EFFECTS OF ALGAE-DERIVED β-GLUCAN SUPPLEMENTATION ON PRODUCTION PERFORMANCES AND SPECIFIC IMMUNE RESPONSES IN NURSERY TO GROWING PIGS

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ผลของการใช้สารเสริมเบต้ากลูแคนจากสาหร่ายในอาหาร ต่อประสิทธิภาพการผลิตและการตอบสนองทางภูมิคุ้มกันแบบจำเพาะในสุกรอนุบาลและสุกรขุนเล็ก

น.ส.ช.บุญนิธิ ช่วยชู

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Thesis Title	EFFECTS OF ALGAE-DERIVED ß-GLUCAN SUPPLEMENTATION ON PRODUCTION PERFORMANCES AND SPECIFIC IMMUNE RESPONSES IN NURSERY TO GROWING PIGS
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ช.บุญนิธิ ช่วยชู : ผลของการใช้สารเสริมเบต้ากลูแคนจากสาหร่ายในอาหาร ต่อประสิทธิภาพการผลิตและการตอบสนองทางภูมิคุ้มกันแบบจำเพาะในสุกรอนุบาลและสุกรขุนเล็ก. (EFFECTS OF ALGAE-DERIVED β-GLUCAN SUPPLEMENTATION ON PRODUCTION PERFORMANCES AND SPECIFIC IMMUNE RESPONSES IN NURSERY TO GROWING PIGS) อ.ที่ปรึกษาหลัก : ศ. น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, อ.ที่ปรึกษาร่วม : อ.น.สพ. ดร.ธีรวุฒิ เนตรอำพันธ์,ศ.เผด็จ ธรรมรักษ์

เบต้ากลูแคนเป็นสารเสริมในอาหารสัตว์ที่มีการใช้กันอย่างแพร่หลายในอุตสาหกรรมการเลี้ยงสุกร ถึงแม้ว่าการเสริมสารเบต้ากลูแคนในสูตรอาหารสุกร จะเชื่อว่าสามารถช่วยพัฒนาการเจริญเติบโตและปรับการทำงานของภูมิคุ้มกันในสุกรได้ แต่อย่างไรก็ตาม ในปัจจุบันยังไม่มีข้อมูลการศึกษาที่ชัดเจนเกี่ยวกับผลของการเสริมเบต้ากลูแคนที่สกัดมาจากสาหร่ายต่ออัตราการเจริญเติบโตและการตอบสนองทางภูมิคุ้มกันโดยเฉพาะระบบภูมิคุ้มกันแบบจำเพาะในสุกร ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินผลของการใช้สารเสริมเบต้ากลูแคนจากสาหร่ายในอาหารสุกรต่อประสิทธิภาพการเจริญเติบโตและการตอบสนองทางภูมิคุ้มกันแบบจำเพาะในสุกรอนุบาลและสุกรขุนเล็ก โดยแบ่งสุกรหย่านมสามสายพันธุ์เป็น 3 กลุ่มการทดลอง ได้แก่ กลุ่มควบคุมที่ได้รับอาหารสูตรมาตรฐาน กลุ่มทดลองที่ 2 และ 3 ได้รับอาหารชนิดเดียวกันที่เสริมด้วยสารเสริมเบต้ากลูแคนจากสาหร่ายในปริมาณ 100 และ 200 กรัมต่อตันตามลำดับ จากการทดลอง ไม่พบความแตกต่างอย่างมีนัยสำคัญของประสิทธิภาพการผลิตและอัตราการเจริญเติบโตของสุกรสุกร การทดสอบการตอบสนองของระบบภูมิคุ้มกันที่จำเพาะหลังการฉีดวัคซีน CSFV และ PRRSV พบว่าสุกรที่ได้รับสารเสริมเบต้ากลูแคนจากสาหร่ายในอาหาร มีระดับ neutralizing antibody ต่อ CSFV สูงขึ้นอย่างมีนัยยะสำคัญ นอกจากนี้ พบการเพิ่มขึ้นของจำนวนเซลล์ CD4+(T lymphocytes) และ CD4+CD8+ (putative memory T lymphocytes) ซึ่งสร้างไซโตคายน์ IFN-g+ ที่มีความจำเพาะต่อ CSFV และ PRRSV ในกลุ่มสุกรที่ได้รับสารเสริมเบต้ากลูแคนจากสาหร่ายอีกด้วย จากการศึกษาสรุปได้ว่า ถึงแม้ว่าการให้สารเสริมเบต้ากลูแคนจะไม่สามารถเสริมสมรรถภาพการผลิตของสุกรได้ แต่มีคุณสมบัติทางชีวภาพในการเป็นสารกระตุ้นภูมิคุ้มกันซึ่งอาจเป็นการเพิ่มความต้านทานโรคติดเชื้อไวรัสในสุกรอนุบาลและสุกรขุนเล็กได้

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Sh.Boonnithi Chuaychu : EFFECTS OF ALGAE-DERIVED ß-GLUCAN SUPPLEMENTATION ON PRODUCTION PERFORMANCES AND SPECIFIC IMMUNE RESPONSES IN NURSERY TO GROWING PIGS. Advisor: Prof. Dr. ROONGROJE THANAWONGNUWECH, D.V.M., Ph.D., D.T.B.V.P Co-advisor: Dr. TEERAWUT NEDUMPUN, D.V.M., Ph.D.,Prof. Dr. PADET TUMMARUK, D.V.M., M.Sc., Ph.D., D.T.B.V.P

ß-glucan has been widely used as a feed additive in pigs. Supplementation of ß-glucan in pig's diet has been believed to improve growth performance and modulate host immunity of the pigs. However, there is no precise information associated with effects of algae-derived ß-glucan supplementation on comprehensive swine health and immunological responses especially the specific immunity in pigs. Hence, the objective of this study was to evaluate the effects of ß-glucan derived from algae supplementation on production performances and specific immunity against swine viral diseases in nursery to growing pigs. The sixty days of trial was done in three hundred and thirty-six crossbreed 4-week-old pigs. Weaning pigs were assigned into 3 groups. Control group was fed a standard feed formular. Treatment group 2 and 3 were fed the same diet supplemented with ß-glucan extracted from algae sources in 100 and 200 grams per ton, respectively (BG 100 and 200 g/ton). No significant difference of production performances was observed among the groups of pigs. In order to examine the specific immune responses following CSFV and PRRSV vaccination, the measurement of neutralizing antibody level and in vitro activating specific T cell were performed. Interestingly, the pigs supplemented with algae-derived ß-glucan showed significant higher level of CSFV neutralizing antibody titers regardless of ß-glucan concentration, as compared to control group. In addition, increased numbers of virus-specific IFN-g^{+} producing CD4^{+} and double CD4^{+}CD8^{+} (putative memory T cell) cells in the ß-glucan-supplemented pigs were significantly observed. Here, the finding confirmed that in addition to supporting of production performances, the algae-derived ß-glucan possesses biological potential as an immunomodulatory substance, which might contribute to disease resistance in the growing pigs.

