90, and the last day of treatment.

Meat: At the end of the raising period, all pigs were slaughtered at 275 days of age in a local commercial slaughterhouse (Ibaraki Chuo Meat Center Co., Ltd., Ibaraki, Japan). Chilled for 24 h, longissimus dorsi was excised between the 11 th and 13 th ribs

from the right half of the carcase. All samples were placed in zipper storage bags and stored at −80 °C until analyses.

5.2.5. Feed analysis

The chemical compositions of diet were measured by the Japan Food Research Laboratories (Tokyo, Japan). Moisture content (atmospheric drying method), crude protein (combustion method, and was calculated by Nitrogen × 6.25), crude fat (diethyl ether extraction method), crude fiber (filtration method) and ash (direct ashing method) of the basal diets and 60% chestnut diets were determined. Then subtracted the sum of moisture, crude protein, fat, fiber, and ash from 100, and the difference is nitrogen free extract. Tannin concentration was measured by Folin-Denis method and expressed as tannic acid equivalents.

5.2.6. Fattening performance

The pigs were weighed at the same time of blood collection, and feed consumption on a pen basis were recorded to calculate ADG, ADFI and FCR.

5.2.7. Blood hematology

Hematological analysis was performed immediately after the collection of whole blood. White blood cells (WBC), lymphocytes, neutrophils, red blood cells (RBC) and platelets (PLT) concentrations in the whole blood were determined using a pocH-100iV Diff hematology analyzer (Sysmex Corp., Kobe, Japan).

5.2.8. Meat Color

After taking the meat sample, measure the color on the remaining freshly cut surface at the 13 th rib immediately. The lightness (L*), redness (a*), and yellowness (b*) values were measured at three different points on each cut surface using a calibrated PCE-TCR 200 colorimeter (PCE Instruments, Hampshire, UK). The mean of the three readings was taken as the sample color value.

To better reflect the color difference, after calculating the average of all samples in each group, the total color difference (ΔE*) between the two groups of meat samples was calculated using the following formula:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)]^{1/2}.$$

where ΔL*, Δa*, and Δb* are the differences of the mean value of L*, a*, and b* between the control and treated samples.

5.2.9. Meat quality evaluation

The meat quality analysis involved meat composition, eating quality (sensory evaluation), fatty acid composition and amino acid profile of longissimus dorsi. Analysis was performed by the Bureau Veritas Japan Co., Ltd. (Shimane, Japan). Briefly, macro-Kjeldahl method was used to measure protein content with the use of the conversion coefficient 6.25. The lipid was measured by ether extraction method. The moisture content was measured by drying at 135 °C for 2 hours using a desiccant, and the ash content was measured by the ashing method. The carbohydrate content is obtained by subtracting the sum of the above four components from 100. The energy value of meat was calculated by multiplying the protein, lipid and carbohydrate content by the energy conversion factors (proteins: 4.22 kcal/g, lipids: 9.41 kcal/g, and carbohydrates: 4.11 kcal/g). The sensory evaluation included water holding capacity, elongation rate, the weight loss of the muscle during heating (cooking loss), as well as the compression loss of the heated meat sample. The fatty acid composition of the longissimus dorsi was analyzed by gas-chromatography and was expressed as percentage content of total fatty acids, included SFA (saturated fatty acids), UFA (unsaturated fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids), ω6 FA (n-6 fatty acids), and ω3 FA (n-3 fatty acids). Finally, high performance liquid chromatography method was adopted to measure the free amino acid profile and expressed as mg/100g of muscle.

5.2.10. Statistical Analysis

Data analysis was performed using JMP Pro software (version 15.2.0, SAS Institute Inc., Cary, NC, USA). The statistical significance between averages of two groups at each stage was determined using the independent sample t test, with the results presented as means ± SEM. *($p < 0.05$) and **($P < 0.01$) respectively indicate statistical significance and extreme significance.

5.3. Results

5.3.1. Chemical compositions of diets

The chemical compositions of diets are presented in **Table 5.2**. The chestnut shells alternative diets contained higher percentages of moisture and crude fiber when compared to the control diets, but it is lower in terms of crude protein, ether extract and nitrogen free extract, and directly causes lower energy. In respect to tannin concentration, tannins were detected in all diets. The addition of chestnut shells increased tannin content in the feed, on a dry matter basis, the tannin content of the control diet was 0.52% and that of the treated diet was 0.96%, so it is equivalent to supplementing 0.44% of the tannins in the case of eating the same amount of dry matter. And it can be calculated that the tannin content in the chestnut shells used in this experiment was 1.44% (dry matter basis).

5.3.2. Fattening performance

The results of the fattening performance of pigs are presented in **Table 5.3**. Despite not being significant, we found that dietary supplementation of chestnut shells had obviously low ADG compared with control group, thus reduced the final body weight. With the increase in feed intake, FCR in treatment group is distinct higher than control group.

5.3.3. Blood hematology

Blood parameters of pigs fed chestnut shells supplement diets are shown in **Table 5.4**. No effect of chestnut shells supplementation on WBC, lymphocyte and neutrophils counts was observed throughout the experiment ($p > 0.05$). Moreover, no diet related changes of RBC and platelets counts were also found during feeding trial.

5.3.4. Meat Color

Table 5.5 shows the effects of chestnut shells supplementation on the meat color of finishing pigs. L* value and b* value in the chestnut shells supplementation group significantly decreased ($p < 0.01$) as compared to the control group. No significant differences ($p > 0.05$) in a* values were observed.

5.3.5. Sensory evaluation of muscle

Table 5.6 summarizes results on sensory evaluation of longissimus dorsi samples. No effect of dietary supplementation with chestnut shells was observed on the water holding capacity and cooking loss ($p > 0.05$). However, chestnut shells supplementation had a very significant effect of increasing elongation rate and compression loss ($p < 0.01$).

5.3.6. Chemical composition of muscle

As show in **Table 5.7**. The supplement of chestnuts shell in the diet has no significant influence on the protein, lipid, carbohydrate, moisture, ash content and energy in muscle samples ($p > 0.05$), however, even if it is not significant, higher protein content and lower lipid content were observed in the T group.

5.3.7. Fatty acids composition of muscle

The data on fatty acids content and composition are summarized in **Table 5.8** and **Figure 5.1**. There were no significant differences ($p > 0.05$) in the fatty acids content or composition between the chestnut shells supplementation group and control group.

5.3.8. Amino acids profile of muscle

The effects of chestnut shells supplement diets on the amino acids content and profile of muscle are detailed in **Table 5.9** and **Figure 5.2**. Compared with pigs fed with basal diet, the content of 14 amino acids in the pigs' muscle of the treatment group were higher ($p < 0.01$ or $p < 0.05$), including aspartic acid, glutamic acid, glycine, alanine, threonine, serine, methionine, lysine, isoleucine, leucine, phenylalanine, valine, histidine, and arginine. In terms of total free amino acids and total peptide constituent amino acids content, the treated group also showed a significant improvement ($p < 0.01$).

5.4. Discussion

In the present trial, the addition of 60% chestnut shells in the commercial diet obviously increases the contents of water and crude fiber, and decreased the contents of crude protein, crude fat, and nitrogen free extract, thereby reducing the energy of the mixed diet. Literature reported that chestnut shells, are mainly composed of lignin, accounting for 41.6%, followed by cellulose, which account for 28.4%, and the moisture, crude protein, crude fat and ash contents were 21.29–38.61%, 2.77–3.13%, 0.15–0.52%, 1.08–1.60%, respectively (Hu et al. 2021). Furthermore, cooking (steaming or boiling) chestnuts in industrial processing is able to change the chemical composition of the chestnut shells, especially the moisture content, thus the observed obvious changes of moisture in mixed diets were unequivocally related to this. Considering the tannin content in feed, it is remarkable that tannins are widely distributed in animal feed, such as corn, wheat and barley. Thus, a certain concentration of tannins can also be detected in the basal diets (Caprarulo et al. 2021). With the addition of tannin-rich chestnut shells could increase the concentrations of tannins in the diets. Lee et al. (2016) extracted and determined the tannin content in the inner and outer shells of chestnuts and found that the tannin content in the outer shell was 0.94% of dry matter, while the tannin content in the inner shell was 2.50%. The chestnut shells used in our experiments were a mixture of inner and outer shells with a tannin content of 1.44% in between this range, which roughly coincided.

The 60% chestnut shells supplementation, while giving the pigs sufficient tannin active substance, also resulted in insufficient gross energy. To replenish required energy, the animal's feed intake increased obviously. However, due to the decline in feed efficiency, even high feed intake has a negative impact on fattening. Lorenzo et al. (2013) added a similar dose of chestnuts (62.50%) to the diet of 12-month-old Celta pigs, and their body weight increased by 4.40% compared with the control group after fattening for 4 months. Compared with chestnut shells, the whole chestnut which contains more than 50% starch, plays a key role in the fattening process of pigs (Lee et al. 2016). However, lignin and cellulose, which are mainly present in chestnut shells, are difficult to be digested and used by pigs, so the value of available energy is very low, and the growth of pigs depends more on energy and nutrients from 40% of the concentrate feed. The average daily intake of pigs in the treatment group was 4.74 kg, including only 1.90 kg of concentrate feed, which was one-third less than the amount of concentrate feed taken by the control group, so the part of easily digestible protein and energy from concentrate feed was also reduced by one-third, which directly led to the decrease of fattening performance of pigs in the treatment group, manifested as the decrease of ADG and the increase of FCR. It can be obtained by calculation that compared with the control group, the daily supplemented tannin content of the pigs in the treatment group accounted for 0.37% of ADFI, which is within the safe range of addition compared to the MGM-P addition experiment, so the negative effect of tannins on fattening performance was not considered.

Measuring hematological parameters is a practical tool for evaluating the pathological conditions of animals and monitoring the health of animal groups (Ježek 2018). Throughout the experimental phase, the counts of WBC including lymphocytes and neutrophils, as well as RBC and platelets were not affected by chestnut shells supplementation. Joo et al. (2018) also reported that adding 3%, 5% and 10% chestnut meal to pig diet, respectively, did not affect the WBC and RBC counts in the blood.

When consumers choose pork products, they are affected by the combination of color, flavor, tenderness and juiciness (Aaslyng et al. 2003). The meat color largely depends on the amount of myoglobin and state of oxidation of the muscle pigment (O'Connor-Shaw and Reyes 1999). The oxidation will result in a conversion of myoglobin to metmyoglobin which then appears a undesirable dull brown color (Listrat et al. 2016). Nevertheless, when this process is suppressed, it usually shows decreases in lightness (L*) and yellowness (b*) when measuring color (LeMaster et al. 2019). Previous studies suggesting that tannin extracts and tannin-rich plants have strong antioxidant potential, and the diet addition of them were shown to delay the oxidation and improves meat color stability (Luciano et al. 2009; Sun et al. 2018). In this experiment, the chestnut shells supplementation also reduced the L* and b* values, which may be related to the antioxidant capacity of the tannins contained in the chestnut shells. ΔE* is a value that can distinguish the overall change of L* and a* and b* values. When it is higher than 1, the color difference between the two samples is detectable with naked eye (Semjon et al. 2020). Thus, the supplementation of chestnut shells caused the visible change in pork color. Elongation rate is a useful measure of the softness of meat during chewing, higher value indicating softer, so addition of chestnut shells to the diet made meat softer. In addition, the chestnut shells supplementation effectively increased the compression loss of meat, allowing more juice to flow out when the meat is chewed and improves the taste and flavor.

The nutritional value of pork is determined by the nutritional composition. High protein and low fat are two key words for consumers to evaluate the health of meat products (Barone et al. 2021). In this study, the chestnut shells supplemented group of pork had the trend of high protein and low fat as well as energy proves that healthier meat was obtained. Pugliese et al. (2007) fed pigs with chestnuts under a traditional extensive system during fattening period and found that both MUFA and PUFA contents in muscle were elevated. The increased UFA content in meat products contributed to prevent the occurrence of coronary heart disease and cancer (Wood et al.

2004). Therefore attempts have been made to investigate the causes of changes in fatty acid composition induced by chestnut supplementation. Bahelka et al. (2021) supplemented the diet of finishing pigs with 0.4% tannin-rich chestnut extract and observed a significant increase in ω6 PUFA and total PUFA content in the longest dorsal muscle, and the authors suggested that the accumulation of PUFA may be due to the influence of tannins on the metabolism of linoleic acid in pigs. Contrarily to our expectations, the supplementation of tannin-rich chestnut shells in this experiment did not affect the fatty acid content of pork. Since tannins in chestnuts are almost exclusively present in the shells, our results demonstrate that fatty acid changes induced by feeding chestnuts may be more influenced by the chestnut kernels. Although phenolic substances with antioxidant properties can improve the antioxidant capacity of the organism and increase the PUFA content in tissues (Youdim and Deans 2000). However, they do not play a significant role in fat deposition, since carbohydrates are the main substrate for fat synthesis, and therefore starch-rich chestnut kernels may play an important role in the deposition of fatty acids. Moreover, fatty acids that cannot be synthesized in tissues, such as C18:2 (ω6) and C18:3 (ω3), are more directly affected by the corresponding content in the diet (Jesús et al. 2016). Pugliese et al. (2013) also concluded that the substitution of commercial mixed diets with chestnuts in the late fattening period resulted in a significant increase in the content of PUFA ω3 and ω6 in pork is related to the higher UFA content in chestnuts.

Free amino acids are not only an important indicator to evaluate the nutritional value of meat, but also are important substances for meat to present the specific flavor (Ma et al. 2020). In fact, many amino acids have more than one taste characteristic. Glutamic acid and aspartic acid have a sour taste, but when the sodium salt is present, it is easy to react and produce an umami taste similar to monosodium glutamate (Chen and Zhang 2007). Glycine, alanine, threonine, serine, and proline are all amino acids contribute to the sweet taste (Luo et al. 2019). Our results showed that the contents of many umami and sweetness related amino acids, such as glutamic acid, aspartic acid,

glycine, alanine, threonine, and serine in the muscles of chestnut shell supplementary group were significantly higher compared with the control group. Their changes also contributed to the increase in total free amino acid content. The free amino acids in meat are released by cathepsins and other proteolytic enzymes after slaughter, which are influenced by many factors such as storage time and temperature (Penet et al. 1983). To our knowledge, very limited data are available on the effect of adding chestnuts and their by-products to the diet on free amino acids in pork. A recent study indicated that adding 1-4% sweet chestnut extract to the diet did not change the content of free amino acids in pork, nor did it affect the protein content of pork (Bahelka et al. 2021). In the absence of additional similar studies, it is difficult to determine the cause of this contrast, but since the actual amount of tannins added in this report was much higher than the safety threshold we inferred in our study, potential antinutritional properties are the likely cause for the absence of changes in protein deposition and free amino acid content in muscle. Given the increased protein content in the muscle of treatment group in present experiment, tannins in chestnut shells may have a regulatory effect on the metabolism of amino acids. Xu et al. (2019) showed a significant increase in total flavor amino acids, total free amino acids, and protein content of dorsal longest muscle after supplementation of 0.08% apple polyphenol powder in fattening pig diets, in addition to a significant increase in the expression of amino acid transporter protein SLC1A2 mRNA, thus inferring that polyphenol supplementation could increase amino acid transporter protein to promote the accumulation of amino acids in muscle and thus protein accumulation. On the other hand, most free amino acid content in meat were correlated negatively with the lipid content, as observed in the study of Ueda et al. (2007), a similar correlation appeared in this experiment, which may be another reason for the increase of free amino acid content. The results have also shown in muscle that the peptide constituent amino acids content increased significantly, and this suggests an increase in peptides content. The accumulation of free amino acids and peptides have a great influence on the palatability, contributing to higher quality of pork. The aroma

flavor characteristics of cooked meat are produced by Maillard reaction of amino acid or peptides with sugars and the interaction between Maillard reaction products and lipid oxidation products during the heating (Ba et al. 2012). Moreover, the levels of free amino acids in cooked meat, are known may be governed by initial levels in raw meat (Penet et al. 1983), and thus, higher free amino acid content implies higher nutritional value. Because we observed the good performance of eight essential amino acids, namely histidine, isoleucine, leucine, valine, lysine, threonine, phenylalanine and methionine, in the pork after dietary treatment.

5.5. Conclusions

Supplementation with 60% chestnut shells increased the tannin content of the feed, but with the decrease in feed protein and energy, may have had a negative impact on growth performance, and hematology was not affected. As chestnuts have often been used in the fattening period to improve pork quality, a particular focus on meat quality has been performed during this study. With the supplement of chestnut shells, stable color, high tenderness and juicy longissimus dorsi were obtained. The profile of fatty acids were not affected by the chestnut shell diet. Importantly, supplementation with 60% chestnut shells could increase the contents of the free amino acids and peptide constitute amino acids in the meat. This will help to improve the palatability and nutrition of pork.

Table 5.1. Ingredients of basal diets (as-fed basis).[1]

Items	Finisher
Ingredients [2] (%)	
Cereals [3]	70
Bran [4]	14
Oil seed meal [5]	13
Others [6]	3

[1] Experimental group diet was based on this diet with the addition of chestnut shells at 60%.

[2] Ingredients are provided by Marubeni Nisshin Feed Co., Ltd., Tokyo.

[3] Composition: milo, corn, wheat, wheat flour, rice, cassava, barley.

[4] Composition: corn gluten feed, wheat bran, rice bran.

[5] Composition: rape seed oil residue, soybean oil residue, corn jam meal.

[6] Composition: calcium carbonate, calcium phosphate, salt, baker's yeast, molasses.

Table 5.2. Chemical compositions of different experimental diets (%).

Item	DM	CP	EE	CF	Ash	NFE	DNE[1]	Tannin[2]
C	87.70	14.50	3.40	2.90	4.50	62.40	3.80	0.46
T	67.60	8.30	1.30	5.40	2.30	50.30	2.73	0.65

DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; NFE, nitrogen free extract; DNE, digestible nutrient energy.

[1] The digestible nutrient energy was calculated with the following equation:

Digestible nutrient energy (Mcal/kg) = [5.67×Crude protein (%)＋9.68×Crude fat (%)＋4.25×Nitrogen free extract (%)]/100

[2] Expressed as tannic acid equivalents.

Table 5.3. Effect of supplementing 60% chestnut shells in the diet on the fattening performance of finishing pigs (throughout the experimental period).

Item	Initial BW (kg)	Final BW (kg)	ADG (kg)	ADFI (kg)	FCR (kg/kg)
C	103.60±2.23	163.87±4.71	0.55±0.03	2.89	5.27
T	103.13±3.94	142.80±11.31	0.36±0.08	4.74	13.14

Values of BW and ADG are expressed as mean ± SEM; n = 3; There were no statistically significant differences between two groups according to the Student's t-test.

Table 5.4. Effects of supplementing 60% chestnut shells in the diet on the blood hematology of finishing pigs.

Item	WBC (×10²/μL)	Lymphocyte (×10²/μL)	Neutrophil (×10²/μL)	RBC (×10⁴/μL)	PLT (×10⁴/μL)
0d					
C	163.00 ±21.46	117.33 ±16.76	29.00 ±2.65	826.00 ±68.11	23.17 ±0.60
T	173.00 ±5.57	126.67 ±9.74	30.33 ±5.24	943.33 ±59.45	28.27 ±1.94
30d					
C	183.67 ±32.69	121.00 ±17.35	47.00 ±12.77	1030.33±99.68	23.13 ±4.58
T	204.00 ±29.50	149.33 ±27.36	42.00 ±1.53	946.00 ±7.37	13.13 ±3.33
60d					
C	191.67 ±16.46	125.33 ±7.22	47.67 ±8.37	931.67 ±50.20	25.10 ±2.90
T	217.33 ±7.88	147.33 ±3.93	50.67 ±7.17	1027.33±115.99	21.70 ±3.29
90d					
C	216.33 ±12.02	133.00 ±6.81	60.33 ±9.49	1128.00±148.59	30.97 ±4.72
T	198.00 ±24.95	129.67 ±23.95	51.33 ±6.69	1234.33±174.41	25.60 ±4.76
110d					
C	186.67 ±11.02	125.33 ±4.10	45.33 ±7.75	1039.33±156.12	21.33 ±1.58
T	210.67 ±12.45	143.33 ±13.68	51.00 ±1.73	940.67 ±24.34	25.57 ±2.61

All data are expressed as mean ± SEM; n = 3; There were no statistically significant differences between two groups according to the Student's t-test.

Table 5.5. Effects of supplementing 60% chestnut shells in the diet on the meat color of finishing pigs.

Item	L*	a*	b*	⊿E
C	55.04±1.24	4.54±0.33	13.13±0.33	5.30
T	50.30±0.73**	4.05±0.44	10.81±0.21**	

L*, lightness; a*, redness; b*, yellowness; ⊿E, the total color difference. The values of L*, a* and b* are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 5.6. Effects of supplementing 60% chestnut shells in the diet on the sensory evaluation of muscle in finishing pigs.

Item	Water holding capacity (%)	Elongation rate (cm^2/g)	Cooking loss (%)	Compression loss (%)
C	81.40±1.47	9.37±0.27	23.00±1.95	35.30±0.72
T	81.00±0.55	12.93±0.37**	25.53±1.00	41.10±0.45**

All data are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 5.7. Effects of supplementing 60% chestnut shells in the diet on the chemical composition of muscle in finishing pigs (g/100g).