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List of abbreviations

ADFI	average daily feed intake
ADG	average daily gain
APP	*Actinobacillus pleuropneumoniae*
BG	ß-glucan
bw	body weight
cm	centimeter
CR	complement receptor
CSFV	classical swine fever virus
ct	cycle threshold
DNA	deoxyribonucleic acid
e.g.,	exampla gratia, for example
et al.	et alii, and others
EU	European union
G	gravity
g	gram (s)
h	hour (s)
FACS	fluorescence-activated cell sorting
FCR	feed conversion ratio
FITC	fluorescein isothiocyanate
FMD	foot and mouth disease
IEC	intestinal epithelial cells
IFN	interferon
IgG	immunoglobulin g
IL	interleukine
IPMA	immunoperoxidase monolayer assay
Kb	kilo base pair
kg	kilogram (s)
L	liter (s)
m	metre (s)
M	molar (s)

mAb	monoclonal antibody
ME	metabolic energy
MEM	minimum essential media
mg	milligram (s)
min	minute (s)
ml	milliliter (s)
m.o.i	multiplicity of infection
mm	millimetre (s)
NDV	Newcastle disease virus
nm	nanometer
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PCV	Porcine circovirus
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PMWS	post-weaning multisystemic wasting syndrome
PRDC	porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
qPCR	quantitative polymerase chain reaction
rt-PCR	reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
rpm	round per minute
rt-PCR	reverse transcriptase polymerase chain reaction
S.D.	standard deviation
sec	second (s)
TCID	tissue culture infective dose
TLR	toll-like receptors
TNF	tumor necrosis factor
USA	the United states of America
%	percentage

°C	degree Celsius
µl	microliter (s)
µm	micrometer (s)

CHAPTER I

INTRODUCTION

Thailand is one of the biggest swine production countries in the South East Asia. Since the regulation of living pig/pork product transportation was implemented during African swine fever virus (ASFV) outbreak in the region, six thousand ASFV- free pigs, including fattening pigs, piglets as well as processed pork product, had been daily exported from Thailand to the neighbouring countries (Anderson, 2020). Due to the increased demand of domestic and international exports, ex-farm pork price was rose dramatically (The Swine Raisers Association of Thailand, 2020). Hence, the swine production in Thailand is needed to be increased.

Nursery period is the most critical stage in the chain of swine production. The piglets are usually suffered by several stressors during transitional/post-weaning phase, including 1) adaptation to a new environment (changing of temperature and humidity), 2) transformation of diet from fresh milk to commercial pellet feed, 3) arrangement of their social hierarchy, and 4) High risk of pathogen exposure during transitional phase, in which piglets are mingled from different litters. These stressor factors could cause decreasing in piglet feed intake during the first week of post-weaning stage (McCracken et al., 1999). Impacts of stressors on physiological function including enzymatic and epithelial functions, as well as mucosal immune responses in the gut of piglets are disrupted resulting in poor growth performance in the overall pig production stages (Campbell et al., 2013). In addition, as the window of susceptibility exists in the nursery period, the piglets are commonly susceptible to various viral pathogens and secondary bacterial infection, which may cause high morbidity and mortality in that affected population. Hence, the principal of swine farming management puts an intensive economical effort on decreasing of the detrimental aspects caused by weaning stressors and infectious disease, especially during the nursery period.

Accordingly, pig producers attempt to notice several health managements and preventive strategies to maintain health and improve the productivity during nursery period. Immunizations provide disease-specific individual and herd immunity, and potentially decrease susceptibility of the infection (Rose and Andraud, 2017). Apart from immunization, solid biosecurity would help to control disease entry and

spreading (Backhans et al., 2015). Since feed additive has been introduced into swine nutritional formula, it has been shown positive effects on the swine health and production performance. Currently, supplementation with antimicrobial drugs as a growth promotor is prohibited (Cromwell, 2002) due to the matter of public health concern regarding antibiotic residues in the meat products. Thus, the pig producers need other alternative approaches to sustain swine production performances. There are several non-antibiotic feed additives, by which they could improve health, enhance immunity and productivity in pigs - for example, acidifiers, minerals, prebiotics, probiotics, yeast products, nucleotides, and plant extracts (Liu et al., 2018). Beta-glucan (ß-glucan) is one of the most alternative feed additives used in the livestock animals. ß-glucan is a natural heterogenous glucose polysaccharide comprise ß-(1,3) glycosidic linkage backbone with various side chains (Kim et al., 2011). ß -glucan can be extracted from cell wall of plant, algae and yeast, which are different structures and (Vetvicka et al., 2008). Previous studies demonstrated that ß-glucan supplementation could improve growth performance and immunostimulatory capacity (Vetvicka and Oliveira, 2014; Vetvicka et al., 2014). Focusing on the pig industry, additional of ß-glucan in diet is an optional management to increase swine production performances. It had been reported that supplementation with ß-glucan could enhance the tight junction protein (claudin, occluding and MUC2) at the intestinal mucosa and, therefore, reducing clinical outcome of the *Escherichia coli*-infected pigs (Kim et al., 2019). In addition, ß-glucan itself would provide immunomodulatory effects on porcine innate (Vetvicka et al., 2014) and adaptive immunity (Wang et al., 2008)

ß-glucan derived from *Euglena gracilis*, is now refined into a commercial dietary supplement in humans and animals. Although, better in health benefit of ß-glucan supplementation has been proven through improving of growth performance and possessing of immunostimulatory capacity in humans, there was no further information associated with effects of algae-derived ß-glucan supplementation on swine health and immunological responses especially adaptive immunity. Therefore, insight in effects of algae-derived ß-glucan on enhancing of production performances and viral specific immunity in growing pigs are of interest. The data paved understanding in effect of ß-glucan supplementation on swine health and specific immunological advantage.