Item	Protein	Lipid	Carbohydrate	Moisture	Ash	Energy (kcal/100g)
C	17.57±1.05	22.23±3.74	0.50±0.17	58.80±2.53	0.90±0.06	285.40±30.32
T	20.87±0.28	17.70±1.79	0.13±0.03	60.23±1.49	1.07±0.07	255.17±15.84

All data are expressed as mean ± SEM; n = 3; There were no statistically significant differences between two groups according to the Student's t-test.

Table 5.8. Effects of supplementing 60% chestnut shells in the diet on the fatty acids content of muscle in finishing pigs.

Item	SFA (%)	UFA (%)	MUFA (%)	PUFA (%)	ω6 FA (%)	ω3 FA (%)
C	37.73±1.14	59.77±0.62	51.70±0.83	8.10±1.22	7.37±1.03	0.70±0.30
T	38.07±1.20	59.23±1.10	51.93±1.19	7.30±0.36	6.87±0.38	0.43±0.03

All data are expressed are expressed as mean ± SEM; n = 3; There were no statistically significant differences between two groups according to the Student's t-test.

Table 5.9. Effect of supplementing 60% chestnut shells in the diet on the amino acids profile of muscle in finishing pigs (mg/100g).

Item	Aspartic acid	Glutamic acid	glutamine	Asparagine	glycine	Alanine	Threonine	Serine	Proline	Methionine	lysine	Isoleucine	Leucine	Phenylalanine	Tyrosine	Valine	Histidine	Arginine
C	3.33±0.33	15.33±0.33	19.00±1.53	2.00±0.00	6.33±0.33	17.33±1.76	2.67±0.33	2.33±0.33	3.00±0.00	1.00±0.00	3.67±0.33	3.33±0.33	5.67±0.33	1.00±0.00	0.00±0.00	4.67±0.33	2.00±0.00	5.33±0.67
T	4.67±0.33*	32.00±3.21*	15.33±3.48	1.33±0.33	10.67±0.33**	28.33±0.88*	5.33±0.33**	8.00±0.58**	3.00±0.00	8.00±0.58**	13.00±0.58**	10.33±0.88**	21.00±1.73*	12.67±0.88**	1.00±0.00	10.00±0.58**	4.67±0.33*	26.67±1.76**

All data are expressed are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

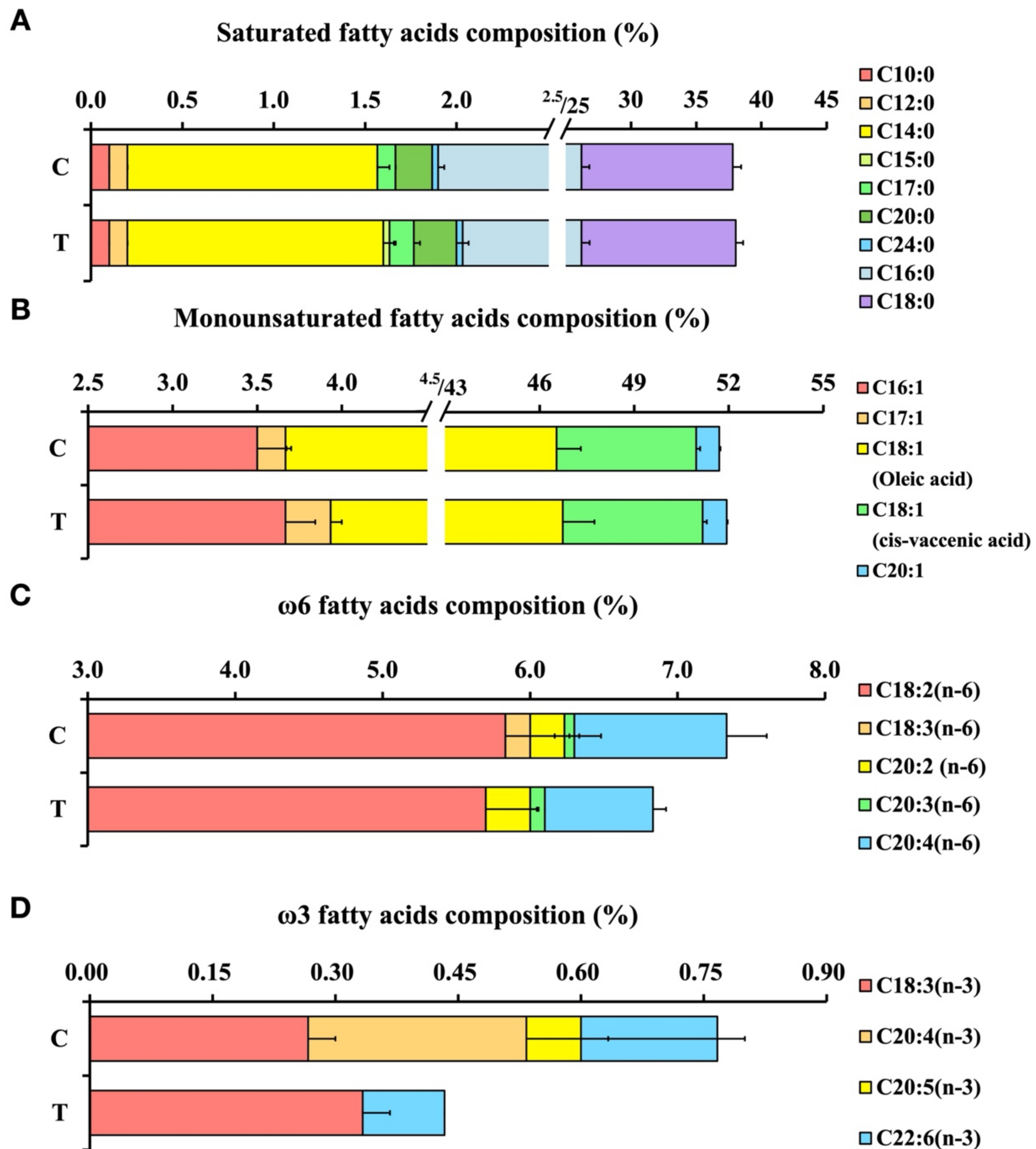


Figure 5.1. Effect of supplementing 60% chestnut shells in the diet on the fatty acids composition of muscle in finishing pigs. All data are expressed as mean ± SEM (n = 3); There were no statistically significant differences between two groups according to the Student's t-test. (**A**) the composition of saturated fatty acids; (**B**) the composition of monounsaturated fatty acids; (**C**) the composition of ω6 fatty acids; (**D**) the composition of ω3 fatty acids.

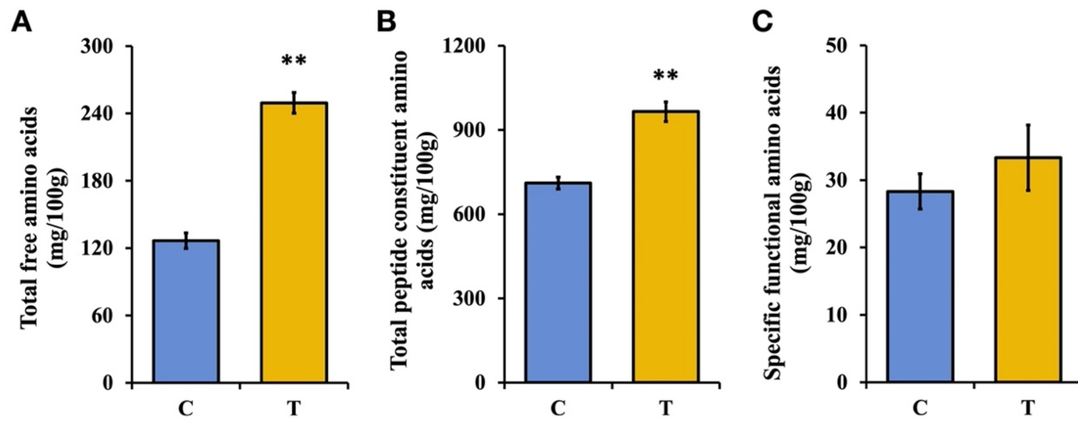


Figure 5.2. Effects of supplementing 60% chestnut shells in the diet on the amino acids content of muscle in finishing pigs. All data are expressed as mean ± SEM (n = 3); Asterisk above bars indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$). (**A**) total free amino acids content; (**B**) total peptide constituent amino acids content; (**C**) specific functional amino acids content.

Chapter 6 Effects of supplementation with silage chestnut by-products on growth performance, blood parameter, and meat quality in finishing pigs

6.1. Introduction

We have established that supplementing 60% of chestnut shells in pig feed during fattening helps to improve pork quality, so it makes sense to try to extend the use of chestnut shells to pig farming, but first of all it is necessary to overcome the extremely seasonal nature of chestnuts.

The harvesting season of chestnuts varies according to the geographical location where they are grown, for example, chestnuts grown in the south of Spain are harvested in the second half of September, while chestnuts from the north are harvested at the beginning of October (Pereira-Lorenzo et al. 2012). Chestnut harvest is usually completed within a month. The factory processes chestnuts while collecting them in large quantities. Some collected chestnuts are too late to be processed will be stored in cold storage. But the initial processing is also usually completed within 3 months, and a large number of chestnut shells are produced during this process.

This extremely seasonal nature makes it difficult to cover the longer feeding period of the pig, so there is a pressing need to develop reasonable storage procedures. Freezing can preserve chestnut shells to prevent deterioration, but it is not suitable for large-scale farming because of its high cost. Silage is a common practice for preserving plant-based feeds and plays an important role in livestock production systems (Jayanegara et al. 2019). However, no relevant studies on chestnut shell silage have been reported, especially the silage duration is still unclear.

Therefore, in the present study, we used two types of silage, short-term (3 months) and long-term (12 months, matching the chestnut production cycle), mixing 60% of the chestnut shells with 40% of the basal diet, and monitored their changes in tannin content and other chemical composition, and explored the feeding value of silage chestnut shells by observing the fattening performance and blood parameters of pigs. Then, the

application potential of silage chestnut shells in pork production was evaluated by investigating its effect on the sensory quality, chemical composition, fatty acid, and amino acid profiles of meat.

6.2. Materials and Methods

6.2.1. Animal ethics

All animal care and handling procedures conformed to the Guidelines for Animal Experiments of the University of Tokyo and were approved (P20-098) by the Animal Care and Use Committee of Life Science, Faculty of Agriculture, University of Tokyo.

6.2.2. Materials

The experiment was conducted at the Animal Resource Science Center of the University of Tokyo (Japan). The chestnut shells were provided by local corporations (Aiki Maron Co., Ltd, Kasama, Japan; Odaki Shoten Co., Ltd., Kasama, Japan), comprise outer shells and inner shells with a little pulp. The collected chestnut shells were shattered using an electric minced machine (PMM-12F, Minato, Fukuoka, Japan), and passed through a 10-mm screen. Every 10 kg of chestnut shell meal was packed into a plastic bag, compacted and sealed. Then divide them into 2 portions and were put into polyethylene bags and air was extracted by vacuum cleaner. Silage was carried out at normal temperature for 3 months and 12 months, respectively.

6.2.3. Animals and experimental design

A total of 12 Duroc finishing pigs (167±2 d of age, 105.62±2.85 kg), including castrated males and females, bred by the Animal Resource Science Center of the University of Tokyo were selected as experimental animals.

With the differences of silage duration, two trials were conducted in two periods, each trial using 6 pigs from the same sow as experimental animals and setting up a separate control and treatment groups (3 pigs each, kept in a pen). The EXP diet

purchased from Marubeni Nisshin Feed Co., Ltd (Tokyo, Japan) was used as the basal diet (**Table 5.1**), and it meets the National Research Council standards (NRC 2012).

In trial 1, the treatment group received a mixed diet of 40% basal diet and 60% chestnut shells silaged for 3 months (S3T), correspondingly, the control group was fed only the basal diet (S3C). In trial 2, pigs in the treatment group received a mixed diet of 40% basal diet and 60% chestnut shells silaged for 12 months (S12T), correspondingly, the control group was fed only the basal diet (S12C). Three pigs in the same group were kept in the same semi-enclosed barn without temperature control and the barn was kept clean daily. All animals were given feed and water using a 4-hole stainless-steel feeder, and a stainless-steel nipple waterer. The raising period lasted for 110 days. Taking account of the energy deficiencies of the alternative diet of chestnut shell meal, all pigs had ad libitum access to food and water was allowed, and the hopper and drinker were constantly inspected to ensure adequate supplies throughout the trial period.

6.2.4. Sample collection

Feed: Two kinds of treated chestnut shell meal were mixed with basal feed well, sampled while feeding, and then quickly placed in a −80°C refrigerator for storage.

Blood: The sampling procedures and methods are the same as we mentioned before (Piao et al. 2020). Blood samples were collected at the same time on days 0 (before treatment), 30, 60, 90, and the last day of treatment.

Meat: At the end of the raising period, all pigs were slaughtered after rearing in a local commercial slaughterhouse (Ibaraki Chuo Meat Center Co., Ltd., Ibaraki, Japan). Chilled for 24 h, longissimus dorsi was excised between the 11 th and 13 th ribs from the right half of the carcase. All samples were placed in vacuum bags and stored at −80 °C until analyses.

6.2.5. Feed analysis

The chemical compositions of diet were measured by the Japan Food Research Laboratories (Tokyo, Japan). Moisture content (atmospheric drying method), crude protein (combustion method, and was calculated by Nitrogen × 6.25), crude fat (diethyl ether extraction method), crude fiber (filtration method) and ash (direct ashing method) of the basal diets and 60% chestnut diets were determined. Then subtracted the sum of moisture, crude protein, fat, fiber, and ash from 100, and the difference is nitrogen free extract. Tannin concentration was measured by Folin-Denis method and expressed as tannic acid equivalents.

6.2.6. Fattening performance

The pigs were weighed at the same time of blood collection, and feed consumption on a pen basis were recorded to calculate ADG, ADFI and FCR.

6.2.7. Blood parameters

Hematological analysis was performed immediately after the collection of whole blood. White blood cells (WBC), lymphocytes, neutrophils, red blood cells (RBC) and platelets (PLT) concentrations in the whole blood were determined using a pocH-100iV Diff hematology analyzer (Sysmex Corp., Kobe, Japan). Plasma was collected after centrifugation at 3,000 rpm for 20 min at 4°C and then used an automatic dry-chemistry analyzer (DRI-CHEM 3500s; Fujifilm, Tokyo, Japan) to assess the concentrations of glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), gamma glutamyl transferase (GGT), ammonia (NH_3), blood urea nitrogen (BUN), amylase (AMYL), glucose (GLU), total protein (TP), albumin (ALB) and triglyceride (TG) in the plasma.

6.2.8. Meat Color

After taking the meat sample, measure the color on the remaining freshly cut surface at the 13 th rib immediately. The lightness (L*), redness (a*), and yellowness (b*) values were measured at three different points on each cut surface using a

calibrated PCE-TCR 200 colorimeter (PCE Instruments, Hampshire, UK). The mean of the three readings was taken as the sample color value.

To better reflect the color difference, after calculating the average of all samples in each group, the total color difference (ΔE*) between the two groups of meat samples was calculated using the following formula:

$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)]^{1/2}$.

where ΔL*, Δa*, and Δb* are the differences of the mean value of L*, a*, and b* between the control and treated samples.

6.2.9. Meat quality evaluation

The meat quality analysis involved meat composition, eating quality (sensory evaluation), fatty acid composition and amino acid profile of longissimus dorsi. Analysis was performed by the Bureau Veritas Japan Co., Ltd. (Shimane, Japan). Briefly, macro-Kjeldahl method was used to measure protein content with the use of the conversion coefficient 6.25. The lipid was measured by ether extraction method. The moisture content was measured by drying at 135 °C for 2 hours using a desiccant, and the ash content was measured by the ashing method. The carbohydrate content is obtained by subtracting the sum of the above four components from 100. The energy value of meat was calculated by multiplying the protein, lipid and carbohydrate content by the energy conversion factors (proteins: 4.22 kcal/g, lipids: 9.41 kcal/g, and carbohydrates: 4.11 kcal/g). The sensory evaluation included water holding capacity, elongation rate, the weight loss of the muscle during heating (cooking loss), as well as the compression loss of the heated meat sample. In addition, the tenderness, pliability, toughness, and brittleness were determined by means of a Tensipresser texture analyzer after heating the meat. The fatty acid composition of the longissimus dorsi was analyzed by gas-chromatography and was expressed as percentage content of total fatty acids, included SFA, UFA, MUFA, PUFA, ω6 FA (n-6 fatty acids), and ω3 FA (n-3 fatty

acids). Finally, high performance liquid chromatography method was adopted to measure the free amino acid profile and expressed as mg/100g of muscle.

6.2.10. Statistical Analysis

Data analysis was performed using JMP Pro software (version 15.2.0, SAS Institute Inc., Cary, NC, USA). The statistical significance between averages of two groups at each stage was determined using the independent sample t test, with the results presented as means ± SEM. *($p < 0.05$) and **($P < 0.01$) respectively indicate statistical significance and extreme significance.

6.3. Results

6.3.1. Chemical compositions of diets

Table 6.1 shows the compositions of different diets. The nutrient concentrations analyzed in the two control diets differed slightly but not obvious, with a 0.12% difference in tannin content and a similar amount of energy from digestible nutrients, about 3.8 Mcal/Kg. The chestnut shell alternative diets contained higher percentages of moisture and crude fiber when compared to the control diets, but it is lower in terms of crude protein, crude fat and nitrogen free extract, and directly causes lower energy. In respect to tannin concentration, tannins were detected in all diets. Based on dry matter, the tannin content of the S3C group diet was 0.39% and that of the S3T group diet was 0.66%, so it was equivalent to a 0.27% tannin supplementation when the same amount of dry matter was consumed. In contrast, the tannin content of the S12C group diet was 0.52% and the S12T group diet was 0.66%, so it was equivalent to 0.14% tannin supplementation for the same amount of dry matter harvested. It was also calculated that the tannin content of chestnut shells in 3 months silage was 0.96% (dry matter basis) and that of chestnut shells in 12 months silage was 0.80% (dry matter basis).

6.3.2. Fattening performance

The results of the fattening performance of pigs are depicted in **Table 6.2**. Dietary supplementation of chestnut shells had no significant effect on body weight throughout the trial period ($p > 0.05$). Pigs fed silage chestnut shells supplement diet had lower ADG and higher ADFI in the experimental phase, which made FCR obviously higher than the control group.

6.3.3. Blood hematology

The changes of blood hematology of pigs fed chestnut shells supplement diets are presented in **Figure 6.1**. Silage chestnut shells supplement did not affect the WBC and lymphocyte counts throughout the experiment ($p > 0.05$). Compared with the control group (S12C), the neutrophils count of S12T group was decreased ($p < 0.05$) on 60 d. Moreover, no diet related changes of RBC and platelets counts were found during feeding trial.

6.3.4. Blood biochemistry

As can be seen from **Table 6.3** and **Figure 6.2**, among the biochemical parameters, the GPT activity in each group were at low levels at the beginning of the trial. After 30 days feeding it had increased significantly ($p < 0.05$) in S3T and S12T groups, and after that, both kept high levels and the differences were significant ($p < 0.01$ or $p < 0.05$). No significant changes were observed in blood plasma GOT and GGT ($p > 0.05$). Low concentrations ($p < 0.05$) of NH_3 were recorded in groups S3T and S12T than in the corresponding control groups (S3C or S12C) on the 60 d of the experiment, and on the 30 d and 110 d of the trial the TP contents of S3T groups were significantly higher than in the S3C group ($p < 0.01$ or $p < 0.05$). In the first phase of the trial, significant decreases ($p < 0.05$) were observed for BUN concentrations in groups S3T and S12T. After that, the concentrations of BUN in the blood collected at 60 d and 90 d in the S3T group were significantly lower ($p < 0.01$ or $p < 0.05$) than control group (S3C), while the S12T group was significantly lower only at 110 d ($p < 0.05$).

6.3.5. Meat color

Data on the meat color of pigs fed the experimental diets is shown in **Table 6.4** The L* value was higher ($p < 0.05$) in muscle from S3T pigs compared with the control group (S3C). No significant differences ($p > 0.05$) in a* and b*values were observed. The 3-month silage chestnut shells supplementation has a greater effect on muscle color because of higher ΔE* value.

6.3.6. Chemical composition of muscle

Data on chemical composition of muscle is shown in **Table 6.5**. Protein, lipid, carbohydrate, ash content and energy in muscle samples did not differ among dietary treatments, however, a lower moisture content was observed in the S3T group ($p < 0.05$).

6.3.7. Sensory evaluation of muscle

The sensory evaluation of longissimus dorsi samples are depicted in **Table 6.6**. No effect of dietary supplementation with 3 months silage chestnut shells was observed on the water holding capacity, elongation rate, cooking loss and compression loss. The meat samples of S12T group analyzed lower water holding capacity ($p < 0.05$), and higher cooking loss ($p < 0.01$), than the meat samples from the control group (S12C). In addition, muscle of pigs fed with 3-month silage chestnut shells supplement diet had lower tenderness and toughness than those fed basic diet ($p < 0.01$ or $p < 0.05$). However, the muscle of 12-month silage chestnut shell group had higher ($p < 0.05$) toughness than that in the control group (S12C).