Objectives of study

1. To evaluate effects of algae-derived ß-glucan supplementation on production performances in growing pigs.
2. To determine effects of algae-derived ß-glucan supplementation on viral specific immune responses in growing pigs.

Question of study

Can supplementation of algae-derived ß-glucan in growing pigs enhance growth performance and virus-specific immune responses?

CHAPTER II

LITERATURE REVIEW

Stressors in nursery pigs

Nursery is a critical period in the swine production chain. The study from Paredes et al. (2012) showed that weaning weight and final weight at the first two weeks of nursery significantly contribute to overall pig's performance. During transitional period, the nursery pigs usually are suffered by several stressors, as the pigs are moved to the new environment. Physical factors including animal handling, transportation, and fluctuation of temperature and humidity in the new housing could induce stress condition in pigs. Since social hierarchy is usually observed among the pigs, co-mingling of the pigs at post-weaning period also increase the stress. (Salazar et al., 2018). Various stressors appeared during nursery period significantly impair swine production performances through decreased diet consumption of the pigs. Transportation and establishing social hierarchy cause low feed in take in early nursery period (Le Dividich and Sève, 2000). The stress and starvation cause decrease feed intake in weaning period contributes to intestinal barrier disruption such as decline paracellular transport and the impairment of mucosal integrity (Spreeuwenberg et al., 2001). Nursery pigs confront with physiological and functional changes of their gastrointestinal system which affect to the digestive and absorption of the nutrient (Pluske et al., 1997). The gut structure and function alterations include villous atrophy, crypt elongation and reduction of intestinal/pancreatic enzyme until the first two weeks post-weaning (Hampson, 1986). Taken together, the intestinal mucosal disfunctions probably cause inflammation at the gastrointestinal system and post weaning diarrhea resulted in poor growth performance in weaning pigs (Lackeyram et al., 2010). So, it is important that some strategy or intervention are needed to ameliorate negative impacts of post-weaning stress, which might enhance productivity and immune responses in the piglets.

Challenging issues of porcine viral infection during nursery period

Nursery piglets encounter to several types of stressors leading to disease susceptibility (Moeser et al., 2017). There are many endemic viral diseases causing high mortality during nursery to growing period. Porcine reproductive and respiratory

syndrome (PRRS) is one of threatening diseases to the swine industry globally. PRRSV is a positive single strand, enveloped virus that is approximately 15.0 kb in length and 50 – 60 nm. in diameter. PRRSV is classified in a member of family *Arteriviridae*, genus Arterivirus. This virus composes of 2 genotypes; Western Europe (genotype 1) and North America (genotype 2) (Kuhn et al., 2015). PRRSV usually plays a role as primary infection in porcine respiratory disease complex (PRDC). PRRSV suppresses the host immune defense mechanism causing secondary or opportunistic infection (Lunney et al., 2016). PRDC is caused by multifactual pathogens resulting in deleterious economic impact to piggeries. PRDC is commonly associated with PRRSV and other viruses such as porcine circovirus and swine influenza virus. Porcine circovirus (PCV) is the smallest, single-stranded non-enveloped DNA virus with circular shape, genomes. The virus is categorized in the family *Circoviridae*, genus circovirus. PCV has been classified into 4 groups including non-pathogenic PCV1, pathogenic PCV2 and PCV3 and recently identified PCV4 with the high prevalence of PCV2 (Zhang et al., 2019). As a non-enveloped virus, PCV2 is resistant to the heat and chemical agents and persists for long-term in the surrounding environment. PCV2 induces lymphoid depletion and impairs pig's immune functions leading to other disease susceptibility. PCV2 has been known as a major causative agent of porcine circovirus-associated diseases (PCVAD) including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), exudative epidermitis, and necrotizing lymphadenopathy as well as reproductive failure. Classical swine fever (CSF) or hog cholera is a major viral disease caused by a small, enveloped RNA virus. CSFV is a member of the family *Flaviviridae*, genus Pestivirus, which is known as a contagious viral disease of domestic and wild pigs. CSFV has many phases of clinical diseases composing acute, subacute, chronic and subclinical forms depending on strains of the virus. PRRSV, PCV2 and CSFV are capable of suppressing host immune system contributing to secondary or opportunistic infection and causing high mortality rate in the growing pigs (Thacker, 2001) These aforementioned viral diseases could be prevented and controlled by adequate immunization (Gebhardt et al., 2020). The growth competency is another concern for comprehensive approach in disease prevention and control. The protective immunity against diseases should be appeared

before the occurrence of natural infection. Therefore, the pigs require effectively viral-specific immune response after vaccination. Accordingly, dietary efficiency also be needed to reach the standard of pig performance. Supplementation with feed additive is alternatively considered as health promotor and immunomodulator, which possibly sustain the pig performances and disease control in order to maximize the profit in the production chain.

Biological structure of ß-glucan

Beta-glucan (ß-glucan) is a natural insoluble polysaccharide of glucose-D monomer containing ß-(1,3) backbone linked by (1,4) and/or (1,6) glycosidic linkage side chain. These ß-glucan can be found in the cell wall of grains (oat and barley), seaweed, fungi (yeast and mushrooms), and some bacteria. Bioactivities among ß-glucan are different depending on molecular weight, sources, solubility, and molecular structure (Akramiene et al., 2007). For example, ß-glucan derived from oat and barley are linear ß-(1,3) linked backbone and short ß-(1,3) side chain, while *Saccharomyces cerevisiae* or baker's yeast has ß-(1,3), (1,6) branched structure. *Euglena gracilis* is a fresh water alga that produces huge of linear ß-glucan which called as paramylon in its cytoplasm. *E. gracilis* can produce and accumulate large amounts ß-glucan as granules in the cytoplasm up to 95% of the cell mass which contain almost 100% glucose (Russo et al., 2016). *E. gracilis* can be cultured by both photosynthetic and heterotrophic conditions, which may result in different quantity of ß-glucan. X-ray diffraction showed the high crystallinity structure of ß-glucan from *E. gracilis* (Barsanti et al., 2011). Therefore, the process of ß-glucan isolation can be done by disrupting the cell membrane and purifying the granule via low concentration of the detergent that makes ß-glucan extracted from euglenoid is quite cheaper than that of other sources (Gissibl et al., 2019).