6.3.8. Fatty acids composition of muscle

As depicted in **Table 6.7**, there were no obvious differences ($p > 0.05$) in the concentration of SFA between the silage chestnut shells supplementation group and control group. However, from **Figure 6.3** it is obvious that among the nine saturated fatty acids, the proportion of C16:0 (palmitic acid) was significantly higher ($p < 0.05$)

in S3T group. No significant differences ($p > 0.05$) were found in proportion of UFA, MUFA and MUFA composition of muscle from the various dietary treatments. On the other hand, the proportion of PUFA was lower ($p < 0.05$) in muscle from pigs fed the 3-month silage chestnut shells supplementary diet than control group (S3C), particularly ω-6 PUFA. Regarding ω-6 PUFA composition, the observed decrease in C18:2 of S3T might be the main reason for the decrease in the proportion of ω-6 PUFA in S3T treatment. Chestnut shell supplement treatments did not differ in ω-3 PUFA, but had a significantly lower ($p < 0.05$) C18:3 in S12T group than that in the control group (S12C).

6.3.9. Amino acids profile of muscle

The amino acids profile of muscle is shown in **Table 6.8**. Muscle in the S3T group had higher contents of glutamine and asparagine ($p < 0.05$), in the S12T group had higher content of aspartic acid, threonine, proline, methionine, phenylalanine and valine ($p < 0.01$ or $p < 0.05$) than those in respective control groups. However, diet supplementation with 3-month silage chestnut shells significantly reduced ($p < 0.01$ or $p < 0.05$) the contents of threonine and tyrosine in the muscle compared to the control group (S3C). As shown in **Figure 6.4**, in terms of total free amino acids content in muscle, the S12T group had higher ($p < 0.05$) value than that of control group (S12C). No significant differences were observed among dietary treatments in total peptide constituent amino acids. With regard to the specific functional amino acids, although there was no significant difference ($p > 0.05$), the S3T group was palpable lower than the control group (S3C).

6.4. Discussion

Besides phenols, chestnut shells mainly contains a lot of cellulose, hemicelluloses, and lignin (Vázquez et al. 2008). With the addition of chestnut shells, the content of water and crude fiber increased, and the content of crude protein and crude fat decreased obviously. Our results corroborate the findings of Rodrigues et al. (2015), who

evaluated nutritional composition of chestnut shells, and observed the lower protein, fat and ash, due to the higher content of fibers. These changes considerably reduced the energy level of the feed. As mentioned in chapter 5, processing (steaming or boiling) will increase the moisture in the chestnut shells, thus we measured a higher moisture content compared to other reports (Rodrigues et al. 2015). He et al. (2018) considered that active substances such as hydrolysable tannins in chestnut shells may be considerably degraded by microorganisms during silage. The tannin contents of various chestnut shells have been previously calculated and are 1.44% (frozen), 0.96% (3-month silage), and 0.80% (12-month silage) on a dry matter basis. From this, it is evident that silage will reduce the tannin levels in chestnut shells, and the effect is enhanced with increasing silage time. Likewise, since tannins are widely distributed in animal feed, such as corn, wheat and barley (Caprarulo et al. 2021). Thus, a certain concentration of tannins can also be detected in the basal diets, and the differences in tannin content in the diets of the two control groups may be related to the plant-derived components in the diet, the growing region, and the harvest season.

The current study confirmed that adding 60% of large proportion silage chestnut shells in commercial diets had little effect on the growth performance of finishing pigs. Because of the partial decomposition during the silage process, the actual amount of tannins added is lower compared to Chapter 5 and within the safe range, therefore the anti-nutritional effect of tannins is not considered. The addition of high-dose chestnut shells resulted in insufficient energy. In the feeding test, the fermented chestnut shells produce fruity/alcoholic odor was found through sensory evaluation, and it was stronger after 3 months of silage. This led to a high intake of the feed containing silage chestnut shells by pigs, and the ADFI of pigs in S3T and S12T groups in this study was 5.3 and 5.0, respectively, the ADFI of pigs on frozen chestnut shells mixed feed in the previous experiment was 4.74. As the percentage of concentrate feed was the same, the elevated intake directly led to the increase of the actual intake of concentrate feed and the corresponding increase of protein and energy supplementation, which was the main

reason for the smaller effect of silage chestnut shells feed on pig growth. Silage may also help to increase the efficiency of nutrient utilization in pigs' diets. We found that the utilization efficiency of the mixed diets gradually increased with the duration of silage, so that the growth performance was almost the same even though the ADFI of the S12T group was slightly lower than that of the S3T group.

The counts of WBC, lymphocytes and neutrophils may indicate the immune status of animals (Liu et al. 2016). Except that the decrease of neutrophils count in the S12T group during a certain period, WBC and lymphocyte counts were not affected by chestnut shells supplementation in this experiment. The low neutrophil count may be related to the relief of inflammation and stress (Ronchetti et al. 2018). However, animals consuming the silage may be more likely to potentiate intestinal inflammation attributed to the mycotoxins constituents present (Grenier and Applegate 2013). It follows that the decline in neutrophils may only herald the weakening of immune function.

Wilkinson (1999) showed that the harmful microorganisms that may appear in the silage process and the undesirable chemicals they produce (e.g. toxins), are potential hazards to the health of animals. Thus, blood biochemical analysis was performed on pigs of the experimental group added with silage chestnut shells. The blood GPT is a highly sensitive and specific clinical indicator of hepatotoxicity (Ozer et al. 2008). A notable finding of this study was that the level of GPT activities in plasma of pigs that had eaten silage chestnut shells increased significantly, and the increases are proportional to the silage time, probably owing to the mycotoxins accumulated during the silage, which were ingested by animals and cause hepatocyte necrosis or deterioration of membrane permeability to release GPT (Tsiouris et al. 2021). Of relevance is the GOT activity in plasma of pigs ingested silage chestnut shells is also higher in most periods, because GOT activity usually increases with GPT activity when liver damage occurs, and will return to normal before GPT after hours to days (Ramaiah 2007). Even so, liver function was maintained because the GGT activity and NH_3

concentration of the S3T and S12T groups were lower in a long period of time, which also coincided with an increase in plasma TP. It was stated that the concentration of BUN is highly correlated with the excretion rate of nitrogen in urine, and the lower BUN concentrations of chestnut shells supplement groups, especially S3T group in current study, indicating high utilization of dietary protein (Kohn et al. 2005). It is undeniable that the urea nitrogen content is closely related to the protein level in the diet and as the protein level of the diet decreases, the urea nitrogen as well as the fecal nitrogen content decreases significantly (O'Connell et al. 2006). So the consistent decrease in urea nitrogen content of pigs in the treatment group after the start of the experiment was related to the decrease in protein content after supplementation of the diet with chestnut shells. In addition, according to Galassi et al. (2010), increasing the fiber content of the diet contributed to a further decrease in nitrogen emissions from livestock fed on a low-protein diet. This further contributed to the emergence of significant differences in urea nitrogen content between the two groups of pigs.

Meat color is a very important sensory attribute, it is directly related to consumers' desire to purchase because they relate color to freshness (Morrissey et al. 1998). The meat color is related to the content of myoglobin and the oxidation state of muscle pigment. (O'Connor-Shaw and Reyes 1999). Nevertheless, compared with beef and mutton, the color of pork is more susceptible to glycolytic fibers (Listrat et al. 2016). After slaughter, the carbohydrates in pork are converted into lactic acid by glycolysis (Chauhan and England 2018). Notably, the L* value was proved to be highly correlated with the lactic acid content in meat, and it increased with increasing lactate levels, and cause paleness of the meat (Mancini and Hunt 2005). Similar changes in the L* value and carbohydrate content of meat samples in our study illustrate this. ΔE* is a value that can distinguish the overall change of L* and a* and b* values. When it is higher than 1, the color difference between the two samples is detectable with naked eye (Semjon et al. 2020). Thus, the supplementation of two kinds of chestnut shells caused

the visible change in pork color, and that of S3T treatment was the most evident and turning an undesirable color.

As for the chemical composition of the meat, the increasing trend in protein content was not as pronounced as in the frozen chestnut shells supplement. Moisture is the main component of meat constituting up to 75% by weight, and its content is inversely related to fat content (Park 2008). We observed that feeding chestnut shells silage for 3 months reduced the moisture content of the meat, and the fat content increased slightly but not significantly and is in agreement with Kim et al. (2005), who found similar changes after adding 7% of the persimmon shell that had been fermented for 60 days. Moisture is easily lost from meat during processing or storage, which will not only decrease the weight and juiciness of meat, but also cause the loss of nutrients such as valuable water-soluble proteins and vitamins, consequently, the fact that water holding capacity is one of the most used characteristics to evaluate meat quality (Webb 2011). After eating chestnut shells silage for 12 months, the water holding capacity of pork decreased significantly, and correspondingly, the cooking loss increased significantly. It is stated that pH decline, ionic strength and oxidation may affect the ability of myofibrillar protein, myofibrils and muscle cells to retain water in meat (Huff-Lonergan and Lonergan 2005). Tenderness is an important meat quality trait valued by consumers, and its influencing factors are quite complex (Warner et al. 2010). However, there is currently no clear definition for tenderness in sensory studies of meat texture (Sasaki et al. 2012). In Japan, a widespread method is to measure the breaking strength and biting resistance of the meat under simulated action, the so-called ‘tenderness’ and ‘toughness’ values, to evaluate the meat tenderness. In porcine diets in which the supplemental 3-month silage chestnut shells, it shows low ‘tenderness’ and ‘toughness’, indicating that the meat has low cohesiveness and is easier to chew. But the S12T diet group shows an opposite trend.

Considering the association between fatty acids and human health, nutritional strategies to enrich pork fatty acids through feed additives have been extensively

studied in recent years (Inserra et al. 2015; Ahmed et al. 2016; Rezar et al. 2017). Iberian pigs are known worldwide for producing pork rich in UFA by feeding on acorns, which are rich in polyphenols that reduce lipid oxidation in Iberian pigs and thus increase the UFA content of pork (Cava et al. 2000). Similar to acorns, the tannins contained in chestnuts are almost exclusively present in the shells, and we learned from feeding experiments supplemented only with chestnut shells that the deposition of UFA in pork does not correlate significantly with the tannins contained. Jesús et al. (2017) believed that these fatty acids were directly derived from chestnuts, who evaluated the fatty acids composition of diet containing 15% chestnut (dry weight) and observed increases in linoleic acid (C18:2 ω6) and linolenic acid (C18:3 ω3) and a decrease in palmitic acid (C16:0). Correspondingly, when the pigs were fed this diet continuously during the growth and fattening phases, consistent significant differences were detected in the loin. Both C18:2 (ω6) and C18:3 (ω3) are essential fatty acids, which cannot be synthesized through enzymatic reactions, so the content in muscle tissue is limited by the content in the diet (Jesús et al. 2017). Only using chestnut shells appears to be an important cause for the different results in this experiment.

As the basic components of proteins, amino acids are not only essential nutrients for human beings, but also important precursors for the special flavor of meat. They can elicit five basic tastes: umami, sweet, bitter, salty and sour (Delompré et al. 2019). The taste of amino acids is far more complex than expected. For example, glutamine has a slightly sweet taste in addition to the rich umami taste. Aspartic acid presents a sour taste while imparting umami to the food. Umami plays a significant role in palatability and can enhance the harmony and mellowness of food (Bao et al. 2020). This indicates that the increased glutamine content in the S3T group and the increased aspartic acid content in the S12T group contribute to the establishment of umami in pork. Threonine, proline, and phenylalanine are all taste-active amino acids associated with sweetness (Bachmanov et al. 2016). Dietary S3T chestnut shells supplementation reduced the threonine content, while S12T chestnut shells supplementation increased

the contents of threonine, proline, and phenylalanine. Cooking process can induce the unique “meat flavor” of meat, but raw meat has only a light salty-sweetish taste (Ngapo and Gariépy 2008). During the cooking process, with Stryker degradation and Maillard reaction, free amino acids in the meat will be degraded to form aldehydes and aminoketones, and then further react with other compounds to contributed to the formation of “meat flavor” (Ba et al. 2012). The sulphur-containing amino acid methionine plays a major role in the acquisition of palatable meat flavor during the Maillard reaction (Feiner 2006). However, only the supplementation of chestnut shells with 12-month silage significantly increased the methionine content of pork. Moreover, one needs to be aware that three of the free amino acids (phenylalanine, threonine, and valine) in the muscle improved by the S12T treatment were essential amino acids. This also indicate that the nutritional value of pork was improved.

Compared with the results in Chapter 5, the tannin content in chestnut shells decreased after silage, and accordingly the influence on the free amino acid and protein content in pork became weaker, which further supports the view of Xu et al. (2019) that tannins may promote the expression of amino acid transporter proteins thereby increasing the deposition of free amino acids and proteins in muscle tissue. However, the difference in tannin content between the S12T and S3T groups was not significant and the improvement in free amino acids of S12T group were more pronounced. This may be due to microorganisms during silage gradually used the indigestible proteins in the chestnut shells to produce more easily digestible substances, such as bacterial proteins. As reported by Lee et al. (2016), the protein digestibility of chestnut shells, especially the outer shell, is almost zero, while the inner shell contains relatively more protein, but the digestibility is only 49.8%. The conversion of these proteins during prolonged silage may promote the accumulation of free amino acids as well as protein in pork.

6.5. Conclusions

The degradation degree of tannins in chestnut shells was enhanced with the duration of silage. However, since the tannin content in 60% chestnut shells was within the safe threshold, different silage chestnut shells did not have different effects on pig fattening performance due to changes in tannin content. The results of the blood parameters indicate that the intake of silage chestnut shells supplementary diet with no adverse effects on normal functions of liver even though has inevitably brought a certain burden. Furthermore, short-term silage chestnut shells supplement (S3T) has the potential to increase tenderness, but lost the function of stabilizing the color of meat. Nevertheless, Long-term silage chestnut shells supplement (S12T) may reduce water holding capacity and increase cooking loss, thus reducing the sensory quality of meat. However, it contributed to optimize the distribution of amino acids in pork, and had a better effect than S3T. Though the effect on meat quality is limited, the silage of chestnut shells has a feeding value in terms of production performance and feed cost.

Table 6.1. Chemical compositions of different experimental diets (%).

(A) Trial 1

Item	DM	CP	EE	CF	Ash	NFE	DNE[1]	Tannin[2]
S3C	87.90	13.60	3.30	3.30	4.20	63.50	3.79	0.34
S3T	67.80	7.70	1.30	5.00	2.20	51.60	2.76	0.45

(B) Trial 2

Item	DM	CP	EE	CF	Ash	NFE	DNE[1]	Tannin[2]
S12C	87.70	14.50	3.40	2.90	4.50	62.40	3.80	0.46
S12T	68.20	8.00	1.50	6.00	2.50	50.20	2.73	0.45

DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; NFE, nitrogen free extract; DNE, digestible nutrient energy.

[1] The digestible nutrient energy was calculated with the following equation:
Digestible nutrient energy (Mcal/kg) = [5.67×Crude protein (%)＋9.68×Crude fat (%)＋4.25×Nitrogen free extract (%)]/100

[2] Expressed as tannic acid equivalents.

Table 6.2. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the fattening performance of finishing pigs (throughout the experimental period).

(A) Trial 1

Item	Initial BW (kg)	Final BW (kg)	ADG (kg)	ADFI (kg)	FCR (kg/kg)
S3C	103.40±7.18	164.80±8.58	0.55±0.03	2.77	5.05
S3T	108.00±4.27	164.40±9.69	0.50±0.05	5.31	10.55

(B) Trial 2

Item	Initial BW (kg)	Final BW (kg)	ADG (kg)	ADFI (kg)	FCR (kg/kg)
S12C	105.33±8.00	165.80±4.69	0.54±0.10	2.86	5.24
S12T	105.73±6.27	161.47±13.92	0.50±0.07	5.00	9.97

Values of BW and ADG are expressed as mean ± SEM; n = 3; There were no statistically significant differences between two groups according to the Student's t-test.

Table 6.3. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the blood biochemical parameters of finishing pigs.

(A) Trial 1

Item	GPT (U/L)	GOT (U/L)	GGT (U/L)	NH_3 (μg/dL)	BUN (mg/dL)	AMYL (U/L)	GLU (mg/dL)	TP (g/dL)	ALB (g/dL)	TG (mg/dL)
0d										
S3C	38.33±3.38	27.33±4.26	37.00±4.00	87.00±13.58	12.40±0.42	342.00±28.88	91.67±8.51	6.23±0.07	3.93±0.29	20.67±3.28
S3T	39.00±1.15	34.00±10.15	32.00±4.04	76.33±2.19	12.63±0.49	302.67±21.06	91.00±4.00	6.10±0.06	3.93±0.03	28.00±9.17
30d										
S3C	44.00±1.73	55.33±9.02	44.00±0.58	96.00±4.73	11.33±1.39	299.67±20.51	99.00±17.04	6.33±0.03	4.07±0.12	25.33±7.36
S3T	59.00±0.58**	29.67±2.03	33.00±4.04	75.00±7.94	5.23*±0.23	316.33±20.74	87.67±4.33	6.63±0.03**	4.20±0.06	42.33±4.67
60d										
S3C	38.00±3.51	22.67±3.18	49.33±6.36	85.67±5.21	13.60±1.01	324.00±23.18	93.00±9.29	6.40±0.06	4.30±0.06	37.00±9.45
S3T	62.67±1.86**	35.00±6.35	33.00±5.69	58.33±4.10*	5.00±0.00*	338.33±14.66	79.67±2.19	6.40±0.00	4.10±0.10	20.67±0.88
90d										
S3C	41.33±2.67	40.67±8.19	44.33±2.03	165.67±12.81	11.73±0.52	393.67±85.31	88.00±20.22	6.9.00±0.46	4.23±0.18	15.33±3.53
S3T	62.67±4.48*	40.67±6.69	36.00±4.73	145.00±11.53	6.17±0.58**	288.00±25.74	78.00±6.56	6.9.00±0.2	4.33±0.15	23.33±2.67
110d										
S3C	38.67±1.20	27.33±3.18	40.00±0.58	79.67±4.37	10.6±0.15	324.33±30.80	85.67±11.78	6.40±0.15	4.33±0.09	15.00±5.00
S3T	57.67±2.40**	40.33±5.21	41.67±2.40	86.00±5.29	8.20±0.85	423.00±57.19	92.00±9.07	7.23±0.19*	4.57±0.03	18.67±2.96

(B) Trial 2

Item	GPT (U/L)	GOT (U/L)	GGT (U/L)	NH_3 (μg/dL)	BUN (mg/dL)	AMYL (U/L)	GLU (mg/dL)	TP (g/dL)	ALB (g/dL)	TG (mg/dL)
0d										
S12C	34.33±2.85	39.00±12.66	18.33±2.33	116.00±16.37	11.47±1.79	380.00±37.45	94.33±22.51	6.60±0.15	4.07±0.20	26.67±11.29
S12T	42.33±0.33	23.33±0.88	18.00±1.15	113.00±4.36	11.80±1.27	408.33±32.27	99.33±10.81	7.30±0.32	4.67±0.09	33.33±10.59
30d										
S12C	35.33±1.20	29.33±3.84	29.00±1.15	72.67±4.10	12.40±1.33	364.00±22.34	76.33±7.22	6.80±0.17	4.23±0.33	21.00±7.94
S12T	72.33±4.63*	36.00±4.36	29.67±2.33	71.67±2.96	5.53±0.19*	406.67±29.85	83.00±2.31	7.07±0.22	4.40±0.06	16.33±4.10
60d										
S12C	38.00±2.65	28.67±5.24	27.67±4.33	79.67±1.20	12.87±0.99	290.67±11.14	81.67±9.53	7.00±0.06	4.37±0.23	38.33±11.68
S12T	78.33±2.67**	36.33±1.67	21.33±3.84	65.67±2.67*	7.67±1.54	330.67±16.91	83.33±8.88	7.07±0.23	4.77±0.18	22.00±8.02
90d										
S12C	39.00±4.93	30.00±1.73	30.33±2.85	73.67±3.18	13.93±1.28	305.33±45.85	97.67±0.33	6.83±0.41	4.40±0.30	53.67±24.10
S12T	76.67±3.18**	28.33±2.03	21.33±3.84	69.00±2.08	8.37±1.83	302.00±14.42	82.67±5.93	7.23±0.18	5.00±0.17	65.33±11.10
110d										
S12C	38.00±0.00	36.00±6.11	35.67±4.26	79.67±7.36	14.63±1.51	261.33±5.24	86.67±3.53	7.17±0.41	4.57±0.26	34.67±15.93
S12T	78.00±4.04*	43.67±1.45	31.67±7.54	69.00±2.08	7.67±0.62*	268.67±5.55	57.33±9.82	7.30±0.25	5.13±0.33	56.33±4.33

Abbreviations: GPT, glutamate-pyruvate transaminase; GOT, glutamate-oxaloacetate transaminase; GGT, gamma-glutamyl transpeptidase; NH_3, ammonia; BUN, blood urea nitrogen; AMYL, amylase; GLU, glucose; TP, total protein; ALB, albumin; TG, triglyceride. All data are expressed as mean ± SEM (n = 3). For a given row, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 6.4. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the meat color of finishing pigs.

(A) Trial 1

Item	L*	a*	b*	⊿E
S3C	45.70±0.69	14.23±1.31	4.45±0.36	8.37
S3T	54.04±1.05**	14.94±1.01	4.58±0.30	

(B) Trial 2

Item	L*	a*	b*	⊿E
S12C	48.37±0.34	8.85±0.65	-0.81±0.22	3.56
S12T	48.40±1.08	12.09±1.77	0.66±0.65	

L*, lightness; a*, redness; b*, yellowness; ⊿E, the total color difference. The values of L*, a* and b* are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 6.5. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the chemical composition of muscle in finishing pigs (g/100g).

(A) Trial 1

Item	Protein	Lipid	Carbohydrate	Moisture	Ash	Energy (kcal/100g)
S3C	22.70±0.57	6.33±0.97	0.10±0.00	69.83±0.49	1.03±0.03	156.00±7.09
S3T	23.80±0.42	7.27±0.45	0.37±0.27	67.47±0.43*	1.10±0.06	162.33±4.18

(B) Trial 2

Item	Protein	Lipid	Carbohydrate	Moisture	Ash	Energy (kcal/100g)
S12C	22.73±0.29	4.10±0.25	0.13±0.03	71.90±0.12	1.13±0.03	135.07±1.33
S12T	23.20±0.38	3.43±0.77	0.13±0.03	72.13±0.47	1.10±0.00	130.77±5.93

All data are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 6.6. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the sensory evaluation of muscle in finishing pigs.

(A) Trial 1

Item	Water holding capacity (%)	Elongation rate (cm^2/g)	Cooking loss (%)	Compression loss (%)	Tenderness ($\times 10^4 gf/cm^2$)	Pliability	Toughness ($\times 10^8 gf/cm^2 \cdot cm$)	Brittleness
S3C	81.20±0.81	14.60±0.06	20.57±1.02	46.30±1.11	5.97±0.13	1.65±0.10	2.71±0.22	1.42±0.05
S3T	80.37±1.11	12.30±0.92	17.77±1.48	39.30±2.79	4.04±0.14**	1.54±0.01	1.33±0.08*	1.57±0.01

(B) Trial 2

Item	Water holding capacity (%)	Elongation rate (cm^2/g)	Cooking loss (%)	Compression loss (%)	Tenderness ($\times 10^4 gf/cm^2$)	Pliability	Toughness ($\times 10^8 gf/cm^2 \cdot cm$)	Brittleness
S12C	80.23±0.64	12.90±0.76	24.70±0.45	39.33±1.05	4.07±0.34	1.44±0.06	1.53±0.28	1.72±0.10
S12T	77.00±0.56*	12.47±0.35	30.73±0.81**	35.73±0.38	4.67±0.24	1.31±0.05	2.69±0.24*	1.66±0.09

All data are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 6.7. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the fatty acids content of muscle in finishing pigs.

(A) Trial 1

Item	SFA (%)	UFA (%)	MUFA (%)	PUFA (%)	ω6 FA (%)	ω3 FA (%)
S3C	38.80±0.74	60.00±0.67	53.30±0.64	6.70±0.06	6.33±0.03	0.37±0.03
S3T	40.63±0.30	58.37±0.23	53.53±0.55	4.83±0.43*	4.50±0.36*	0.33±0.07

(B) Trial 2

Item	SFA (%)	UFA (%)	MUFA (%)	PUFA (%)	ω6 FA (%)	ω3 FA (%)
S12C	41.13±0.56	57.17±0.70	48.57±0.84	8.60±0.44	8.17±0.38	0.43±0.09
S12T	41.07±0.94	57.53±0.90	49.17±0.64	8.40±1.52	8.20±1.46	0.17±0.07

All data are expressed are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

Table 6.8. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the amino acids profile of muscle in finishing pigs(mg/100g).

(A) Trial 1

Item	Aspartic acid	Glutamic acid	glutamine	Asparagine	glycine	Alanine	Threonine	Serine	Proline	Methionine	lysine	Isoleucine	Leucine	Phenylalanine	Tyrosine	Valine	Histidine	Arginine
S3C	2.67±1.45	21.67±6.33	5.67±2.91	1.00±0.58	12.33±0.67	31.00±2.31	8.33±0.33	5.67±2.40	5.00±1.00	7.00±0.58	12.33±0.67	8.33±1.45	16.67±2.03	12.33±1.45	2.00±0.00	11.00±0.58	5.67±0.33	13.00±2.52
S3T	1.67±0.67	32.67±1.45	23.00±1.53*	4.00±0.58*	12.67±0.67	26.00±2.52	5.33±0.33**	5.67±0.67	5.67±0.67	5.67±0.33	12.67±0.67	9.33±0.33	17.67±1.20	13.00±0.00	0.33±0.33*	10.67±0.67	5.33±0.33	20.00±1.73

(B) Trial 2

Item	Aspartic acid	Glutamic acid	glutamine	Asparagine	glycine	Alanine	Threonine	Serine	Proline	Methionine	lysine	Isoleucine	Leucine	Phenylalanine	Tyrosine	Valine	Histidine	Arginine
S12C	8.00±0.58	19.33±1.67	18.00±0.58	1.67±0.33	9.00±1.00	18.67±2.91	4.67±0.67	3.33±0.88	2.67±0.33	1.33±0.33	3.33±0.88	3.33±0.67	6.33±1.20	1.33±0.33	0.00±0.00	6.33±0.67	3.00±0.00	6.67±2.19
S12T	20.00±1.00**	19.33±2.33	24.67±3.48	1.67±0.33	7.67±0.33	17.00±0.00	9.33±0.88*	2.67±0.33	15.00±0.58**	3.33±0.33*	5.00±0.58	4.67±0.33	8.00±0.58	5.00±0.58*	0.00±0.00	18.33±1.33**	2.00±0.58	8.00±1.15

All data are expressed are expressed as mean ± SEM; n = 3; For a given column, mean values with asterisk indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$).

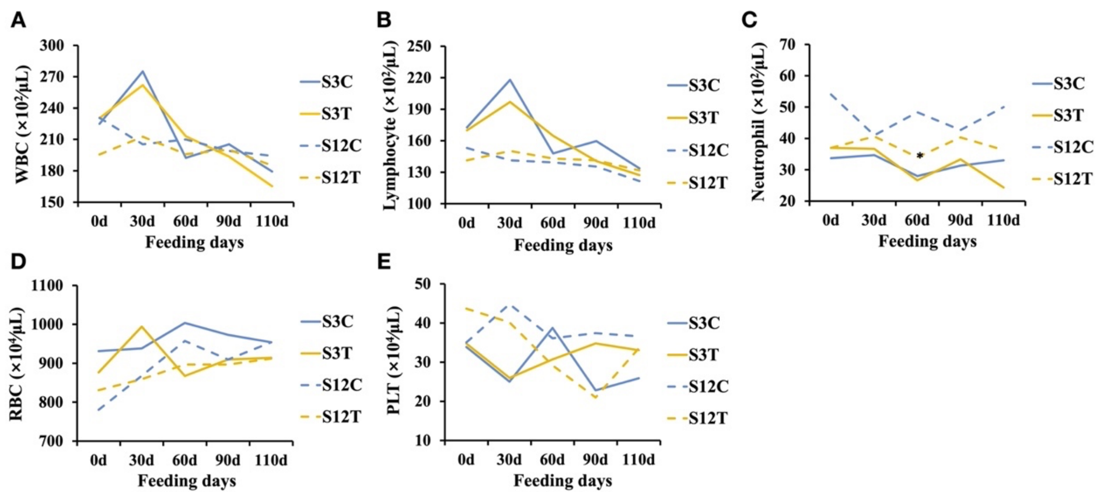


Figure 6.1. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the blood hematology of finishing pigs. All data are expressed as mean (n = 3). Asterisk on the node indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$). (**A**) the changes of the white blood cell count; (**B**) the changes of the lymphocyte count; (**C**) the changes of the neutrophil count; (**D**) the changes of the red blood cell count; (**E**) the changes of the platelet count.

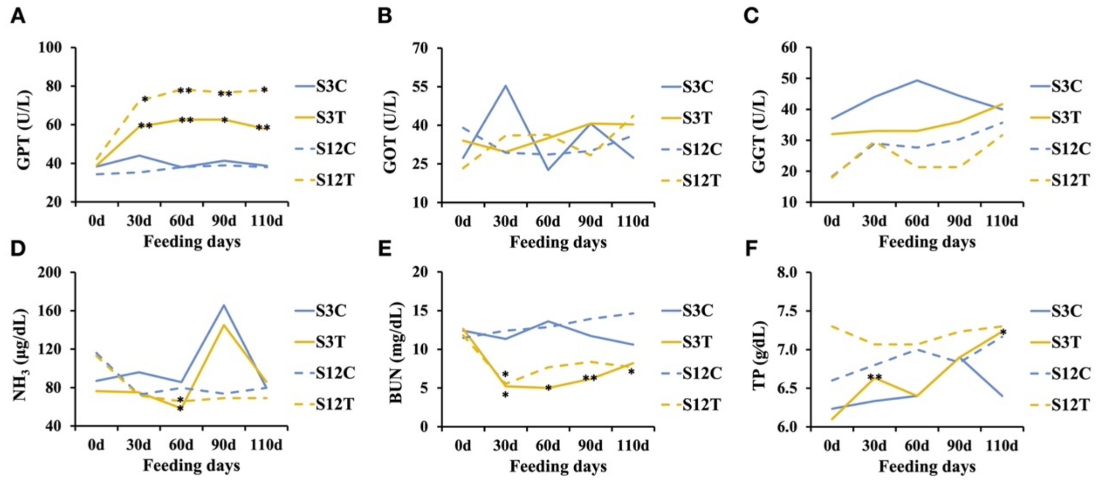


Figure 6.2. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the blood biochemistry of finishing pigs. All data are expressed as mean (n = 3). Asterisk on or near the node indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$). (**A**) the changes of the glutamic pyruvic transaminase level; (**B**) the changes of the glutamic oxaloacetic transaminase level; (**C**) the changes of the gamma glutamyl transferase level; (**D**) the changes of the blood ammonia concentration; (**E**) the changes of the urea nitrogen concentration; (**F**) the changes of the total protein concentration.

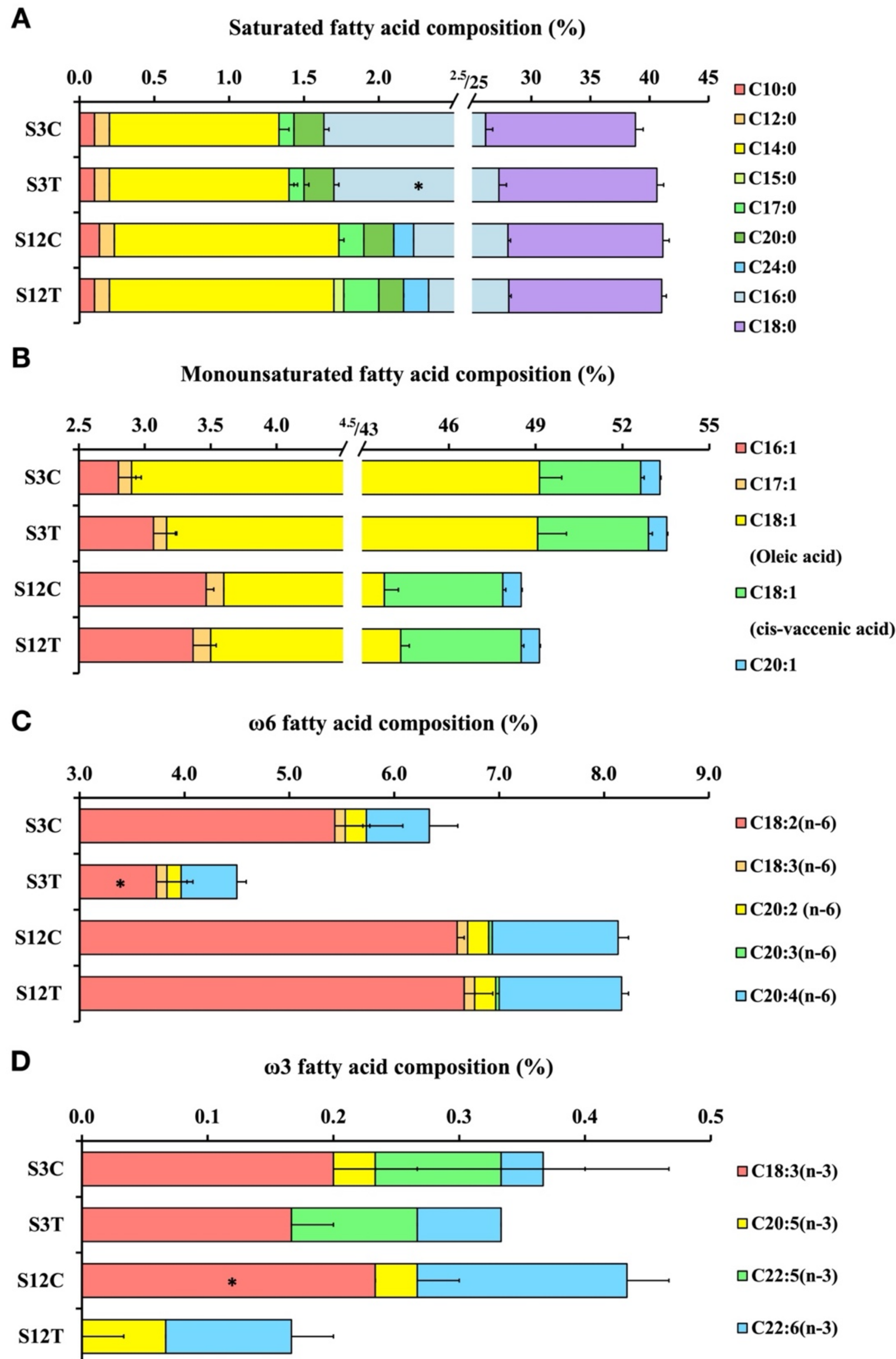


Figure 6.3. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the fatty acids composition of muscle in finishing pigs. All data are expressed as mean ± SEM (n = 3); Asterisk on bars indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$). (Due to the small value of S12T in **D**, the asterisk is marked on the bar of S12C). (**A**) the composition of saturated fatty acids; (**B**) the composition of monounsaturated fatty acids; (**C**) the composition of ω6 fatty acids s; (**D**) the composition of ω3 fatty acids.

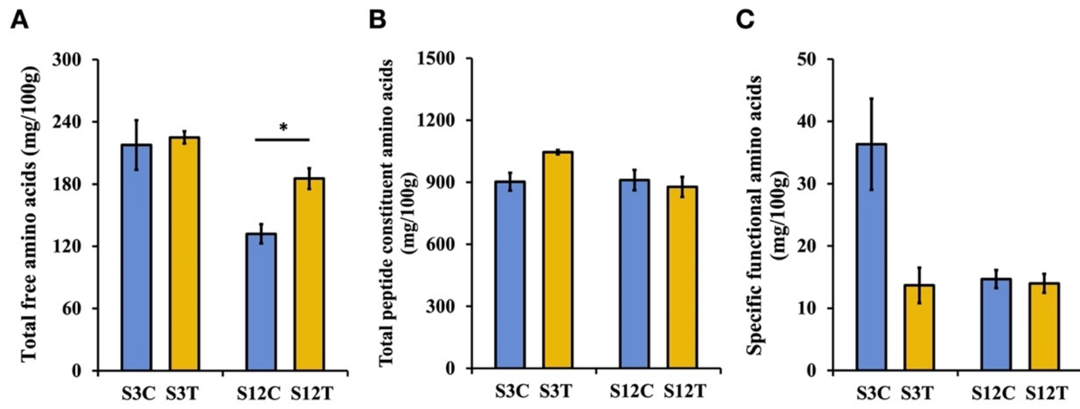


Figure 6.4. Effect of supplementing 60% chestnut shells with different silage treatments in the diet on the amino acids content of muscle in finishing pigs. All data are expressed as mean ± SEM (n = 3); Asterisk above bars indicate significant differences according to the Student's t-test (*$p < 0.05$, ** $p < 0.01$). (**A**) total free amino acids content; (**B**) total peptide constituent amino acids content; (**C**) specific functional amino acids content.

Chapter 7 General discussion

In pig farming, antibiotics are often added to the diet to protect pigs from intestinal pathogens, especially in some risk stages, such as the weaning stage of piglets, where antibiotics play an important role in preventing PWD. However, with the popularization of intensive production and the continuous expansion of production scale, antibiotic was soon regarded as essential feed additives to the smooth operation of production and improvement of productivity as well as competitiveness, and were used throughout the entire pig's development, the abuse of antibiotics has spread rapidly around the world (Kirchhelle 2018).

It has been estimated that the global average annual consumption of antibiotics per kilogram of pig production is 172 $mg \cdot kg^{-1}$, which is much higher than that of other livestock (Boeckel et al. 2015). Due to excessive antibiotics usage in the breeding industry, an emergence of multidrug-resistant microbes has been accelerated, such as resistance in E. coli and *Enterococcus* (Witte 2000). These potentially harmful microorganisms debilitate the curative effectiveness of clinically important antibiotics in human and animal medicine, threatening human health (Gong et al. 2013). Thus, our concerted efforts are urgent required to curb the current abuse of antibiotics. Under such circumstances, it is important to find effective alternatives to antibiotics. Among them, phytochemicals have been extensively studied because of their green, non-toxic, no side-effect, and non-residual characteristics.

Tannin, as a representative phytochemical, has been widely used in traditional human medicine to fight chronic diarrhea because of their abilities to prevent intestinal bacteria and parasites (Lewis and Elvin-Lewis 2003; Parisi et al. 2018). However, tannins have always been presumed to constitute anti-nutritional substances because they can precipitate proteins, inhibit digestive enzymes, and reduce nutrient use (Amarowicz 2007). In fact, there have been successful applications of tannins in pig breeding in the past. In the Mediterranean region, Iberian pigs will eat a lot of

hazelnuts rich in tannins during the grazing, thus producing high-quality meat products (Mueller-Harvey 2006). This historical application basis broke the one-sided perception of tannin as an anti-nutritional substance to a certain extent. The ideal antibiotic substitute should have the same or similar antibacterial effect as antibiotics (Huyghebaert et al. 2011). In addition to natural antibacterial effect (Scalbert 1991), tannins also have antiparasitic (Williams et al. 2014a), anti-oxidative (Rice-Evans et al. 1996), and anti-inflammatory (M. L. R. Mota et al. 1985) properties, which may help improve intestinal barrier function and health of pigs, reduce excess energy consumption thus promote growth.

It is noteworthy that for the development of resistance to antibiotics is due to mutations in genetic material which cause a specific target is modified or the formation of efflux pumps or biofilms to prevent antibiotics from entering the cell and approaching the target (Gupta and Birdi 2017). By contrast, the antimicrobial properties of tannins is attributed to its ability to combine with extracellular microbial enzymes to inhibit its activity (Hassan et al. 2020). This process has neither a specific target nor access to the inside of the cell, so it is relatively difficult to cause drug resistance. These accurately proved that tannins may offer alternatives to antibiotics for the prevention of diarrhea in early-weaned piglets.

Taking into account that tannins need to maintain structural integrity firstly to function in feed or in vivo, and secondly that the breakdown of tannins into small molecules may have toxic effects on animal livers, we selected a common, difficult-to-hydrolyze condensed tannin product, MGM-P (QT). It is known from previous experience that the antinutritional properties and toxicity of tannins are usually associated with large amounts of consumption, therefore, the feasibility of low addition concentrations of 0.2% or 0.3% was first tried. In Chapter 2 it was found that low dose addition had no negative effect on growth and was effective in alleviating the increase in leukocytes after weaning, which may be related to the relief of inflammation. As a result, it was also found to have some therapeutic effect on

diarrhea, and the effect of 0.3% MGM-P addition was found to be stronger, so the effects of higher additions (0.5% and 1.0%) were tried immediately in Chapter 3 with the addition of Flavomycin, which is often used in conventional production, as an antibiotic control group. It was found that 0.5% MGM-P supplementation showed similar functions as after antibiotic supplementation and improved feed efficiency from 0-14 d. The occurrence of diarrhea was not observed throughout the experiment, and accordingly, no effect of both levels of MGM-P supplementation on leukocyte and neutrophil counts was detected. Since the control group also did not develop diarrhea, it was considered that the slight change in the feeding environment caused the difference, such as a smaller rearing density. Aside from that, no liver or kidney damage or abnormal blood indicators were examined. Therefore, it can be determined that MGM-P supplementation up to 1.0% is safe, but the addition of higher concentrations of tannins may have somewhat affected the utilization and absorption of amino acids such as arginine in piglets, resulting in a slight disadvantage in growth performance. The presence of anti-nutritional effects leads to more protein-tannin complex macromolecules passing through the intestine, and the irritating effect on the intestine leads to higher fecal fraction. Considering the positive effects of 0.5% MGM-P supplementation in terms of feed efficiency as well as piglet health, the 0.5% addition level may be closer to the appropriate addition level.

To further verify the effect of QT on growth performance, and the safety of long-term QT supplementation, in Chapter 4 we investigated the effect of long-term supplementation with 0.5% MGM-P during the grow-finish period on the growth and health of pigs. After the addition, a significant increase in ADG was observed between the 8th and 12th week. At the end of the experiment the GOT concentrations in the blood of pigs were significantly decreased due to the addition of 0.5% MGM-P, which suggests a hepatoprotective effect of QT supplementation. Furthermore, there was a significant increase in kidney weight. All these reasons could potentially promote growth performance in pigs.