Potential mechanisms of ß-glucan

Following consumption of ß-glucan, undigested ß-glucan passes from stomach to the intestinal epithelium. The main localization sites of ß-glucan are at the intestinal epithelial cells (IECs) and microfold (M) cells within the Peyer's patches. ß-glucan is considered as a microbial product, known as a pathogen-associated molecular pattern (PAMP). Pattern recognition receptors (PRRs) on the surface of innate immune cells

(macrophage, dendritic cell, monocyte and epithelial cell) recognizes the repeating pattern of ß-glucan structure (Sonck et al., 2010). Dectin-1 plays a major role as a receptor at IECs, while M-cells appear to be dependent on both dectin-1 and toll-like receptor 2 (TLR2) (Rice et al., 2005). Then, ß-glucan is recognized by CXCR3 macrophages or CD103 of dendritic cells. These cells migrate from bloodstream or lymph vessel throughout the body to enhance the biological activities of bone marrow and lymphoid tissues resulting in effector cell differentiation. In addition, macrophages can breakdown ß-glucan particle into small particles. The fractions of particles are secreted from macrophages, and subsequently recognized by complement receptor 3 (CR3) on phagocytic cells. These activated phagocytes secrete various pro-inflammatory cytokines to activate adaptive immunity and T-cell proliferation (Hong et al., 2004). Although the immunological modulatory roles of ß-glucan in human had been proven, the direct evidence of adaptive immune induction in ß-glucan supplemented animals remains inconclusive.

ß-glucan usage in pigs

Effects of ß-glucan on health performances

ß-glucan supplementation has been used as a feed additive in swine intensive farming. Previous studies demonstrated that ß-(1,3) glucan had an immunomodulatory effect and increased growth performance of pig (Wu et al., 2018). In consideration of swine productivity, additional of ß-glucan as a diet supplementary could increase body weight, average daily gain and feed conversion ratio (Park et al., 2018). ß-glucan dietary supplement improved ADG and FCR and also dry matter digestibility in starter to finishing pigs (Zhao et al., 2012; Lee et al., 2016; Luo et al., 2019b). Besides, supplementation with 100 mg/kg ß-glucan in diet of the finishing pigs could increase ADG, FCR, carcass length and meat quality (Luo et al., 2019c). Previous studies report that ß-glucan has beneficial effects on swine intestinal health. ß-glucan supplementation could decline the number of coliform bacteria (Zhou et al., 2013), while it increased beneficial microorganism subpopulation in the weaning pigs (Murphy et al., 2012). Likewise, ß-glucan dietary supplement combined with medical supportive treatment for rotavirus infection significantly decreased intestinal injury and restored fecal score of the weaning pigs (Chethan et al., 2017). Although pigs are grown in low

sanitary environment, supplementation with yeast ß-glucan does mitigate the frequency of diarrhea by increasing in colonization of lactobacilli. The lactic acid bacteria can interfere with pathogen overgrowth and promoting of intestinal integrity. Interestingly, ß-glucans have been known as nutrient sources for intestinal microbiota. ß-glucan also has impact on the increase the number of beneficial bacteria colonization in the intestine. The gut microorganisms ferments ß-glucans and produce short chain fatty acid (SCFA) in the caecum and colon which can be energy source for intestinal epithelium (Thandapilly et al., 2018). Interestingly, consumption of algae-derived ß-glucans effect on the intestinal barrier by improving tight junctions and decrease gut permeability (Kim et al., 2019). ß-glucan could play a role as prebiotic, by which it can regulate a gut microbiota leading to improve animal health and performance (Jayachandran et al., 2018).

Effects of ß-glucan on immunological responses

Many studies have reported several positive effects of ß-glucan supplementation on immunological properties of the pigs. ß-glucan modulated immune response by down-regulation of serum haptoglobin, cortisol and pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) (Kim et al., 2019). Down-regulation of pro-inflammatory cytokine (TNF-α, IFN-γ, IL-6, TLR-2) in ß-glucan-supplemented diet significantly decreased post-weaning stress-induced physiological disorder in piglets (Upadhaya et al., 2019). Moreover, the algae-derived ß-glucan plays a role as an immunostimulant, as ß-glucan is recognized by the receptors of innate immune cells, subsequently enhances non-specific immune functions (Baert et al., 2015). Pigs supplemented with insoluble ß-glucan showed significantly higher phagocytic activity of macrophages and neutrophils (Vetvicka and Oliveira, 2014). The study of Sonck et al. (2010) demonstrated that ß-glucan fr*om E. gracilis* facilitated lymphocyte proliferation *in vitro*. In addition, the weaning pigs fed with ß-glucan exhibited higher T-cell proliferation and significantly increased CSFV-specific antibody following vaccination (Wang et al., 2008). Recently, there was a significant enhanced of antibody response against CSFV in starter to growing pig supplemented with ß-glucan rich molasses yeast powder diet (Pornanek and Phoemchalard, 2020).

As nursery period is a critical period in the chain of swine production, physiological and biological stressors dramatically contribute to disease susceptibility in the growing pigs. Using of antibiotic as a growth promoter in nursery pigs was prohibited in EU since 2006 onwards (Anadon, 2006) and this issue had been implemented in Thailand. Thus, a feed additive will be an alternative approach to enhance swine production. From aforementioned above, supplementation of ß-glucan might provide promising advantages on swine health. Nevertheless, the effect of algae-derived ß-glucan supplementation on both production performances and systemic immunological responses in the nursery pigs still be limited. Thus, our study aimed to investigate the effects of algae derived ß-glucan as a dietary supplementary on swine production performances and viral specific immune responses. The data could pave understanding in effect of ß-glucan supplementation on swine health and specific immunological advantage.

CHAPTER III

MATERIALS AND METHODS

Experimental designs

The experiment was applied a randomized complete block design by sex and initial weight. A total of 336 crossbreed 4-week-old pigs (Landrace x Large white x Duroc) with average of initial body weight 6.8 ± 1.2 kg were randomly assigned into 3 experimental groups with 14 pen per treatment containing 8 piglets in each pen. The experiment lasted for 60 days from 4 to 12 weeks old of age. The control group were given standard feed formula, which is routinely used in the farm. β-glucan supplement, Aleta™ algae-derived β-glucan (Kemin Industries, Inc., Des Moines, IA; effective constituent is 50% β-1,3-glucan) at 100 g/ton and 200 g/ton in treatment group 2 and 3, respectively, were given to the pigs at the fourth day of the experiment after being acclimatized.