These positive results suggests that the perception of tannins as anti-nutritional factors has been one-sided for many years, and confirms the two aspects we considered: tannins type and additive amount are crucial in the practical application of tannins; the 0.5% MGM-P addition level in this study had a stage-specific effect on feed efficiency in weaned piglets and ADG in grow-finish pigs, so QT has the potential to replace antibiotic additives for modern pig farming. Further studies on the mechanism of action of QT additives are needed to confirm our findings. Furthermore, special attention should be paid as tannins research and application cases increase in the future, strict regulation and control of its key components and use may be required, as even natural substances from plants may develop drug-resistant bacteria in long-term applications.

Feed costs are a large part of the cost of farming. With urbanization and population booms worldwide, available arable land is diminishing. In this year's Global Report on Food Crises - 2021, jointly released by the Food and Agriculture Organization of the United Nations, WFP, and the European Union, it is also mentioned that the food crisis is worsening due to a series of severe climate changes resulting from the deterioration of environmental pollution problems ("World Food Programme" 2021). Against this backdrop, a large number of crops originally used for food and feed are being invested in new energy research and development industries that offer higher returns, resulting in a reduction in the number of crops used for feed and high prices. In this situation, how to meet the growing demand of livestock for food and feed with fewer resources and reduce the competition between animal and human nutrition. It is an urgent issue for current livestock practitioners to address (Pinotti et al. 2021).

Ibaraki Prefecture is the largest producer of chestnuts in Japan, and every year from early September to late October, chestnut factories discard large amounts of chestnut shells during chestnut processing. Chestnut shells have been shown by Vekiari Fields (2008) to be rich in ellagitannins, which have biological activities such

as antioxidant, anti-inflammatory, anti-malarial and anti-microbial activities (Reddy et al. 2007; Barreira et al. 2008; Bazylko et al. 2013; Granica et al. 2015). The antioxidant activity of tannins is said to contribute to the antioxidant capacity and unsaturated fatty acid ratio of pork (Decker et al. 2000). As an example, it is because Iberian pigs are fed tannin-rich hazelnuts or chestnuts during the fattening period that the high-quality Iberian pork is produced. Pugliese (2013) in a study in pigs (*Cinta Senese*), pointed out feeding with chestnuts (90%) and bran during the last month of the fattening period can help increase content of intermuscular fat and the UFA. Tomažin (2020) reported that supplemented with 3% chestnut tannin extract in diet helped delay the oxidation of pig ham. Although a few literatures mention the use of chestnuts in pig breeding, the research on the use of chestnut shells is very limited.

We therefore became interested in the reuse of chestnut by-products, hoping to produce high quality pork and promote a sustainable economy by exploring the possibility of using these wastes as feed for finishing pigs. we also tried to silage them to overcome the extremely seasonal nature of this by-product, and results showed that silage could keep the chestnut shells from decaying better, but the tannin content of the by-products decreased linearly with fermentation time.

In order to study the availability of these chestnut shells in pig farming, experiments were conducted by supplementing the feed of finishing pigs with 60% of chestnut shells. The results of feeding experiments revealed that the supplementation of frozen chestnut shells had some negative effect on the fattening performance of pigs, while the supplementation of silage chestnut shells had a small effect on the fattening performance of pigs. Because chestnut shells produce a strong fruit aroma during the silage process, feed intake increased, with a corresponding increase in concentrate intake at the same proportion. The pigs therefore gained more protein and energy, and the performance disadvantage to the control group was reduced. An improvement in feed efficiency was also observed as silage time increased, which may be due to the fact that some of the indigestible nutrients in chestnut shells are

converted to digestible nutrients during the silage process. Thus, the feeding value of silage chestnut shell has been proven in terms of both breeding cost and benefits.

Cava et al. (2000) suggested that the tannins in acorns or chestnuts could reduce lipid oxidation in Iberian pigs and thus increase the UFA content of pork. Since tannins from these nuts are found almost exclusively in the shells, feeding only chestnut shells helps to illustrate the correlation between tannins and fatty acid content in pork. Supplementation of frozen chestnut shells in this study did not cause significant changes in fatty acid content of pork, while supplementation of silage chestnut shells caused small changes but did not agree with the results of previous studies in which whole chestnuts were added, suggesting that fatty acid deposition in pork is not much related to tannins in chestnut shells and is mainly influenced by chestnut kernels. On the one hand, the starch present in abundance in chestnut kernels is an important substrate required for fatty acid metabolism, and on the other hand, the deposition of essential fatty acids, such as (C18:2 ω6) and (C18:3 ω3), in pork is mainly dependent on the direct supply of chestnut diet, since pigs cannot synthesize them themselves, whereas chestnut kernels are rich in these fatty acids (Jesús et al. 2017).

The supplementation of the diet with chestnut shells had a positive effect on the color stability and tenderness of the meat. The stability of flesh color may be related to the tannins contained in the chestnut shells, since only pigs that were fed frozen chestnut shells containing higher levels of tannins was observed to have a more stable color. As for the relationship between meat tenderness and tannins in chestnut shells, although it could not be determined, a certain correlation still could be found. Because only the pork of the frozen or short-term silage chestnut shells supplemented group was more tender, while the pork of the 12-month silage chestnut shells supplemented group with the lowest tannin content was much less tender.

Interestingly, we also found that the free amino acid content of pork increased after supplementation with frozen chestnut shells. Dietary supplementation with 3-

month silage chestnut shells did not increase the free amino acid content of muscle obviously, which seems to prove that the changes in amino acids are related to tannins in the feed. Xu et al. (2019) suggested that dietary supplementation with polyphenols promotes the expression of amino acid transporter proteins and increases the accumulation of amino acids in muscle, ultimately leading to the accumulation of muscle protein. The consistent changes in muscle free amino acid and protein content in the present study seem to support the possibility that such a mechanism exists. It is worth noting that the tannin content in the diet after supplementing 12-month silage chestnut shells and 3-month silage chestnut shells was similar, but the changes of free amino acids and protein contents in the former pork were more obvious, which could be related to the shift from unusable nitrogen-containing compounds to usable nitrogen-containing compounds during the longer silage period, and consistent with the increase in feed efficiency after silage.

The increased content of free amino acids contributes to the nutritional value of meat products, and they not only play an important role in the flavor expression of raw meat, but are also the main substrate for the Maillard reaction during cooking of meat, which is equally important for the flavor of cooked meat (Ba et al. 2012). Thus, the positive effects of adding 60% frozen chestnut shells to pig diets on pork quality was confirmed in this study, which shows that chestnut shells have a strong feeding value.

Chapter 8 Conclusion

1. This study confirms the usefulness of QT as feed additives. The 0.5% MGM-P supplement provided pigs with the right amount of QT to improve feed efficiency and health status of early-weaned piglets and promotes the growth of grow-finish pigs without affecting their health in long-term feeding, thus having the potential to replace antibiotic additives.
2. To our knowledge, this is the first study that revealed the effect of supplementing chestnut shells in diet on pork quality. The chestnut shell by-products to replace part of the feed has the ability to improve pork quality, which is Largely related to its tannin-rich content. Silage leads to a reduction in tannin content, so after silage, the ability of chestnut shells to improve pork quality is relatively diminished. However, it also has some feeding value due to the increased feed intake and feed efficiency after silage.

Acknowledgement

I have many good memories, as well as many difficulties and failures during my PhD studies and life. But I was so lucky to receive so many helps and encouragements from a lot of people. The 4.5 years have gone by in a flash, and I am upset to say goodbye to these precious people in my life.

Looking back on the past few years of Ph.D. study. I cannot concentrate on my own studies and experiments without worrying about my livelihood, without the financial support from my home country and the Japanese Ministry of Education, and the best study conditions and environment provided by my university.

But no matter how good the conditions or environment are, for a novice to learn without someone in front to guide the way is like moving forward blindfolded. So first and foremost, I want to thank my instructors, Prof. Masayoshi Kuwahara and associate Prof. Junyou Li. They are the best teachers I have ever met. It is hard to imagine that I could have successfully completed my Ph.D. study without their help. So kind they were, that my fears and apprehensions of being new to an unfamiliar environment were quickly calmed. Prof. Kuwahara will always show up wherever I need his help without hesitation. What I can hardly forget is that even at the graduation party of Japanese language course, he came specifically to support me. He gave me a pair of wings, which is the greatest freedom and the best environment for me to find and practice my favorite research without worries, and to enjoy the pursuit of truth to the fullest. There is a Japanese proverb that says, " Better than a thousand days of diligent study is one day with a great teacher," Having such a precious opportunity to be one of his students is so honored in my life.

I spend most of my time on the ranch because of experiments. And this is the luckiest thing that I think I have. A very average person can suddenly be mentored by two excellent teachers from the top university in Japan. Although China and Japan are both in the East Asian cultural circle, there are still cultural differences in many

aspects, leading to inevitable misunderstandings in my interactions with others. At such moment a lot of help usually can be received from associate Prof. Li, who not only guided my experiments carefully with his extensive knowledge and great enthusiasm for research, but also proficient in the culture and language of China, Japan, and Korea, and gave me a great deal help and care in life. In the Book of Rites, it is said that a teacher is also a person who teaches things and explains the virtues. Associate Prof. Li taught me not only knowledge and experimental skills, but also how to break through and realize my own value. I can always be inspired by his working attitude and his lifestyle.

My greatest thankfulness also goes to associate Prof. Sekizawa, an elegant gentleman with well-spoken and enchanting smile, gave me great encouragement and help when I was very worried about my entrance exam.

I don’t know how to express my gratitude to everyone at the ranch, especially the workers in the pig house, Mr. izuka, Mr. Hasegawa, Mr. Ikeda, Mr. Enomoto and Mr. Takahashi. Without their support, I would never have been able to finish my graduation experiment, they are my colleagues as well as my friends.

I am also highly indebted to Kawamura Ltd. for their financial support and associate Prof. Kazuyuki Uchida, assistant Prof. James K. Chambers for their substantial contributions to our research.

Finally, I would like to thank my dear family. My parents have always done their best to support me, to give me the most encouragement when I am lost, and most importantly, they have given me life that allows me to write these words of thanks at this moment. Food is the best way to heal the soul. I would also like to thank my wife, although not good at words, but she can always use her food to relieve my attention from the current troubles and discover the charm of life.

The sudden "COVID-19" pandemic is a great test for people. Many innocent lives were brutally taken away. So here I would like to offer my condolences to those who have passed away, and to express my gratitude to healthcare workers who are

still on the front lines of epidemic prevention, and all researchers who are still working on vaccines and therapeutic drugs.

References

Aaslyng, M. D., C. Bejerholm, P. Ertbjerg, H. C. Bertram, and H. J. Andersen. 2003. Cooking loss and juiciness of pork in relation to raw meat quality and cooking procedure. Food Quality and Preference 14(4):277–288.

Adjiri-Awere, A., and T. A. Van Lunen. 2005. Subtherapeutic use of antibiotics in pork production: risks and alternatives. Canadian Journal of Animal Science 85(2):117–130.

Adnan, S.-N.-A., N. Ibrahim, and W. A. Yaacob. 2017. Disruption of methicillin-resistant Staphylococcus aureus protein synthesis by tannins. Germs 7(4):186–192.

Ahmed, S. T., H.-S. Mun, Md. M. Islam, S.-Y. Ko, and C.-J. Yang. 2016. Effects of dietary natural and fermented herb combination on growth performance, carcass traits and meat quality in grower-finisher pigs. Meat Science 122:7–15.

Aldridge, D. R., E. J. Tranah, and D. L. Shawcross. 2015. Pathogenesis of hepatic encephalopathy: role of ammonia and systemic inflammation. Journal of Clinical and Experimental Hepatology 5(Sup1):S7–S20.

Alternatives to Antibiotics. 2019. . https://www.ars.usda.gov/alternativestoantibiotics/Symposium2019/index.html.

Amarowicz, R. 2007. Tannins: the new natural antioxidants. European Journal of Lipid Science and Technology 109(6):549–551.

Anjorin, T. S., E. A. Salako, and H. A. Makun. 2013. Control of toxigenic fungi and mycotoxins with phytochemicals: potentials and challenges. Pages 181–202 *in* Hussaini Makun, editor. Mycotoxin and Food Safety in Developing Countries. IntechOpen.

Ao, X., L. Yan, Q. W. Meng, T. X. Zhou, J. P. Wang, H. J. Kim, J. H. Cho, and I. H. Kim. 2011. Effects of saururus chinensis extract supplementation on growth performance, meat quality and slurry noxious gas emission in finishing pigs. Livestock Science 138(1):187–192.

Arbenz, A., and L. Avérous. 2015. Chemical modification of tannins to elaborate aromatic biobased macromolecular architectures. Green Chemistry 17(5):2626–2646.

Ba, H. V., I. Hwang, D. Jeong, and A. Touseef. 2012. Principle of Meat Aroma Flavors and Future Prospect. IntechOpen.

Baas, T., and P. Thacker. 1996. Impact of gastric pH on dietary enzyme activity and survivability in swine fed β-glucanase supplemented diets. Canadian Journal of Animal Science 76(2):245–252.

Bachmanov, A. A., N. P. Bosak, J. I. Glendinning, M. Inoue, X. Li, S. Manita, S. A. McCaughey, Y. Murata, D. R. Reed, M. G. Tordoff, and G. K. Beauchamp. 2016. Genetics of amino acid taste and appetite. Advances in Nutrition 7(4):806S-822S.

Bahelka, I., O. Bučko, and P. Fľak. 2021. Can Hydrolysable Tannins in Diet of Entire Male Pigs Affect Carcass, Pork Quality Traits, Amino and Fatty Acid Profiles, and Boar Taint, Skatole and Androstenone Levels? Animals 11(3):896.

Banso, A., and S. O. Adeyemo. 2007. Evaluation of antibacterial properties of tannins isolated from *Dichrostachys cinerea*. African Journal of Biotechnology 6(15):1785–1787.

Bao, X., S. Ma, Y. Fu, J. Wu, and M. Zhang. 2020. Sensory and structural characterization of umami peptides derived from sunflower seed. CyTA - Journal of Food 18(1):485–492.

Barbehenn, R. V., and C. Peter Constabel. 2011. Tannins in plant–herbivore interactions. Phytochemistry 72(13):1551–1565.

Barone, A. M., M. Banovic, D. Asioli, E. Wallace, C. Ruiz-Capillas, and S. Grasso. 2021. The usual suspect: how to co-create healthier meat products. Food Research International 143:110304.

Barreira, J. C. M., S. Casal, I. C. F. R. Ferreira, M. B. P. P. Oliveira, and J. A. Pereira. 2009. Nutritional, fatty acid and triacylglycerol profiles of castanea sativa mill. cultivars: a compositional and chemometric approach. Journal of Agricultural and Food Chemistry 57(7):2836–2842.

Barreira, J. C. M., I. C. F. R. Ferreira, M. B. P. P. Oliveira, and J. A. Pereira. 2008. Antioxidant activities of the extracts from chestnut flower, leaf, skins and fruit. Food Chemistry 107(3):1106–1113.

Barry, T. N. 1989. Condensed tannins their role in ruminant protein and carbohydrate digestion and possible effects upon the rumen ecosystem. Pages 153–169 *in* J. V. Nolan, R. A. Leng, and D. I. Demeyer, editors. The Roles of Protozoa and Fungi in Ruminant Digestion. Penambul Books, Armidale, NSW, Australia.

Bazylko, A., J. P. Piwowarski, A. Filipek, J. Bonarewicz, and M. Tomczyk. 2013. In vitro antioxidant and anti-inflammatory activities of extracts from Potentilla recta and its main ellagitannin, agrimoniin. Journal of Ethnopharmacology 149(1):222–227.

Beauchemin K. A., McGinn S. M., Martinez T. F., and McAllister T. A. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. Journal of Animal Science 85(8):1990–1996.

Bělková, J., E. Václavková, M. Rozkot, and S. Kuchařová. 2017. Acorns and chestnuts as important commodities in organic pig farming. Research in Pig Breeding 11(1):7–12.

Bellotti, N., C. Deyá, B. del Amo, and R. Romagnoli. 2012. "Quebracho" tannin derivative and boosters biocides for new antifouling formulations. Journal of Coatings Technology and Research 9(5):551–559.

Biagi, G., I. Cipollini, B. R. Paulicks, and F. X. Roth. 2010. Effect of tannins on growth performance and intestinal ecosystem in weaned piglets. Archives of Animal Nutrition 64(2):121–135.

Bilić-Šobot, D., V. Kubale, M. Škrlep, M. Čandek-Potokar, M. P. Povše, G. Fazarinc, and D. Škorjanc. 2016. Effect of hydrolysable tannins on intestinal morphology, proliferation and apoptosis in entire male pigs. Archives of Animal Nutrition 70(5):378–388.

Biswas, S. K. 2016. Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? Oxidative Medicine and Cellular Longevity 2016:5698931.

Boeckel, T. P. V., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences 112(18):5649–5654.

de Boer, H., C. Vongsombath, K. Pålsson, L. Björk, and T. G. T. Jaenson. 2010. Botanical repellents and pesticides traditionally used against hematophagous invertebrates in lao people's democratic republic: a comparative study of plants used in 66 villages. Journal of Medical Entomology 47(3):400–414.

van den Bogaard, A. E., M. Hazen, M. Hoyer, P. Oostenbach, and E. E. Stobberingh. 2002. Effects of flavophospholipol on resistance in fecal escherichia coli and enterococci of fattening pigs. Antimicrobial Agents and Chemotherapy 46(1):110–118.

Bolling, B. W., D. L. McKay, and J. B. Blumberg. 2010. The phytochemical composition and antioxidant actions of tree nuts. Asia Pacific journal of clinical nutrition 19(1):117–123.

Bone, K., and S. Mills, editors. 2013. 2 - Principles of herbal pharmacology. Pages 17–82 Principles and Practice of Phytotherapy (Second Edition). Churchill Livingstone, Saint Louis.

Bottoms, G. D., O. F. Roesel, F. D. Rausch, and E. L. Akins. 1972. Circadian variation in plasma cortisol and corticosterone in pigs and mares. American Journal of Veterinary Research 33(4):785–790.

Bottos L. C. 2019. Race and process: certifying iberian pigs and invisibilising humans. Norsk antropologisk tidsskrift 30(03–04):258–273.

Brillouet, J.-M., C. Romieu, B. Schoefs, K. Solymosi, V. Cheynier, H. Fulcrand, J.-L. Verdeil, and G. Conéjéro. 2013. The tannosome is an organelle forming condensed tannins in the chlorophyllous organs of Tracheophyta. Annals of Botany 112(6):1003–1014.

Buccioni, A., M. Pauselli, S. Minieri, V. Roscini, F. Mannelli, S. Rapaccini, P. Lupi, G. Conte, A. Serra, A. Cappucci, L. Brufani, F. Ciucci, and M. Mele. 2017. Chestnut or quebracho tannins in the diet of grazing ewes supplemented with soybean oil: effects on animal performances, blood parameters and fatty acid composition of plasma and milk lipids. Small Ruminant Research 153:23–30.

Butaye, P., L. A. Devriese, and F. Haesebrouck. 2003. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. Clinical Microbiology Reviews 16(2):175–188.

Cairo, P. L. G., F. D. Gois, M. Sbardella, H. Silveira, R. M. de Oliveira, I. B. Allaman, V. S. Cantarelli, and L. B. Costa. 2018. Effects of dietary supplementation of red pepper (Schinus terebinthifolius Raddi) essential oil on performance, small intestinal morphology and microbial counts of weanling pigs. Journal of the Science of Food and Agriculture 98(2):541–548.

Callaway, T. R., H. Lillehoj, R. Chuanchuen, and C. G. Gay. 2021. Alternatives to antibiotics: a symposium on the challenges and solutions for animal health and production. Antibiotics 10(5):471.

Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. Journal of Animal Science and Biotechnology 4(1):19.

Caprarulo, V., C. Giromini, and L. Rossi. 2021. Review: Chestnut and quebracho tannins in pig nutrition: the effects on performance and intestinal health. Animal 15(1):100064.

Caprarulo, V., M. Hejna, C. Giromini, Y. Liu, M. Dell'Anno, S. Sotira, S. Reggi, C. A. Sgoifo-Rossi, M. L. Callegari, and L. Rossi. 2020. Evaluation of dietary administration of chestnut and quebracho tannins on growth, serum metabolites and fecal parameters of weaned piglets. Animals 10(11):1945.

Carlson, M. S., G. M. Hill, and J. E. Link. 1999. Early- and traditionally weaned nursery pigs benefit from phase-feeding pharmacological concentrations of zinc oxide: effect on metallothionein and mineral concentrations. Journal of Animal Science 77(5):1199–1207.

Castro-Montoya, J., E. Westreicher-Kristen, A. Henke, M. Diaby, A. Susenbeth, and U. Dickhoefer. 2018. In vitro microbial protein synthesis, ruminal degradation and post-ruminal digestibility of crude protein of dairy rations containing Quebracho tannin extract. Journal of Animal Physiology and Animal Nutrition 102(1):e77–e86.

Cava, R., J. Ventanas, J. Florencio Tejeda, J. Ruiz, and T. Antequera. 2000. Effect of free-range rearing and α-tocopherol and copper supplementation on fatty acid profiles and susceptibility to lipid oxidation of fresh meat from Iberian pigs. Food Chemistry 68(1):51–59.

Chauhan, S. S., and E. M. England. 2018. Postmortem glycolysis and glycogenolysis: insights from species comparisons. Meat Science 144:118–126.

Chen, D.-W., and M. Zhang. 2007. Non-volatile taste active compounds in the meat of Chinese mitten crab (Eriocheir sinensis). Food Chemistry 104(3):1200–1205.

Chen, J., B. Kang, K. Yao, C. Fu, and Y. Zhao. 2019a. Effects of dietary Macleaya cordata extract on growth performance, immune responses, antioxidant capacity, and intestinal development in weaned piglets. Journal of Applied Animal Research 47(1):349–356.

Chen, S., B. Tan, Y. Xia, S. Liao, M. Wang, J. Yin, J. Wang, H. Xiao, M. Qi, P. Bin, G. Liu, W. Ren, and Y. Yin. 2019b. Effects of dietary gamma-aminobutyric acid supplementation on the intestinal functions in weaning piglets. Food & Function 10(1):366–378.

Chen, X., Z. Zeng, Z. Huang, D. Chen, J. He, H. Chen, B. Yu, J. Yu, J. Luo, Y. Luo, and P. Zheng. 2021. Effects of dietary resveratrol supplementation on immunity, antioxidative capacity and intestinal barrier function in weaning piglets. Animal Biotechnology 32(2):240–245.

Cheng, G., H. Hao, S. Xie, X. Wang, M. Dai, L. Huang, and Z. Yuan. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Frontiers in Microbiology 5:217.

Chengwen, S. U., X. U. Xiangting, and Z. Song. 2016. Study on quebracho tannin on production performance and immunity of nursing pigs. China Feed (21):23–32.

China, C. R., S. S. Nyandoro, J. J. E. Munissi, M. M. Maguta, M. Meyer, and M. Schroepfer. 2021. Tanning capacity of Tessmannia burttii extracts: the potential eco-friendly tanning agents for the leather industry. Journal of Leather Science and Engineering 3(1):13.

Chu, X., H. Wang, Y. Jiang, Y. Zhang, Y. Bao, X. Zhang, J. Zhang, H. Guo, F. Yang, Y. Luan, and Y. Dong. 2016. Ameliorative effects of tannic acid on carbon tetrachloride-induced liver fibrosis in vivo and in vitro. Journal of Pharmacological Sciences 130(1):15–23.

Cirkovic Velickovic, T. D., and D. J. Stanic-Vucinic. 2018. The role of dietary phenolic compounds in protein digestion and processing technologies to improve their antinutritive properties. Comprehensive Reviews in Food Science and Food Safety 17(1):82–103.

Clifford, M. N., J. J. van der Hooft, and A. Crozier. 2013. Human studies on the absorption, distribution, metabolism, and excretion of tea polyphenols. The American Journal of Clinical Nutrition 98(6):1619S-1630S.

Coates M. E., Davies M. K., and Kon S. K. 1955. The effect of antibiotics on the intestine of the chick. British Journal of Nutrition 9(1):110–119.

Coates, M. E., R. Fuller, G. F. Harrison, M. Lev, and S. F. Suffolk. 1963. A comparision of the growth of chicks in the Gustafsson germ-free apparatus and

in a conventional environment, with and without dietary supplements of penicillin. British Journal of Nutrition 17(1):141–150.

Cowan, M. M. 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews 12(4):564–582.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production. Animal Biotechnology 13(1):7–27.

Czech, A., E. Grela, R. Klebaniuk, K. Ognik, and W. Samolińska. 2018. Polish crossbred pigs' blood haematological parameters depending on their age and physiological state. Annals of Warsaw University of Life Sciences - SGGW - Animal Science 56(2):185–195.

Dahmer, P. L., and C. K. Jones. 2021. Evaluating dietary acidifiers as alternatives for conventional feed-based antibiotics in nursery pig diets. Translational Animal Science 5(2):txab040.

Davis M. E., Sears S. C., Apple J. K., Maxwell C. V., and Johnson Z. B. 2006. Effect of weaning age and commingling after the nursery phase of pigs in a wean-to-finish facility on growth, and humoral and behavioral indicators of well-being,. Journal of Animal Science 84(3):743–756.

Dealy, J., and M. W. Moeller. 1976. Influence of bambermycin on salmonella infectiion and antibiotic resistance in swine. Journal of Animal Science 42(5):1331–1336.

Decker, E. A., C. Faustman, and C. J. Lopez-Bote. 2000. Antioxidants in Muscle Foods: Nutritional Strategies to Improve Quality. John Wiley & Sons, New York.

Dell'Agli, M., G. V. Galli, Y. Corbett, D. Taramelli, L. Lucantoni, A. Habluetzel, O. Maschi, D. Caruso, F. Giavarini, S. Romeo, D. Bhattacharya, and E. Bosisio.

2009. Antiplasmodial activity of Punica granatum L. fruit rind. Journal of Ethnopharmacology 125(2):279–285.

Delompré, T., E. Guichard, L. Briand, and C. Salles. 2019. Taste perception of nutrients found in nutritional supplements: a review. Nutrients 11(9):2050.

Díaz, O., C. Ros, A. Veiga, and Á. Cobos. 2009. Including chestnuts and sugar beet pulp in diets for pigs: the effects on the quality of pork meat and the sensory properties of dry-cured sausage (chorizo gallego). Journal of Muscle Foods 20(4):449–464.

Domínguez, R., S. Martínez, M. Gómez, J. Carballo, and I. Franco. 2015. Fatty acids, retinol and cholesterol composition in various fatty tissues of celta pig breed: effect of the use of chestnuts in the finishing diet. Journal of Food Composition and Analysis 37:104–111.

Dong, G., H. Liu, X. Yu, X. Zhang, H. Lu, T. Zhou, and J. Cao. 2018. Antimicrobial and anti-biofilm activity of tannic acid against Staphylococcus aureus. Natural Product Research 32(18):2225–2228.

Dransfield, E., T. M. Ngapo, N. A. Nielsen, L. Bredahl, P. O. Sjödén, M. Magnusson, M. M. Campo, and G. R. Nute. 2005. Consumer choice and suggested price for pork as influenced by its appearance, taste and information concerning country of origin and organic pig production. Meat Science 69(1):61–70.

Dubreuil, J. D. 2013. Antibacterial and antidiarrheal activities of plant products against enterotoxinogenic Escherichia coli. Toxins 5(11):2009–2041.

Dw, W., S. Rj, O. St, J. Do, Z. Tr, and W. Xd. 1988. The gut: a central organ after surgical stress. Surgery 104(5):917–923.

Echegaray, N., P. E. S. Munekata, J. A. Centeno, R. Domínguez, M. Pateiro, J. Carballo, and J. M. Lorenzo. 2020a. Total phenol content and antioxidant

activity of different celta pig carcass locations as affected by the finishing diet (chestnuts or commercial feed). Antioxidants 10(1):5.

Echegaray, N., M. Paterio, R. Domínguez, L. Purriños, R. Bermúdez, J. Carballo, and J. M. Lorenzo. 2020b. Effects of different cooking methods and of the inclusion of chestnut (Castanea sativa Miller) in the finishing diet of Celta pig breed on the physicochemical parameters and volatile profile of Longissimus thoracis et lumborum muscle. Food Research International 137:109407.

Ekakoro, J. E., M. Caldwell, E. B. Strand, and C. C. Okafor. 2019. Perceptions of Tennessee cattle producers regarding the Veterinary Feed Directive. PLOS ONE 14(5):e0217773.

Elizondo, A. M., E. C. Mercado, B. C. Rabinovitz, and M. E. Fernandez-Miyakawa. 2010. Effect of tannins on the in vitro growth of Clostridium perfringens. Veterinary Microbiology 145(3):308–314.

Etheridge, R. D., R. W. Seerley, and R. D. Wyatt. 1984. The effect of diet on performance, digestibility, blood composition and intestinal microflora of weaned pigs. Journal of Animal Science 58(6):1396–1402.

Etuk, E. B., N. J. Okeudo, B. O. Esonu, and A. B. I. Udedibie. 2012. Antinutritional factors in sorghum: chemistry, mode of action and effects on livestock and poultry. Online Journal of Animal and Feed Research 2(2):113–119.

Exton, J. H., L. E. Mallette, L. S. Jefferson, E. H. A. Wong, N. Friedmann, T. B. Miller, and C. R. Park. 1970. The hormonal control of hepatic gluconeogenesis. Pages 411–461 *in* E. B. Astwood, editor. Proceedings of the 1969 Laurentian Hormone Conference. Academic Press, Boston.

Fairbrother, J. M., É. Nadeau, and C. L. Gyles. 2005. Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Animal Health Research Reviews 6(1):17–39.

Falcão, L., and M. E. M. Araújo. 2018. Vegetable tannins used in the manufacture of historic leathers. Molecules 23(5):1081.

Feiner, G. 2006. 4 - Definitions of terms used in meat science and technology. Pages 46–71 *in* G. Feiner, editor. Meat Products Handbook. Woodhead Publishing.

Felig, P. 1973. The glucose-alanine cycle. Metabolism - Clinical and Experimental 22(2):179–207.

Fernandes, Â., A. L. Antonio, L. Barros, J. C. M. Barreira, A. Bento, M. L. Botelho, and I. C. F. R. Ferreira. 2011. Low dose γ-irradiation as a suitable solution for chestnut (Castanea sativa Miller) conservation: effects on sugars, fatty acids, and tocopherols. Journal of Agricultural and Food Chemistry 59(18):10028–10033.

Fiesel, A., D. K. Gessner, E. Most, and K. Eder. 2014. Effects of dietary polyphenol-rich plant products from grape or hop on pro-inflammatory gene expression in the intestine, nutrient digestibility and faecal microbiota of weaned pigs. BMC Veterinary Research 10:196.

Filippich, L. J., J. Zhu, P. Oelrichs, M. T. Alsalami, A. J. Doig, G. R. Cao, and P. B. English. 1991. Hepatotoxic and nephrotoxic principles in terminalia oblongata. Research in Veterinary Science 50(2):170–177.

Fournier, B. M., and C. A. Parkos. 2012. The role of neutrophils during intestinal inflammation. Mucosal Immunology 5(4):354–366.

Franklin, M. A., A. G. Mathew, J. R. Vickers, and R. A. Clift. 2002. Characterization of microbial populations and volatile fatty acid concentrations in the jejunum,

ileum, and cecum of pigs weaned at 17 vs 24 days of age. Journal of Animal Science 80(11):2904–2910.

Freeman, B. M., A. C. C. Manning, G. F. Harrison, and M. E. Coates. 1975. Dietary Aureomycin and the response of the fowl to stressors. British Poultry Science 16(4):395–404.

Freudenberg, K. 1920. Die Chemie der Natürlichen Gerbstoffe. Springer, Berlin, Heidelberg.

Fruet, A. P. B., F. M. Giotto, M. A. Fonseca, J. L. Nörnberg, and A. S. De Mello. 2020. Effects of the incorporation of tannin extract from quebracho colorado wood on color parameters, lipid oxidation, and sensory attributes of beef patties. Foods 9(5):667.

Frutos, P., G. Hervás, F. J. Giráldez, M. Fernández, and A. R. Mantecón. 2000. Digestive utilization of quebracho-treated soya bean meals in sheep. The Journal of Agricultural Science 134(1):101–108.

Frutos, P., G. Hervás, A. Natalello, G. Luciano, M. Fondevila, A. Priolo, and P. G. Toral. 2020. Ability of tannins to modulate ruminal lipid metabolism and milk and meat fatty acid profiles. Animal Feed Science and Technology 269:114623.

Fujimoto, D., and S. Kanaya. 1973. Cuticlin: a noncollagen structural protein from Ascaris cuticle. Archives of Biochemistry and Biophysics 157(1):1–6.

G. Papatsiros, V. PapatsirosV., and C. Billinis. 2012. The prophylactic use of acidifiers as antibacterial agents in swine. Pages 295–310 *in* V. Bobbarala, editor. Antimicrobial Agents. IntechOpen.

Galassi, G., S. Colombini, L. Malagutti, G. M. Crovetto, and L. Rapetti. 2010. Effects of high fibre and low protein diets on performance, digestibility, nitrogen

excretion and ammonia emission in the heavy pig. Animal Feed Science and Technology 161(3):140–148.

Galassi, G., F. Mason, L. Rapetti, G. M. Crovetto, and M. Spanghero. 2019. Digestibility and metabolic utilisation of diets containing chestnut tannins and their effects on growth and slaughter traits of heavy pigs. Italian Journal of Animal Science 18(1):746–753.

Garg, S. K., H. P. Makkar, K. B. Nagal, S. K. Sharma, D. R. Wadhwa, and B. Singh. 1992. Oak (Quercus incana) leaf poisoning in cattle. Veterinary and Human Toxicology 34(2):161–164.

Gaskins, H. R., C. T. Collier, and D. B. Anderson. 2002. Antibiotics as growth promotants:mode of action. Animal Biotechnology 13(1):29–42.

Gaskins, H. R., and K. W. Kelley. 1995. Immunology and neonatal mortality. Pages 39–55 *in* M. A. Varley, editor. The Neonatal Pig. Development and Survival. Oxon: CAB International, Wallingford.

Gessner, D. K., M. Bonarius, E. Most, A. Fiesel, and K. Eder. 2017. Effects of polyphenol-rich plant products from grape or hop as feed supplements on the expression of inflammatory, antioxidative, cytoprotective and endoplasmic reticulum stress-related genes and the antioxidative status in the liver of piglets. Journal of Animal Physiology and Animal Nutrition 101(5):e185–e194.

Girard M., and Bee G. 2020. Invited review: tannins as a potential alternative to antibiotics to prevent coliform diarrhea in weaned pigs. animal 14(1):95–107.

Gong, J., F. Yin, Y. Hou, and Y. Yin. 2013. Review: chinese herbs as alternatives to antibiotics in feed for swine and poultry production: potential and challenges in application. Canadian Journal of Animal Science 94(2):223–241.

Granica, S., J. P. Piwowarski, and A. K. Kiss. 2015. Ellagitannins modulate the inflammatory response of human neutrophils ex vivo. Phytomedicine 22(14):1215–1222.

Grenier, B., and T. J. Applegate. 2013. Modulation of intestinal functions following mycotoxin ingestion: meta-analysis of published experiments in animals. Toxins 5(2):396–430.

Gullon Patricia, Eibes Gemma, Davila Izaskun, Moreira Maria T., Labidi Jalel, and Gullon Beatriz. 2018. Manufacture of nutraceutical compounds from chestnut shells by hydrothermal processing. Chemical Engineering Transactions 70:1705–1710.

Gupta, P. D., and T. J. Birdi. 2017. Development of botanicals to combat antibiotic resistance. Journal of Ayurveda and Integrative Medicine 8(4):266–275.

Gxasheka, M., T. Tyasi, N. Qin, and Z.-C. Lyu. 2015. An overview of tannins rich plants as alternative supplementation on ruminant animals: a review. International Journal of Agricultural Research and Review 3:343–349.

Hancock, R. E. 2001. Cationic peptides: effectors in innate immunity and novel antimicrobials. The Lancet Infectious Diseases 1(3):156–164.

Hancock, R. E. W., and H.-G. Sahl. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nature Biotechnology 24(12):1551–1557.

Harper, A. F., E. T. Kornegay, K. L. Bryant, and H. R. Thomas. 1983. Efficacy of virginiamycin and a commercially-available lactobacillus probiotic in swine diets. Animal Feed Science and Technology 8(1):69–76.

Hashemi, S. R., and H. Davoodi. 2011. Herbal plants and their derivatives as growth and health promoters in animal nutrition. Veterinary Research Communications 35(3):169–180.

Haslam, E. 1989. Plant Polyphenols: Vegetable Tannins Revisited. Cambridge University Press, Cambridge.

Hassan, Z. M., T. G. Manyelo, L. Selaledi, and M. Mabelebele. 2020. The effects of tannins in monogastric animals with special reference to alternative feed ingredients. Molecules 25(20):4680.

Hayes D. J., Jensen H. H., and Fabiosa J. F. 2000. Economic Impact of a Ban on Use of Antibiotics in U.S. Swine Rations. Swine Research Report 1999. 17.

He, L., W. Zhou, Y. Wang, C. Wang, X. Chen, and Q. Zhang. 2018. Effect of applying lactic acid bacteria and cellulase on the fermentation quality, nutritive value, tannins profile and in vitro digestibility of Neolamarckia cadamba leaves silage. Journal of Animal Physiology and Animal Nutrition 102(6):1429–1436.

Hedemann, M. S., S. Højsgaard, and B. B. Jensen. 2003. Small intestinal morphology and activity of intestinal peptidases in piglets around weaning. Journal of Animal Physiology and Animal Nutrition 87(1–2):32–41.

Henry R. W., Pickard D. W., and Hughes P. E. 1985. Citric acid and fumaric acid as food additives for early-weaned piglets. Animal Science 40(3):505–509.

Heo, J. M., F. O. Opapeju, J. R. Pluske, J. C. Kim, D. J. Hampson, and C. M. Nyachoti. 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. Journal of Animal Physiology and Animal Nutrition 97(2):207–237.

Hoste, H., F. Jackson, S. Athanasiadou, Stig. M. Thamsborg, and S. O. Hoskin. 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends in Parasitology 22(6):253–261.

Hu, M., X. Yang, and X. Chang. 2021. Bioactive phenolic components and potential health effects of chestnut shell: a review. Journal of Food Biochemistry 45(4):e13696.

Huang, C., S. Qiao, D. Li, X. Piao, and J. Ren. 2004. Effects of lactobacilli on the performance, diarrhea incidence, VFA concentration and gastrointestinal microbial flora of weaning pigs. Asian-Australasian Journal of Animal Sciences 17(3):401–409.

Huang, Q., X. Liu, G. Zhao, T. Hu, and Y. Wang. 2018. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. Animal Nutrition 4(2):137–150.

Huang, X.-J., Y.-K. Choi, H.-S. Im, O. Yarimaga, E. Yoon, and H.-S. Kim. 2006. Aspartate aminotransferase (ast/got) and alanine aminotransferase (alt/gpt) detection techniques. Sensors (Basel, Switzerland) 6(7):756–782.

Huff-Lonergan, E., and S. M. Lonergan. 2005. Mechanisms of water-holding capacity of meat: the role of postmortem biochemical and structural changes. Meat Science 71(1):194–204.

Huyghebaert, G., R. Ducatelle, and F. V. Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal 187(2):182–188.

Inserra, L., G. Luciano, M. Bella, M. Scerra, C. Cilione, P. Basile, M. Lanza, and A. Priolo. 2015. Effect of including carob pulp in the diet of fattening pigs on the

fatty acid composition and oxidative stability of pork. Meat Science 100:256–261.

Jansman, A. J. M. 1993. Tannins in feedstuffs for simple-stomached animals. Nutrition Research Reviews 6(1):209–236.

Jayanegara, A., T. U. P. Sujarnoko, M. Ridla, M. Kondo, and M. Kreuzer. 2019. Silage quality as influenced by concentration and type of tannins present in the material ensiled: a meta-analysis. Journal of Animal Physiology and Animal Nutrition 103(2):456–465.

Jerez, M., S. Touriño, J. Sineiro, J. L. Torres, and M. J. Núñez. 2007. Procyanidins from pine bark: relationships between structure, composition and antiradical activity. Food Chemistry 104(2):518–527.

Jesús, C. D., R. Domínguez, J. Cantalapiedra, A. Iglesias, and J. M. L. Rodríguez. 2016. Effect of chestnuts level in the formulation of the commercial feed on carcass characteristics and meat quality of Celta pig breed. Spanish journal of agricultural research 14(2):7.

Jesús, M. C. de, R. Domínguez, J. Cantalapiedra, A. Iglesias, and J. M. Lorenzo. 2017. Effect the inclusion of chestnut in feed formulation on carcass and meat quality of industrial pig. ITEA 113(1):36–51.

Ježek, J. 2018. The influence of age, farm, and physiological status on pig hematological profiles. Journal of Swine Health and Production 26(2):72–78.

Joo, Y. H., I. H. Choi, D. H. Kim, H. J. Lee, S. M. Amanullah, H. S. Yang, S. C. Kim, Y. H. Joo, I. H. Choi, D. H. Kim, H. J. Lee, S. M. Amanullah, H. S. Yang, and S. C. Kim. 2018. Effects of chestnut (Castanea sativa) meal supplementation on growth performance, carcass characteristics, and meat quality of pigs. Revista Brasileira de Zootecnia 47.

Jouany, J.-P., and D. P. Morgavi. 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. animal 1(10):1443–1466.

Juhász, Á., Z. Mayer, V. Szentpéteri, and K. Posta. 2019. Effect of feed supplemented with plant extract and zinc oxide on coliform and lactic acid bacteria in pigs. Pages 130–131 Abstract book of the 18th Alps-Adria Scientific Workshop. Szent István Egyetemi Kiadó Nonprofit Kft.

Khanbabaee, K., and T. van Ree. 2001. Tannins: classification and definition. Natural Product Reports 18(6):641–649.

Khanyile, M., S. P. Ndou, and M. Chimonyo. 2017. Influence of Acacia tortilis leaf meal-based diet on serum biochemistry, carcass characteristics and internal organs of finishing pigs. Animal Production Science 57(4):675–682.