Animals, housing and management

The study was conducted in the SPM Agro testing station, a commercial swine herd in the western region of Thailand. The pigs were raised in a conventional evaporative cooling system and were housed in 190 x 200 x 50 cm solid bottom with open top cage. The temperature and relative humidity were maintained at 28 – 32°C and 60 – 80 %, respectively. Weaning pigs were kept under the same conditions and fed with the same proper diet for 3 days before starting the experiment. The pigs were fed with 3 standard feed formula which were different by age according to the farm procedure. Phase 1 was from 4- 8 weeks old, phase 2 was from 8-10 weeks old and phase 3 was from 10-13 weeks old. All pigs allowed to access ad libitum on feed and water. The vaccination program was also provided by the testing station protocol as shown in Table 1.

All methods and animal care used in this study were conducted under the approval of Chulalongkorn University Animal Care and Use Committee Animal Care and Use Committee. The number of project approval is 2031007.

Table 1 Vaccination program in the animal experiment.

Age (week)	Vaccine	Product
2	porcine reproductive and respiratory syndrome virus (PRRSV)	Ingelvac® PRRS MLV, Boehringer Ingelheim, St.Joseph, Missouri 64506 USA
3	Porcine circovirus 2 (PCV2)	Porcine Circovirus Vaccine, Type 2, killed Baculovirus vector, Intervet lnc. Delaware, USA
	Classical swine fever (CSF)	COGLAPEST®, Ceva, S.A.
5	Classical swine fever (CSF)	COGLAPEST®, Ceva, S.A.
7	Porcine circovirus 2 (PCV2)	Porcine Circovirus Vaccine, Type 2, killed Baculovirus vector, Intervet lnc. Delaware, USA
8	*Actinobacillus pleuropneumoniae* (APP)	COGLAPIX®, Ceva, S.A.
9	Foot and mouth disease (FMD)	DLD, Thailand
10	*Actinobacillus pleuropneumoniae* (APP)	COGLAPIX®, Ceva, S.A.
11	Pseudorabies (AD)	Porcilis® BEGONIA, Intervet International B.V., BOXMEER - HOLLAND

Clinical observation and production performances parameters

General health of the experimental pigs was monitored daily by a field veterinarian. Fecal scores of each pen were recorded daily. The four fecal consistency categories ranged from 1 – 4 (normal, cream, loose, watery). Body weight and feed intake were collected and used for estimating the production performance of the pigs. Average daily gain (ADG), feed gain (FG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were defined as the period of feed formula (1: 4 - 8 weeks old,

2: 8 -10 weeks old and 3: 10 – 13 weeks old, respectively) and throughout experimental period. Body weight of pigs were individually measured at 4, 8, 10 and 13 weeks old using digital scale. Average daily weight gain (ADG, g/day) of the pigs were calculated as: ADG (g/day) = (body weight at the last day of feed formula – body weight at starting period/ duration of feed formula) x 1000. Furthermore, feed intake in each pen were collected daily and the average daily feed intake (ADFI) of the pigs were an average amount of feed intake per day divided by the number of lived pigs each day. Besides, feed gain of the pigs was individually calculated by feed intake divided by weight gain. And, feed conversion ratio (FCR) at each phase were calculated by dividing the feed intake through the weight gain of the pigs. Lastly, the number of removal pigs were used for calculating mortality rate in each phase.

Cells and viruses

CSFV (strain ALD) and PRRSV (strain 01NP01) were kindly given from the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). Virus propagation and titration for PRRSV and CSFV were performed in MARC-145 cell and SK-6 cell, respectively. Mock-infected cell lysates (mock) were prepared from MARC-145 (for PRRSV) and SK6 (for CSFV) cells.

Antibodies and secondary conjugates

Anti-porcine CD3-FITC monoclonal antibody (mAb) (IgG2b, BB23-8E6) was purchased from Southern Biotech (Birmingham, AL, USA). Anti-porcine CD4-PE mAb (IgG2b, 74-12-4), purified anti-porcine CD8 mAb (IgG2a, 295/33-25) and biotinylated anti-porcine IFN-γ mAb (IgG2a, P2C11) were purchased from BD Bioscience (San Jose, CA, USA). For secondary conjugates, PE-conjugated goat anti-mouse IgG was purchased from Abcam (Cambridge, MA, USA). Streptavidin APC/Cy 7 was purchased from Biolegend (San Diego, CA, USA).

Sample collection for immunological studies

Blood collections were performed at 4, 8, 10 and 13 weeks old (14 samples per group, randomly 1 pig per pen). Blood samples were collected by venipuncture of jugular vein using 18-gauge needle. Five to six ml of whole blood were kept in lithium heparinized blood tube for PBMCs isolation. The sera were also obtained, aliquoted

and stored at -80°C for virological and immunological studies.

Virological studies

Quantitative real-time PCR for porcine circovirus 2 (PCV2) detection

Virus DNA samples were extracted from 200 µl of serum using Nucleospin DNA extraction kit (Macherey–Nagel, Duren, Germany) according to the manufacturer's instructions. Quantification of PCV2 ORF1 gene in serum was performed by a QuantStudio™ 5 real-time PCR System (Applied Biosystems, Foster City, CA, USA). The primers and probes have been described in the previous study (Wang et al., 2019). The amplification reaction was as the following, initial step 95°C for 3 min, and denaturation step at 95°C for 30 sec for 40 cycles, annealing at 51°C for 30 sec, extension at 72°C for 1 min and final extension step at 72°C for 10 min.

Quantitative real-time PCR for porcine reproductive and respiratory syndrome virus (PRRSV) detection

Total viral RNA samples were extracted from 200 µl of serum sample using Nucleospin RNA extraction kit (Macherey–Nagel, Duren, Germany) according to the manufacturer's instructions. The RNA quantification was assessed by QuantStudio™ 5 real-time PCR System (Applied Biosystems, Foster City, CA, USA) as previously published (Egli et al., 2001).

Immunological studies

The sera preparation for humoral immune (HI) responses

Two hundred µl of sera (total of 168 samples at 4 different time points) were used for downstream assay of serum PRRSV and CSFV neutralization. The serum inactivation was performed by heating at 56°C for 30 min.