Khondker, A., and M. C. Rheinstädter. 2020. How do bacterial membranes resist polymyxin antibiotics? Communications Biology 3(1):1–4.

Kil, D. Y., W. B. Kwon, and B. G. Kim. 2011. Dietary acidifiers in weanling pig diets: a review. Revista Colombiana de Ciencias Pecuarias 24(3):231–247.

Kil, D. Y., L. G. Piao, H. F. Long, J. S. Lim, M. S. Yun, C. S. Kong, W. S. Ju, H. B. Lee, and Y. Y. Kim. 2005. Effects of organic or inorganic acid supplementation on growth performance, nutrient digestibility and white blood cell counts in weanling pigs. Asian-Australasian Journal of Animal Sciences 19(2):252–261.

Kim B. G. and M.D. Lindemann. 2007. A spreadsheet method for experimental animal allotment. undefined 85(2):112.

Kim, H.-Y., Y.-M. Song, S.-K. Jin, I.-S. Kim, Y.-S. Kang, S.-D. Lee, R. Chowdappa, J.-H. Ha, and S.-M. Kang. 2005. The effect of change in meat quality parameters on pig longissimus dorsi muscle by the addition of fermented persimmon shell diet. Asian-Australasian Journal of Animal Sciences 19(2):286–291.

Kirchhelle, C. 2018. Pharming animals: a global history of antibiotics in food production (1935–2017). Palgrave Communications 4(1):1–13.

Kitabatake, M., Y. Matsumura, N. Ouji-Sageshima, T. Nishioka, A. Hara, S. Kayano, and T. Ito. 2021. Persimmon-derived tannin ameliorates the pathogenesis of ulcerative colitis in a murine model through inhibition of the inflammatory response and alteration of microbiota. Scientific Reports 11(1):7286.

Kohanski, M. A., D. J. Dwyer, and J. J. Collins. 2010. How antibiotics kill bacteria: from targets to networks. Nature reviews. Microbiology 8(6):423–435.

Kohn, R. A., M. M. Dinneen, and E. Russek-Cohen. 2005. Using blood urea nitrogen to predict nitrogen excretion and efficiency of nitrogen utilization in cattle, sheep, goats, horses, pigs, and rats. Journal of Animal Science 83(4):879–889.

Krzyzowska, M., E. Tomaszewska, K. Ranoszek-Soliwoda, K. Bien, P. Orlowski, G. Celichowski, and J. Grobelny. 2017. Tannic acid modification of metal nanoparticles: possibility for new antiviral applications. Pages 335–363 *in* E. Andronescu and A. M. Grumezescu, editors. Nanostructures for Oral Medicine. Elsevier, Amsterdam, Netherlands.

Kuang, Y., Y. Wang, Y. Zhang, Y. Song, X. Zhang, Y. Lin, L. Che, S. Xu, D. Wu, B. Xue, and Z. Fang. 2015. Effects of dietary combinations of organic acids and medium chain fatty acids as a replacement of zinc oxide on growth, digestibility and immunity of weaned pigs. Animal Feed Science and Technology 208:145–157.

Kumar, K. 2011. Role of tannins on animal performance: an overview. Journal of Natural Remedies 11:82–89.

Kumar, R., and M. Singh. 1984. Tannins: their adverse role in ruminant nutrition. Journal of Agricultural and Food Chemistry 32(3):447–453.

Kusuda, M., K. Inada, T. Ogawa, T. Yoshida, S. Shiota, T. Tsuchiya, and T. Hatano. 2006. Polyphenolic constituent structures of zanthoxylum piperitum fruit and the antibacterial effects of its polymeric procyanidin on methicillin-resistant staphylococcus aureus. Bioscience, Biotechnology, and Biochemistry 70(6):1423–1431.

L, W., H. Y, Y. D, D. B, Z. D, W. Z, Z. H, L. Y, G. J, A. H, and W. G. 2015. Beneficial roles of dietary oleum cinnamomi in alleviating intestinal injury. Frontiers in Bioscience (Landmark Edition) 20:814–828.

Laine, T. M., T. Lyytikäinen, M. Yliaho, and M. Anttila. 2008. Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. Acta Veterinaria Scandinavica 50(1):21.

Landete, J. M. 2011. Ellagitannins, ellagic acid and their derived metabolites: a review about source, metabolism, functions and health. Food Research International 44(5):1150–1160.

de Lange, C. F. M., J. Pluske, J. Gong, and C. M. Nyachoti. 2010. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livestock Science 134(1):124–134.

Lebret B. 2008. Effects of feeding and rearing systems on growth, carcass composition and meat quality in pigs. animal 2(10):1548–1558.

Lee, H. J., I. H. Choi, D. H. Kim, S. M. Amanullah, and S. C. Kim. 2016. Nutritional characterization of tannin rich chestnut (Castanea) and its meal for pig. Journal of Applied Animal Research 44(1):258–262.

LEE, K.-M., W.-S. KIM, J. LIM, S. NAM, M. YOUN, S.-W. NAM, Y. KIM, S.-H. KIM, W. PARK, and S. PARK. 2009. Antipathogenic properties of green tea polyphenol epigallocatechin gallate at concentrations below the MIC against

enterohemorrhagic Escherichia coli O157:H7. Journal of Food Protection 72(2):325–331.

LeMaster, M. N., S. S. Chauhan, M. P. Wick, D. L. Clark, and E. M. England. 2019. Potassium carbonate improves fresh pork quality characteristics. Meat Science 156:222–230.

Levy, S. B., and B. Marshall. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature Medicine 10(12):S122–S129.

Lewis, W. H., and M. P. F. Elvin-Lewis. 2003. Medical Botany: Plants Affecting Human Health. John Wiley & Sons, Hoboken, New Jersey.

Li, J. 2017. Current status and prospects for in-feed antibiotics in the different stages of pork production — a review. Asian-Australasian Journal of Animal Sciences 30(12):1667–1673.

Li, P., X. Piao, Y. Ru, X. Han, L. Xue, and H. Zhang. 2012. Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response and intestinal health. Asian-Australasian Journal of Animal Sciences 25(11):1617–1626.

Listrat, A., B. Lebret, I. Louveau, T. Astruc, M. Bonnet, L. Lefaucheur, B. Picard, and J. Bugeon. 2016. How muscle structure and composition influence meat and flesh quality. The Scientific World Journal 2016:e3182746.

Liu, G., L. Yu, Y. Martínez, W. Ren, H. Ni, N. Abdullah Al-Dhabi, V. Duraipandiyan, and Y. Yin. 2017. Dietary saccharomyces cerevisiae cell wall extract supplementation alleviates oxidative stress and modulates serum amino acids profiles in weaned piglets. Oxidative Medicine and Cellular Longevity 2017:3967439.

Liu, H., J. Hu, S. Mahfuz, and X. Piao. 2020. Effects of hydrolysable tannins as zinc oxide substitutes on antioxidant status, immune function, intestinal morphology, and digestive enzyme activities in weaned piglets. Animals 10(5):757.

Liu, H., K. Li, L. Mingbin, J. Zhao, and B. Xiong. 2016. Effects of chestnut tannins on the meat quality, welfare, and antioxidant status of heat-stressed lambs. Meat Science 116:236–242.

Liu, H., D. Zhou, J. Tong, and V. Vaddella. 2012. Influence of chestnut tannins on welfare, carcass characteristics, meat quality, and lipid oxidation in rabbits under high ambient temperature. Meat Science 90(1):164–169.

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, and H. H. Stein. 2018. Non-antibiotic feed additives in diets for pigs: a review. Animal Nutrition 4(2):113–125.

Lizardo, R., J. Peiniau, and A. Aumaitre. 1995. Effect of sorghum on performance, digestibility of dietary components and activities of pancreatic and intestinal enzymes in the weaned piglet. Animal Feed Science and Technology 56(1):67–82.

Lopez-Bote, C. J. 1998. Sustained utilization of the Iberian pig breed. Meat Science 49:S17–S27.

Lorenzo, J. M., J. Carballo, and D. Franco. 2013. Effect of the inclusion of chestnut in the finishing diet on volatile compounds of dry-cured ham from Celta pig breed. Journal of Integrative Agriculture 12(11):2002–2012.

Low, C. X., L. T.-H. Tan, N.-S. Ab Mutalib, P. Pusparajah, B.-H. Goh, K.-G. Chan, V. Letchumanan, and L.-H. Lee. 2021. Unveiling the impact of antibiotics and alternative methods for animal husbandry: a review. Antibiotics 10(5):578.

Luciano, G., F. J. Monahan, V. Vasta, L. Biondi, M. Lanza, and A. Priolo. 2009. Dietary tannins improve lamb meat colour stability. Meat Science 81(1):120–125.

Luo, J., W. Yan, M. M. Nasiru, H. Zhuang, G. Zhou, and J. Zhang. 2019. Evaluation of physicochemical properties and volatile compounds of Chinese dried pork loin curing with plasma-treated water brine. Scientific Reports 9(1):13793.

M. Ellin Doyle. 2001. Alternatives to antibiotic use for growth promotion in animal husbandry. Food Research:1–17.

M. L. R. Mota, G. Thomas, and J. M. Barbosa Filho. 1985. Anti-inflammatory actions of tannins isolated from the bark of Anacardwm occidentale L. Journal of Ethnopharmacology 13(3):289–300.

Ma, X., M. Yu, Z. Liu, D. Deng, Y. Cui, Z. Tian, and G. Wang. 2020. Effect of amino acids and their derivatives on meat quality of finishing pigs. Journal of Food Science and Technology 57(2):404–412.

Mancini, R. A., and M. C. Hunt. 2005. Current research in meat color. Meat Science 71(1):100–121.

Mancini, S., R. Moruzzo, S. Minieri, B. Turchi, D. Cerri, D. Gatta, S. Sagona, A. Felicioli, and G. Paci. 2019. Dietary supplementation of quebracho and chestnut tannins mix in rabbit: effects on live performances, digestibility, carcase traits, antioxidant status, faecal microbial load and economic value. Italian Journal of Animal Science 18(1):621–629.

Mariscal-Landín, G., J. H. Avellaneda, T. C. Reis de Souza, A. Aguilera, G. A. Borbolla, and B. Mar. 2004. Effect of tannins in sorghum on amino acid ileal digestibility and on trypsin (E.C.2.4.21.4) and chymotrypsin (E.C.2.4.21.1) activity of growing pigs. Animal Feed Science and Technology 117(3):245–264.

Martens, S. D., T. T. Tiemann, J. Bindelle, M. Peters, and C. E. Lascano. 2012. Alternative plant protein sources for pigs and chickens in the tropics – nutritional value and constraints: a review. Journal of Agriculture and Rural Development in the Tropics and Subtropics (JARTS) 113(2):101–123.

McDougall, G. J., F. Shpiro, P. Dobson, P. Smith, A. Blake, and D. Stewart. 2005. Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. Journal of Agricultural and Food Chemistry 53(7):2760–2766.

McEwen S. A., and Fedorka-Cray P. J. 2002. Antimicrobial use and resistance in animals. Clinical Infectious Diseases 34(Sup3):S93–S106.

McLaren, D. G., P. J. Bechtel, G. L. Grebner, J. Novakofski, F. K. McKeith, R. W. Jones, R. H. Dalrymple, and R. A. Easter. 1990. Dose response in growth of pigs injected daily with porcine somatotropin from 57 to 103 kilograms. Journal of Animal Science 68(3):640–651.

Mehansho, H., L. G. Butler, and D. M. Carlson. 1987. Dietary tannins and salivary proline-rich proteins: interactions, induction, and defense mechanisms. Annual Review of Nutrition 7(1):423–440.

Mehdi, Y., M.-P. Létourneau-Montminy, M.-L. Gaucher, Y. Chorfi, G. Suresh, T. Rouissi, S. K. Brar, C. Côté, A. A. Ramirez, and S. Godbout. 2018. Use of antibiotics in broiler production: Global impacts and alternatives. Animal Nutrition 4(2):170–178.

Melkebeek, V., B. M. Goddeeris, and E. Cox. 2013. ETEC vaccination in pigs. Veterinary Immunology and Immunopathology 152(1):37–42.

Mesonero-Escuredo, S., K. Strutzberg-Minder, C. Casanovas, and J. Segalés. 2018. Viral and bacterial investigations on the aetiology of recurrent pig neonatal diarrhoea cases in Spain. Porcine Health Management 4:5.

Meyers, K. J., T. J. Swiecki, and A. E. Mitchell. 2006. Understanding the native Californian diet: identification of condensed and hydrolyzable tannins in tanoak acorns (Lithocarpus densiflorus). Journal of Agricultural and Food Chemistry 54(20):7686–7691.

Min, B. R., T. N. Barry, G. T. Attwood, and W. C. McNabb. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Animal Feed Science and Technology 106(1):3–19.

Mócsai, A. 2013. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. The Journal of Experimental Medicine 210(7):1283–1299.

Montagne L., Boudry G., Favier C., Huërou-Luron I. L., Lallès J.-P., and Sève B. 2007. Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. British Journal of Nutrition 97(1):45–57.

Morrissey, P. A., P. J. A. Sheehy, K. Galvin, J. P. Kerry, and D. J. Buckley. 1998. Lipid stability in meat and meat products. Meat Science 49:S73–S86.

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. Journal of the Science of Food and Agriculture 86(13):2010–2037.

Newberry, R. C., and D. G. M. Wood-Gush. 1985. The suckling behaviour of domestic pigs in a semi-natural environment. Behaviour 95(1–2):11–25.

Ngapo, T. M., and C. Gariépy. 2008. Factors affecting the eating quality of pork. Critical Reviews in Food Science and Nutrition 48(7):599–633.

Nostro, A. 2006. Activity of plant extracts and plant-derived compounds against drug-resistant microorganisms. Pages 199–231 *in* I. Ahmad, F. Aqil, and M. Owais, editors. Modern Phytomedicine. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

NRC. 2012. Nutrient requirements of swine. 11th rev. ed. National Academies Press, Washington, DC.

Ochetim, S., and I. D. Odur. 1979. Response of young pigs to Flavomycin. East African Agricultural and Forestry Journal 44(1):31–33.

O'Connell, J. M., J. J. Callan, and J. V. O'Doherty. 2006. The effect of dietary crude protein level, cereal type and exogenous enzyme supplementation on nutrient digestibility, nitrogen excretion, faecal volatile fatty acid concentration and ammonia emissions from pigs. Animal Feed Science and Technology 127(1):73–88.

O'Connor-Shaw, R. E., and V. G. Reyes. 1999. Chilled storage of foods | use of modified-atmosphere packaging. Pages 410–416 *in* R. K. Robinson, editor. Encyclopedia of Food Microbiology. Elsevier, Oxford.

Ozer, J., M. Ratner, M. Shaw, W. Bailey, and S. Schomaker. 2008. The current state of serum biomarkers of hepatotoxicity. Toxicology 245(3):194–205.

Papatsiros, V. G., G. Christodoulopoulos, and L. C. Filippopoulos. 2012. The use of organic acids in monogastric animals (swine and rabbits). Journal of Cell and Animal Biology 6(10):154–159.

Parisi, F., S. Mancini, M. Mazzei, M. Forzan, B. Turchi, S. Perrucci, A. Poli, and G. Paci. 2018. Effect of dietary supplementation of a mix of chestnut and quebracho tannins on intestinal morphology, bacterial load, eimeria spp oocyst excretion and immune response after vaccination in rabbits. American Journal of Animal and Veterinary Sciences 13(3):94–103.

Park, M., H. Cho, H. Jung, H. Lee, and K. T. Hwang. 2014. Antioxidant and anti-inflammatory activities of tannin fraction of the extract from black raspberry seeds compared to grape seeds. Journal of Food Biochemistry 38(3):259–270.

Park, Y. 2008. Moisture and Water Activity. Pages 35–67 *in* L. Nollet and F. Toldra, editors. Handbook of Processed Meats and Poultry Analysis. CRC Press.

Partanen K. H., and Mroz Z. 1999. Organic acids for performance enhancement in pig diets. Nutrition Research Reviews 12(1):117–145.

Penet, C. S., R. E. Worthington, R. D. Phillips, and N. J. Moon. 1983. Free amino acids of raw and cooked ground beef and pork. Journal of Food Science 48(1):298–299.

Peng, Z., A. Wang, L. Xie, W. Song, J. Wang, Z. Yin, D. Zhou, and F. Li. 2016. Use of recombinant porcine β-defensin 2 as a medicated feed additive for weaned piglets. Scientific Reports 6(1):1–8.

Pereira-Lorenzo, S., A. Ballester, E. Corredoira, A. M. Vieitez, S. Agnanostakis, R. Costa, G. Bounous, R. Botta, G. L. Beccaro, T. L. Kubisiak, M. Conedera, P. Krebs, T. Yamamoto, Y. Sawamura, N. Takada, J. Gomes-Laranjo, and A. M. Ramos-Cabrer. 2012. Chestnut. Pages 729–769 *in* M. L. Badenes and D. H. Byrne, editors. Fruit Breeding. Springer US, Boston, MA.

Pettey, L. A., S. D. Carter, B. W. Senne, and J. A. Shriver. 2002. Effects of beta-mannanase addition to corn-soybean meal diets on growth performance, carcass traits, and nutrient digestibility of weanling and growing-finishing pigs. Journal of Animal Science 80(4):1012–1019.

Piao C., Ma M., Chambers J. K., Uchida K., Ikeda M., Kobayashi N. I., Hirose A., Tanoi K., Kuwahara M., and Li J. 2020. Radiocesium transfer rates among pigs fed haylage contaminated with low levels of cesium at two differentiation stages. PLOS ONE 15(9):e0237977.

Piluzza, G., L. Sulas, and S. Bullitta. 2014. Tannins in forage plants and their role in animal husbandry and environmental sustainability: a review. Grass and Forage Science 69(1):32–48.

Piñeiro-Vázquez, A. T., G. Jiménez-Ferrer, J. A. Alayon-Gamboa, A. J. Chay-Canul, A. J. Ayala-Burgos, C. F. Aguilar-Pérez, and J. C. Ku-Vera. 2018. Effects of quebracho tannin extract on intake, digestibility, rumen fermentation, and methane production in crossbred heifers fed low-quality tropical grass. Tropical Animal Health and Production 50(1):29–36.

Pinotti, L., A. Luciano, M. Ottoboni, M. Manoni, L. Ferrari, D. Marchis, and M. Tretola. 2021. Recycling food leftovers in feed as opportunity to increase the sustainability of livestock production. Journal of Cleaner Production 294:126290.

Pinto, D., A. M. Silva, V. Freitas, A. Vallverdú-Queralt, C. Delerue-Matos, and F. Rodrigues. 2021. Microwave-assisted extraction as a green technology approach to recover polyphenols from castanea sativa shells. ACS Food Science & Technology 1(2):229–241.

Piwowarski, J. P., S. Granica, M. Zwierzyńska, J. Stefańska, P. Schopohl, M. F. Melzig, and A. K. Kiss. 2014. Role of human gut microbiota metabolism in the anti-inflammatory effect of traditionally used ellagitannin-rich plant materials. Journal of Ethnopharmacology 155(1):801–809.

Potter A., Gerdts V., and Hurk S. van D. L. 2008. Veterinary vaccines: alternatives to antibiotics? Animal Health Research Reviews 9(2):187–199.

Poulsen, H. D. 1995. Zinc oxide for weanling piglets. Acta Agriculturae Scandinavica, Section A-Animal Science 45(3):159–167.

Prescott J.F. and Baggot J.D. 1993. Antimicrobial Therapy in Veterinary Medicine2nd edition. Iowa State University Press, Amsterdam.

Prichard, R. K. 2007. Ivermectin resistance and overview of the Consortium for Anthelmintic Resistance SNPs. Expert Opinion on Drug Discovery 2(sup1):S41–S52.

Pugliese, C., L. Pianaccioli, F. Sirtori, A. Acciaioli, R. Bozzi, and O. Franci. 2007. Effect of pasture on chestnut woods on meat quality and fatty acid composition of fat in Cinta Senese pigs. Pages 263–267 Options Méditerranéennes. Série A : Séminaires Méditerranéens (CIHEAM). CIHEAM-IAMZ.

Pugliese, C., F. Sirtori, A. Acciaioli, R. Bozzi, G. Campodoni, and O. Franci. 2013. Quality of fresh and seasoned fat of Cinta Senese pigs as affected by fattening with chestnut. Meat Science 93(1):92–97.

Pugliese, C., F. Sirtori, S. D’Adorante, S. Parenti, A. Rey, C. Lopez-Bote, and O. Franci. 2009. Effect of pasture in oak and chestnut groves on chemical and sensorial traits of cured lard of Cinta Senese pigs. Italian Journal of Animal Science 8(2):131–142.

Quideau, S., D. Deffieux, C. Douat-Casassus, and L. Pouységu. 2011. Plant polyphenols: chemical properties, biological activities, and synthesis. Angewandte Chemie International Edition 50(3):586–621.

Ramaiah, S. K. 2007. A toxicologist guide to the diagnostic interpretation of hepatic biochemical parameters. Food and Chemical Toxicology 45(9):1551–1557.

Rao, M., A. Bast, and A. de Boer. 2021. Valorized food processing by-products in the eu: finding the balance between safety, nutrition, and sustainability. Sustainability 13(8):4428.