Serum neutralization (SN) for porcine reproductive and respiratory syndrome (PRRSV)

The sera were diluted into two-fold dilution in Minimum Essential Medium Eagle medium (MEM) (Gibco, Carlsbad, CA, USA) before titration. The serial diluted the sera were mixed with the same volume of 200 $TCID_{50}$ PRRSV, 01NP1 strain and incubated at 37°C for 1 hour. Then, the mixtures were transferred to 70 - 80 % confluent MARC-145 cell in 96 flat-bottomed microtiter plates and again incubated for

further 72 hours in 5% CO_2, 37°C incubator. The titers of PRRSV neutralizing antibodies ≥1:2 (1log2) were considered as positive. To confirm the presence of PRRSV, IPMA assay was performed as described earlier (Thanawongnuwech et al., 1998).

Serum neutralization (SN) for Classical swine fever virus (CSFV)

The sera were diluted into two-fold dilution manner in MEM (Minimum Essential Medium Eagle) medium before the titration. The serial diluted the sera were mixed with the same volume of 300 $TCID_{50}$ CSFV, ALD strain following by incubation at 37°C for 1 hour. Then, the mixtures were transferred to 70 - 80% confluent SK-6 cell in 96 flat-bottomed microtiter plates and incubated for further 72 hours in 5% CO_2, 37°C incubator. CSFV detection was performed as previously described (Panyasing et al., 2018). The titers of CSFV neutralizing antibodies ≥1:5 (1log2) were considered as positive protection.

Isolation of peripheral blood mononuclear cell (PBMCs) for evaluation of cell-mediated immune (CMI) responses

PBMCs were isolated based on the previous protocols (Suradhat et al., 2015). The Beckman Coulter Z2 Particle Counter (Beckman Coulter, CA, USA) was used for cell counting. Then, 5 x 10^5 cells per100 µl of PBMCs diluted in RPMI medium were plated into a flat-bottomed 96-well microtiter plates.

***In vitro* activation**

To determine PRRSV and CSFV-specific immune responses after vaccination, *in vitro* activation was performed in a flat-bottomed 96-well plate by plating 5 × 10^5 PBMCs into each well and then cultured with 0.1 multiplicity of infection (m.o.i.) of each virus and mock at 100 µl/well. The culture was then incubated at 37°C in CO_2 incubator for 48 hours. following the incubation, 1 µl/ml of GolgiPlug™ (protein transport Inhibitor, BD Biosciences, San Jose, CA, USA) was added for 4 hours to block protein secretion prior to harvesting and immunofluorescent staining.

Immunofluorescent staining for flow cytometric analyses

The cells were harvested and transferred into 96-well round bottom plates and then washed twice with FACs buffer. Different types of isotype control were prepared to set background cut-off. For surface immunofluorescent staining; CD3, CD4 and CD8. The concentration of primary mAbs were performed using 1:50 of anti-porcine CD3-

FITC, 1:50 of anti-porcine CD4 PE, and 1:50 anti-porcine purified CD8, diluted in FACS buffer at final volume 50 µL/reaction, were added and further incubated in the dark at 4°C for 30 min. For secondary staining, 1:100 of goat anti-mouse PE conjugate, was diluted in FACS buffer and added at 4°C in the dark for 20 min.

For intracellular cytokine staining, the cells were fixed with 100 µL of BD Cytofix™ (BD Biosciences, San Jose, CA, USA) in the dark at room temperature for 30 min. Permeabilization step, the cells were incubated with BD Perm/wash buffer (BD Biosciences, San Jose, CA, USA) in the dark at 4°C for 15 min. For primary staining, 1:50 of biotinylated anti-IFN-γ diluted in BD perm/wash buffer (BD Biosciences, San Jose, CA, USA) was added to the cells following by incubated in the dark at 4°C for 30 min. For secondary staining, 1:200 of streptavidin APC/Cy 7 was diluted in BD perm/wash buffer (BD Biosciences, San Jose, CA, USA) and incubated the cells in the dark at 4°C for 20 min. Sample analysis performed by gating the cells at least 1×10^5 cells/event. Flow cytometric analyses were performed using FC 500 MPL (Beckman Coulter, CA, USA).

Statistical analysis

The statistical analyses on production performances including fecal score, body weight, feed intake, ADG, ADFI, feed gain, FCR and mortality rate were analysed using SAS (version 9.0, SAS Institute Inc., Cary, NC, USA). Descriptive statistics were generated using the MEANS procedure of SAS. Pen was considered as an experimental unit. The piglets were randomly introduced into each group. Virological and specific immune response statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software Incorporated, San Diego, CA, USA). Multiple comparison tests composing of Tukey's multiple comparison tests and Kruskal Wallis were used for identification the significant differences between treatments for the data that do not normally distributed. P value < 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

Fecal score of the growing pigs

To investigate the effect of algae-derived ß-glucan supplementation on fecal characteristic in the nursery to growing pigs, 100 or 200 g/ton of ß-glucan from algae source was supplemented into the feed from 4 to 13 weeks old. The piglets in control and ß-glucan-supplemented groups exhibited diarrhea in the first two weeks after moving to the nursery units. The fecal scores of those piglets were restored to normal feces at 6 weeks old. However, the results showed no significant differences in fecal scores among the experimental groups ($P > 0.05$) (Figure 1).

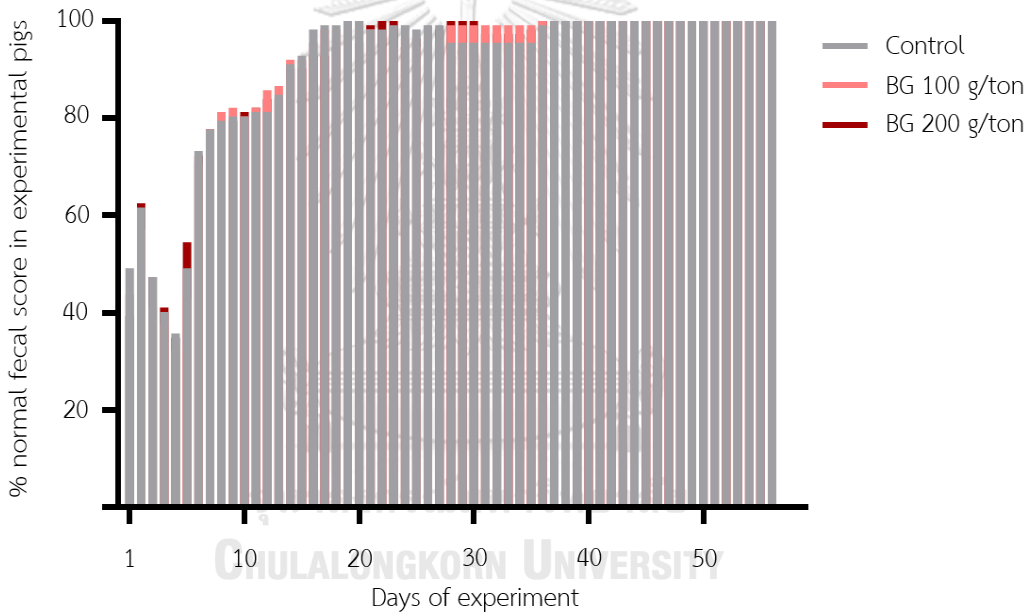


Figure 1 Daily percentage of normal fecal score between control and algae derived ß-glucan supplemented 100 and 200 g/ton groups.
Each bar represents the percentage of normal fecal score of each experimental group. Statistical analyses were done using one-way ANOVA. No significant difference was observed throughout the experimental period ($P > 0.05$).

Production performances of the growing pigs

To examine the effect of algae-derived ß-glucan supplementation 100 and 200 g/ton on production performances in nursery to growing pigs. There were no significant

differences on average weights and ADG (Table 2). Furthermore, no difference was found in feed intake, feed gain, ADFI and FCR of pigs throughout the experiment among the experimental groups. And, no significant difference mortality rate differences between control and treatment groups were found (P > 0.05) (Table 3).

Table 2 Complete descriptive statistics of algae-derived β-glucan supplementation on production performances (mean ± SD).

	Groups			
	Control	**BG 100 g/ton**	**BG 200 g/ton**	***P* - value**
Average body weight, kg				
4 weeks	6.84 ± 1.20	6.84 ± 1.03	6.84 ± 1.10	*P* > 0.99
8 weeks	15.67 ± 1.94	16.10 ± 2.37	16.03 ± 2.40	*P* > 0.99
10 weeks	27.95 ± 2.46	28.34 ± 3.34	28.16 ± 2.88	*P* > 0.99
13 weeks	37.65 ± 3.06	37.94 ± 3.62	37.49 ± 3.83	*P* > 0.99
ADG, g/d				
4 - 8 weeks	0.28 ± 0.05	0.29 ± 0.05	0.29 ± 0.06	*P* = 0.82
8 - 10 weeks	0.50 ± 0.06	0.52 ± 0.08	0.50 ± 0.08	*P* = 0.79
10 - 13 weeks	0.75 ± 0.10	0.71 ± 0.08	0.72 ± 0.10	*P* = 0.55
4 - 13 weeks	0.42 ± 0.06	0.49 ± 0.05	0.43 ± 0.06	*P* = 0.90

Table 3 Complete descriptive statistics of algae-derived β-glucan supplementation on production performances (mean ± SD).

	Groups			
	Control	**BG 100 g/ton**	**BG 200 g/ton**	***P* - value**
Feed intake, g/d				
4 - 8 weeks	66.2 ± 18.66	71.97 ± 11.12	70.06 ± 12.14	*P* = 0.82
8 - 10 weeks	96.81 ± 24.76	101.39 ± 25.82	97.95 ± 17.32	*P* = 0.79
10 - 13 weeks	80.95 ± 25.86	82.89 ± 14.32	80.10 ± 16.70	*P* = 0.55

Feed gain				
4 - 8 weeks	1.05 ± 0.18	1.12 ± 0.06	1.11 ± 0.15	*P* = 0.28
8 - 10 weeks	1.51 ± 0.09	1.46 ± 0.16	1.53 ± 0.16	*P* = 0.42
10 - 13 weeks	1.52 ± 0.13	1.62 ± 0.16	1.57 ± 0.14	*P* = 0.25
4 - 13 weeks	1.35 ± 0.09	1.38 ± 0.07	1.38 ± 0.08	*P* = 0.38
ADFI, g/d				
4 - 8 weeks	0.30 ± 0.08	0.32 ± 0.05	0.32 ± 0.05	*P* = 0.44
8 - 10 weeks	0.75 ± 0.09	0.76 ± 0.15	0.77 ± 0.11	*P* = 0.95
10 - 13 weeks	1.14 ± 0.16	1.14 ± 0.11	1.12 ± 0.16	*P* = 0.97
4 - 13 weeks	0.57 ± 0.09	0.60 ± 0.08	0.59 ± 0.08	*P* = 0.67
FCR				
4 - 8 weeks	1.04 ± 0.34	1.20 ± 0.30	1.15 ± 0.30	*P* = 0.39
8 - 10 weeks	1.37 ± 0.34	1.62 ± 0.41	1.67 ± 0.51	*P* = 0.15
10 - 13 weeks	1.48 ± 0.54	1.87 ± 0.60	1.67 ± 0.68	*P* = 0.25

Table 4 Descriptive statistics of algae-derived ß-glucan supplementation on the percentage of mortality in experimental pigs (mean ± SD).

	Groups			
	Control	**BG 100 g/ton**	**BG 200 g/ton**	***P* - value**
Mortality, %				
4 - 8 weeks	14.29 ± 9.63	15.179 ± 8.74	16.96 ± 6.21	*P* = 0.69
8 - 10 weeks	16.62 ± 17.89	15.58 ± 12.29	16.68 ± 15.69	*P* = 0.98
10 - 13 weeks	0	2.39 ± 6.01	0	*P* = 0.97
4 - 13 weeks	33.04 ± 21.15	30.36 ± 12.7	31.25 ± 11.76	*P* = 0.90

Quantification of PCV2-genomic DNA in sera of the growing pigs

To investigate the effect of ß-glucan supplementation on PCV2 viremia, DNA genomic DNA of PCV2 from experimental pigs were quantitated by real-time PCR. Presence of PCV2 genomic DNA in sera of the pigs were observed throughout the observation period and the peak of viremia were appeared at 8 weeks old.

Nevertheless, the level of PCV2 viremia were not significant differences among the experimental groups ($P > 0.05$) (Figure 2). The results suggested that the experimental pigs were exposed with PCV2 during the period of observation. In addition, supplementation of algae-derived ß-glucan barely controlled PCV2 viremia of the growing pigs.