Reddy, M. K., S. K. Gupta, M. R. Jacob, S. I. Khan, and D. Ferreira. 2007. Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from punica granatum l. Planta Medica 53(5):461–467.

Redondo, L. M., P. A. Chacana, J. E. Dominguez, and M. E. D. Fernandez Miyakawa. 2014. Perspectives in the use of tannins as alternative to antimicrobial growth promoter factors in poultry. Frontiers in Microbiology 5.

Reggi, S., C. Giromini, M. Dell'Anno, A. Baldi, R. Rebucci, and L. Rossi. 2020. In vitro digestion of chestnut and quebracho tannin extracts: antimicrobial effect, antioxidant capacity and cytomodulatory activity in swine intestinal IPEC-J2 cells. Animals 10(2):195.

Rey, A. I., B. Isabel, R. Cava, and C. J. Lopez-Bote. 1998. Dietary acorns provide a source of gamma-tocopherol to pigs raised extensively. Canadian Journal of Animal Science 78(3):441–443.

Rey A. I., Lopez-Bote C. J., and Arias R. S. 1997. Effect of extensive feeding on α-tocopherol concentration and oxidative stability of muscle microsomes from Iberian pigs. Animal Science 65(3):515–520.

Rezaei, R., W. Wang, Z. Wu, Z. Dai, J. Wang, and G. Wu. 2013. Biochemical and physiological bases for utilization of dietary amino acids by young Pigs. Journal of Animal Science and Biotechnology 4(1):7.

Rezar, V., J. Salobir, A. Levart, U. Tomažin, M. Škrlep, N. Batorek Lukač, and M. Čandek-Potokar. 2017. Supplementing entire male pig diet with hydrolysable tannins: effect on carcass traits, meat quality and oxidative stability. Meat Science 133:95–102.

Rhouma, M., J. M. Fairbrother, F. Beaudry, and A. Letellier. 2017. Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. Acta Veterinaria Scandinavica 59(1):31.

Rice-Evans, C. A., N. J. Miller, and G. Paganga. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20(7):933–956.

Riksdagsförvaltningen. 1997. sou 1997 132 d1 Statens offentliga utredningar 1997:132 - Riksdagen. https://www.riksdagen.se/sv/dokument-lagar/dokument/statens-offentliga-utredningar/sou-1997-132-d1_GLB3132d1.

Ritchie, H., and M. Roser. 2017. Meat and Dairy Production. Our World in Data.

Rodrigues, F., J. Santos, F. B. Pimentel, N. Braga, A. Palmeira-de-Oliveira, and M. B. P. P. Oliveira. 2015. Promising new applications of Castanea sativa shell: nutritional composition, antioxidant activity, amino acids and vitamin E profile. Food & Function 6(8):2854–2860.

Ronchetti, S., E. Ricci, G. Migliorati, M. Gentili, and C. Riccardi. 2018. How glucocorticoids affect the neutrophil life. International Journal of Molecular Sciences 19(12):4090.

Sasaki, K., M. Motoyama, and T. Narita. 2012. Increased intramuscular fat improves both ‘chewiness’ and ‘hardness’ as defined in ISO5492:1992 of beef Longissimus muscle of Holstein × Japanese Black F1 steers. Animal Science Journal 83(4):338–343.

Scalbert, A. 1991. Antimicrobial properties of tannins. Phytochemistry 30(12):3875–3883.

Semjon, B., E. Dudriková, I. Jaďďuttová, M. Bartkovský, T. Klempová, D. Marcinčáková, O. Slaný, and S. Marcinčák. 2020. Effect of supplementation

with solid-state fermented feed in the diet of laying hens on egg qualitative variables. Potravinarstvo Slovak Journal of Food Sciences 14:519–527.

Serrano, J., R. Puupponen-Pimiä, A. Dauer, A.-M. Aura, and F. Saura-Calixto. 2009. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. Molecular Nutrition & Food Research 53(S2):S310–S329.

Shah, M. A., S. J. D. Bosco, and S. A. Mir. 2014. Plant extracts as natural antioxidants in meat and meat products. Meat Science 98(1):21–33.

Shahidi, F., and P. Ambigaipalan. 2015. Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects – a review. Journal of Functional Foods 18:820–897.

Sharma, K., V. Kumar, J. Kaur, B. Tanwar, A. Goyal, R. Sharma, Y. Gat, and A. Kumar. 2019. Health effects, sources, utilization and safety of tannins: a critical review. Toxin Reviews 40(4):432–444.

Shoop, W. L. 1993. Ivermectin resistance. Parasitology Today 9(5):154–159.

Sieniawska, E. 2015. Activities of tannins – from in vitro studies to clinical trials. Natural Product Communications 10(11):1934578X1501001118.

Smeriglio, A., D. Barreca, E. Bellocco, and D. Trombetta. 2017. Proanthocyanidins and hydrolysable tannins: occurrence, dietary intake and pharmacological effects. British Journal of Pharmacology 174(11):1244–1262.

Soares, S., E. Brandão, N. Mateus, and V. de Freitas. 2017. Sensorial properties of red wine polyphenols: astringency and bitterness. Critical Reviews in Food Science and Nutrition 57(5):937–948.

Sobeh, M., M. F. Mahmoud, M. A. O. Abdelfattah, H. A. El-Beshbishy, A. M. El-Shazly, and M. Wink. 2017. Hepatoprotective and hypoglycemic effects of a

tannin rich extract from Ximenia americana var. caffra root. Phytomedicine 33:36–42.

Soliman, A., V. De Sanctis, N. Alaaraj, S. Ahmed, F. Alyafei, N. Hamed, and N. Soliman. 2021. Early and long-term consequences of nutritional stunting: from childhood to adulthood. Acta Bio Medica : Atenei Parmensis 92(1):e2021168.

Soyocak, A., H. Kurt, D. T. Cosan, F. Saydam, I. Calis, U. Kolac, Z. O. Koroglu, I. Degirmenci, F. S. Mutlu, and H. Gunes. 2019. Tannic acid exhibits anti-inflammatory effects on formalin-induced paw edema model of inflammation in rats. Human & Experimental Toxicology 38(11):1296–1301.

Stein, H. H., and D. Y. Kil. 2006. Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, part 2. Animal Biotechnology 17(2):217–231.

SU, C., Z. SONG, X. XU, Y. Chen, Y. Ding, H. Guo, and J. Huang. 2019. Effects of quebracho tannin on growth performance and pork shelf life of finishing pigs. Chinese Journal of Animal Nutrition 31(10):4834–4842.

Su C., Xu X., Song Z., Zhang J., and Huang Z. 2016. The effect of quebracho tannin on the production performance of nursery pigs and the impact of immune function. China Feed (Chinese) (21):23–32.

Su, G., X. Zhou, Y. Wang, D. Chen, G. Chen, Y. Li, and J. He. 2018. Effects of plant essential oil supplementation on growth performance, immune function and antioxidant activities in weaned pigs. Lipids in Health and Disease 17(1):139.

Sugiharto, S., M. S. Hedemann, and C. Lauridsen. 2014. Plasma metabolomic profiles and immune responses of piglets after weaning and challenge with E. coli. Journal of Animal Science and Biotechnology 5(1):17.

Sukhotnik, I., J. Mogilner, M. M. Krausz, M. Lurie, M. Hirsh, A. G. Coran, and E. Shiloni. 2004. Oral arginine reduces gut mucosal injury caused by

lipopolysaccharide endotoxemia in rat. Journal of Surgical Research 122(2):256–262.

Sun, H. X., T. S. Gao, R. Z. Zhong, Y. Fang, G. L. Di, and D. W. Zhou. 2018. Effects of corn replacement by sorghum in diets on performance, nutrient utilization, blood parameters, antioxidant status, and meat colour stability in lambs. Canadian Journal of Animal Science 98(4):723–731.

Tejeda, J. F., A. Hernández-Matamoros, M. Paniagua, and E. González. 2020. Effect of free-range and low-protein concentrated diets on growth performance, carcass traits, and meat composition of iberian pig. Animals 10(2):273.

Terlizzi, M. E., A. Occhipinti, A. Luganini, M. E. Maffei, and G. Gribaudo. 2016. Inhibition of herpes simplex type 1 and type 2 infections by Oximacro®, a cranberry extract with a high content of A-type proanthocyanidins (PACs-A). Antiviral Research 132:154–164.

Theisen, L. L., C. A. J. Erdelmeier, G. A. Spoden, F. Boukhallouk, A. Sausy, L. Florin, and C. P. Muller. 2014. Tannins from hamamelis virginiana bark extract: characterization and improvement of the antiviral efficacy against influenza a virus and human papillomavirus. PLoS ONE 9(1):e88062.

Tintino, S. R., C. D. M. Oliveira-Tintino, F. F. Campina, R. L. P. Silva, M. do S. Costa, I. R. A. Menezes, J. T. Calixto-Júnior, J. P. Siqueira-Junior, H. D. M. Coutinho, T. C. Leal-Balbino, and V. Q. Balbino. 2016. Evaluation of the tannic acid inhibitory effect against the NorA efflux pump of Staphylococcus aureus. Microbial Pathogenesis 97:9–13.

Tomažin, U., M. Škrlep, M. Prevolnik Povše, N. Batorek-Lukač, D. Karolyi, M. Červek, and M. Čandek-Potokar. 2020. The effect of supplementing pig diet with

chestnut wood extract or hops on fresh meat and dry-cured products. Applied Sciences 10(19):6922.

Tretola, M., F. Maghin, P. Silacci, S. Ampuero, and G. Bee. 2019. Effect of supplementing hydrolysable tannins to a grower–finisher diet containing divergent PUFA levels on growth performance, boar taint levels in back fat and intestinal microbiota of entire males. Animals 9(12):1063.

Tsao, R. 2010. Chemistry and Biochemistry of Dietary Polyphenols. Nutrients 2(12):1231–1246.

Tsiouris, V., P. Tassis, J. Raj, T. Mantzios, K. Kiskinis, M. Vasiljević, N. Delić, E. Petridou, G. D. Brellou, Z. Polizopoulou, N. Mittas, and I. Georgopoulou. 2021. Investigation of a novel multicomponent mycotoxin detoxifying agent in amelioration of mycotoxicosis induced by Aflatoxin-B1 and Ochratoxin A in broiler chicks. Toxins 13(6):367.

Ueda, K., R. Kawabata, T. Irie, Y. Nakai, Y. Tohya, and T. Sakaguchi. 2013. Inactivation of pathogenic viruses by plant-derived tannins: strong effects of extracts from persimmon (Diospyros kaki) on a broad range of viruses. PLoS ONE 8(1):e55343.

Ueda, Y., A. Watanabe, M. Higuchi, H. Shingu, S. Kushibiki, and M. Shinoda. 2007. Effects of intramuscular fat deposition on the beef traits of Japanese Black steers (Wagyu). Animal Science Journal 78(2):189–194.

Upadhyay, R. K. 2011. Plant natural products: their pharmaceutical potential against disease and drug resistant microbial pathogens. The Journal of Pharmacy 4(4):1179–1185.

Van Boeckel, T. P., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in

antimicrobial use in food animals. Proceedings of the National Academy of Sciences of the United States of America 112(18):5649–5654.

Van der Stede, Y., E. Cox, F. Verdonck, S. Vancaeneghem, and B. M. Goddeeris. 2003. Reduced faecal excretion of F4+-E. coli by the intramuscular immunisation of suckling piglets by the addition of 1α,25-dihydroxyvitamin D3 or CpG-oligodeoxynucleotides. Vaccine 21(9):1023–1032.

Vázquez, G., E. Fontenla, J. Santos, M. S. Freire, J. González-Álvarez, and G. Antorrena. 2008. Antioxidant activity and phenolic content of chestnut (Castanea sativa) shell and eucalyptus (Eucalyptus globulus) bark extracts. Industrial Crops and Products 28(3):279–285.

Vekiari, S. A., M. H. Gordon, P. García-Macías, and H. Labrinea. 2008. Extraction and determination of ellagic acid contentin chestnut bark and fruit. Food Chemistry 110(4):1007–1011.

Verhelst, R., M. Schroyen, N. Buys, and T. Niewold. 2014. Dietary polyphenols reduce diarrhea in enterotoxigenic Escherichia coli (ETEC) infected post-weaning piglets. Livestock Science 160:138–140.

Vilhelmova-Ilieva, N., A. S. Galabov, and M. Mileva. 2019. Tannins as Antiviral Agents. Page Tannins - Structural Properties, Biological Properties and Current Knowledge. IntechOpen.

Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. Animal Feed Science and Technology 147(1):116–139.

Wang, K., H. Zhang, Q. Han, J. Lan, G. Chen, G. Cao, and C. Yang. 2020a. Effects of astragalus and ginseng polysaccharides on growth performance, immune function and intestinal barrier in weaned piglets challenged with

lipopolysaccharide. Journal of Animal Physiology and Animal Nutrition 104(4):1096–1105.

Wang, M., H. Huang, Y. Hu, J. Huang, H. Yang, L. Wang, S. Chen, C. Chen, and S. He. 2020b. Effects of dietary microencapsulated tannic acid supplementation on the growth performance, intestinal morphology, and intestinal microbiota in weaning piglets. Journal of Animal Science 98(5).

Wang, M., H. Huang, Y. Hu, Y. Liu, X. Zeng, Y. Zhuang, H. Yang, L. Wang, S. Chen, L. Yin, S. He, S. Zhang, X. Li, and S. He. 2020c. Effects of dietary supplementation with herbal extract mixture on growth performance, organ weight and intestinal morphology in weaning piglets. Journal of Animal Physiology and Animal Nutrition 104(5):1462–1470.

Wang, W. W., S. Y. Qiao, and D. F. Li. 2009. Amino acids and gut function. Amino Acids 37(1):105–110.

Wapnir, R. A. 2000. Zinc deficiency, malnutrition and the gastrointestinal tract. The Journal of Nutrition 130(5):1388S-1392S.

Warner, R. D., P. L. Greenwood, D. W. Pethick, and D. M. Ferguson. 2010. Genetic and environmental effects on meat quality. Meat Science 86(1):171–183.

Webb, O. M., Isam T. Kadim and Edward. 2011. Goat Meat Production and Quality. CABI.

Webber, M. A., and L. J. V. Piddock. 2003. The importance of efflux pumps in bacterial antibiotic resistance. Journal of Antimicrobial Chemotherapy 51(1):9–11.

Wei, H.-K., H.-X. Xue, Z. X. Zhou, and J. Peng. 2017. A carvacrol–thymol blend decreased intestinal oxidative stress and influenced selected microbes without

changing the messenger RNA levels of tight junction proteins in jejunal mucosa of weaning piglets. animal 11(2):193–201.

Wilkinson, J. M. 1999. Silage and animal health. Natural Toxins 7(6):221–232.

Williams A. R., Fryganas C., Ramsay A., Mueller-Harvey I., and Thamsborg S. M. 2014a. Direct anthelmintic effects of condensed tannins from diverse plant sources against ascaris suum. PLOS ONE 9(5):e97053.

Williams, A. R., H. M. Ropiak, C. Fryganas, O. Desrues, I. Mueller-Harvey, and S. M. Thamsborg. 2014b. Assessment of the anthelmintic activity of medicinal plant extracts and purified condensed tannins against free-living and parasitic stages of Oesophagostomum dentatum. Parasites & Vectors 7(1):518.

Witte, W. 2000. Selective pressure by antibiotic use in livestock. International Journal of Antimicrobial Agents 16:19–24.

Wood, J. D., R. I. Richardson, G. R. Nute, A. V. Fisher, M. M. Campo, E. Kasapidou, P. R. Sheard, and M. Enser. 2004. Effects of fatty acids on meat quality: a review. Meat Science 66(1):21–32.

World Food Programme. 2021. . https://www.wfp.org/publications/global-report-food-crises-2021.

Wright, G. D. 2005. Bacterial resistance to antibiotics: enzymatic degradation and modification. Advanced Drug Delivery Reviews 57(10):1451–1470.

Wu, G., F. W. Bazer, Z. Dai, D. Li, J. Wang, and Z. Wu. 2014. Amino acid nutrition in animals: protein synthesis and beyond. Annual Review of Animal Biosciences 2(1):387–417.

Wu, G., F. W. Bazer, T. A. Davis, S. W. Kim, P. Li, J. Marc Rhoads, M. Carey Satterfield, S. B. Smith, T. E. Spencer, and Y. Yin. 2009. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37(1):153–168.

Wu, S., F. Zhang, Z. Huang, H. Liu, C. Xie, J. Zhang, P. A. Thacker, and S. Qiao. 2012. Effects of the antimicrobial peptide cecropin AD on performance and intestinal health in weaned piglets challenged with Escherichia coli. Peptides 35(2):225–230.

Wu, Y., J. Zhao, C. Xu, N. Ma, T. He, J. Zhao, X. Ma, and P. A. Thacker. 2020. Progress towards pig nutrition in the last 27 years. Journal of the Science of Food and Agriculture 100(14):5102–5110.

Xia, T., W. Lai, M. Han, M. Han, X. Ma, and L. Zhang. 2017. Dietary ZnO nanoparticles alters intestinal microbiota and inflammation response in weaned piglets. Oncotarget 8(39):64878–64891.

Xie, Y., L. Wang, H. Sun, Y. Wang, Z. Yang, G. Zhang, and W. Yang. 2019. Immunomodulatory, antioxidant and intestinal morphology-regulating activities of alfalfa polysaccharides in mice. International Journal of Biological Macromolecules 133:1107–1114.

Xiong, X., B. Tan, M. Song, P. Ji, K. Kim, Y. Yin, and Y. Liu. 2019. Nutritional intervention for the intestinal development and health of weaned pigs. Frontiers in Veterinary Science 6:46.

Xu, X., X. Chen, D. Chen, B. Yu, J. Yin, and Z. Huang. 2019. Effects of dietary apple polyphenol supplementation on carcass traits, meat quality, muscle amino acid and fatty acid composition in finishing pigs. Food & Function 10(11):7426–7434.

Yi, H., D. Jiang, L. Zhang, H. Xiong, F. Han, and Y. Wang. 2016. Developmental expression of STATs, nuclear factor-κB and inflammatory genes in the jejunum of piglets during weaning. International Immunopharmacology 36:199–204.

Yi, H., C. Yu, H. Zhang, D. Song, D. Jiang, H. Du, and Y. Wang. 2015. Cathelicidin-BF suppresses intestinal inflammation by inhibiting the nuclear factor-κB signaling pathway and enhancing the phagocytosis of immune cells via STAT-1 in weanling piglets. International Immunopharmacology 28(1):61–69.

Yin J., Wu M. M., Xiao H., Ren W. K., Duan J. L., Yang G., Li T. J., and Yin Y. L. 2014. Development of an antioxidant system after early weaning in piglets. Journal of Animal Science 92(2):612–619.

Yin, T.-P., L. Cai, Y. Chen, Y. Li, Y.-R. Wang, C.-S. Liu, and Z.-T. Ding. 2015. Tannins and antioxidant activities of the walnut (Juglans regia) pellicle. Natural Product Communications 10(12):1934578X1501001232.

Youdim K. A., and Deans S. G. 2000. Effect of thyme oil and thymol dietary supplementation on the antioxidant status and fatty acid composition of the ageing rat brain. British Journal of Nutrition 83(1):87–93.

Yu, M., C. Mu, Y. Yang, C. Zhang, Y. Su, Z. Huang, K. Yu, and W. Zhu. 2017. Increases in circulating amino acids with in-feed antibiotics correlated with gene expression of intestinal amino acid transporters in piglets. Amino Acids 49(9):1587–1599.

Zhang, J., Q. Song, X. Han, Y. Zhang, Y. Zhang, X. Zhang, X. Chu, F. Zhang, and L. Chu. 2017. Multi-targeted protection of acetaminophen-induced hepatotoxicity in mice by tannic acid. International Immunopharmacology 47:95–105.

Zhang, L., Y. Huang, Y. Zhou, T. Buckley, and H. H. Wang. 2013. Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota. Antimicrobial Agents and Chemotherapy 57(8):3659–3666.

Zhang, P., X. Jiang, X. Nie, Y. Huang, F. Zeng, X. Xia, and S. Wu. 2016. A two-photon fluorescent sensor revealing drug-induced liver injury via tracking γ-glutamyltranspeptidase (GGT) level in vivo. Biomaterials 80:46–56.

Zhang, W., H. Liu, K. Xie, L. Yin, Y. Li, C. L. Kwik-Uribe, and X. Zhu. 2006. Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells. Biochemical and Biophysical Research Communications 345(1):508–515.

Zhang, Y., J. Lu, Y. Yan, J. Liu, and M. Wang. 2021. Antibiotic residues in cattle and sheep meat and human exposure assessment in southern Xinjiang, China. Food Science & Nutrition 9(11):6152–6161.

Zhou, L., L. Fang, Y. Sun, Y. Su, and W. Zhu. 2016. Effects of the dietary protein level on the microbial composition and metabolomic profile in the hindgut of the pig. Anaerobe 38:61–69.

Zhu L. H., Zhao K. L., Chen X. L., and Xu J. X. 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. Journal of Animal Science 90(8):2581–2589.

Zotte, A. D., and M. E. Cossu. 2009. Dietary inclusion of tannin extract from red quebracho trees (Schinopsis spp.) in the rabbit meat production. Italian Journal of Animal Science 8(sup2):784–786.