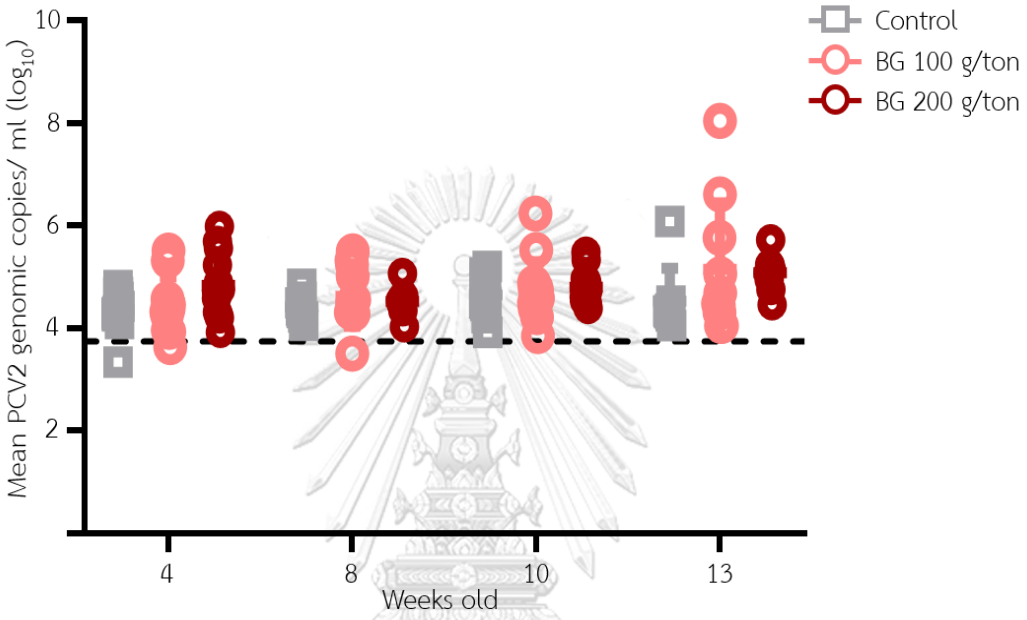


Figure 2 Mean values of PCV2 DNA genomic copy number (log_{10}) in the sera from each treatment group.

All pigs were immunized PCV2 vaccine at 3rd and 7th weeks old. After supplementation with algae-derived ß-glucan, blood sample were collected at 4, 8, 10 and 13 weeks old. Statistical analyses were done using one-way ANOVA.

Quantification of PRRSV-genomic RNA in sera of the growing pigs

To examine PRRSV viremic status in the experimental pigs, PRRSV genomic RNA in sera was quantitated by real-time RT-PCR. The PRRSV infection was observed in all experimental groups at the 8 weeks old of the experimental pigs. Although levels of PRRSV-genomic RNA between control and ß-glucan-treated groups were not significant, 100 g/ton ß-glucan supplementation tended to restrain PRRSV viremia, as observed lower number of PRRSV-genomic RNA at 8 weeks old. At the end of observation, the data showed that most of experimental pigs were negative for PRRSV-genomic RNA

(Figure 3). The results indicated that supplementation of algae-derived ß-glucan might has minimal effect on control of PRRSV viremia in the growing pigs.

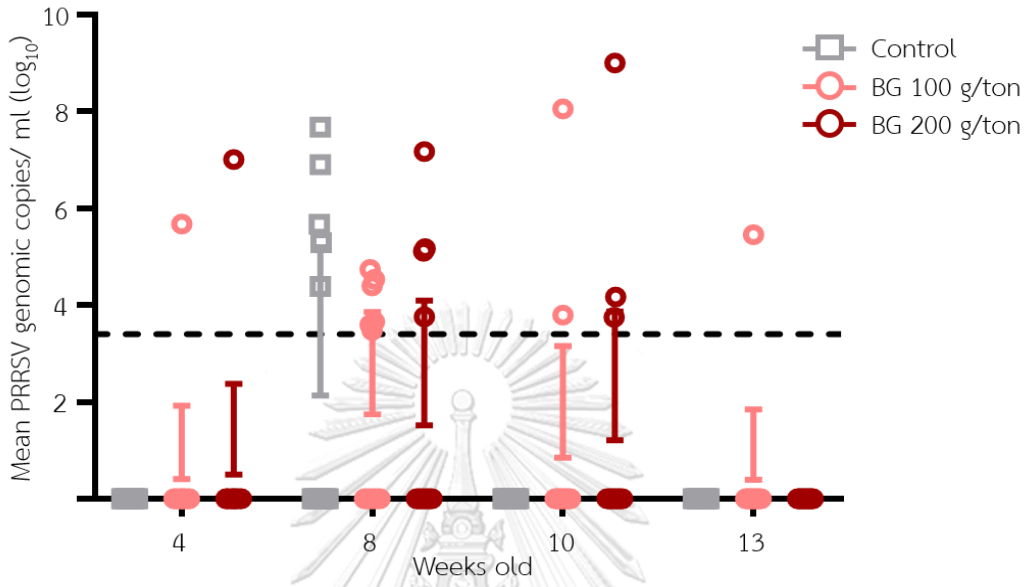


Figure 3 Mean values of PRRSV genomic copy numbers (log_{10}) in the sera from each treatment group.

All pigs were immunized PRRS vaccine at 2nd weeks old. After supplementation with algae-derived ß-glucan, blood sample were collected at 4, 8, 10 and 13 weeks old. Statistical analyses were analyzed using one-way ANOVA. The data were expressed as the mean ± S.D. of the numbers of pigs alive at the time of the sample collection. Dot line represents the cut off of PCR results.

The immunomodulatory effects of algae-derived ß-glucan supplementation on neutralizing antibodies against PRRSV and CSFV

To investigate the immunomodulatory effect of algae-derived ß-glucan supplementation on neutralizing antibodies, the level of PRRSV and CSFV-specific neutralizing antibodies were evaluated following 2 weeks post-PRRSV/CSFV immunizations. PRRSV infection usually delayed neutralizing antibody and cytotoxic T cell response (Lopez and Osorio, 2004), As expected, PRRSV-specific neutralizing antibodies (SN) were not appeared during our observation (Figure 4). Interestingly, supplementation with 100 and 200 g/ton of ß-glucan significantly exhibited higher level

of CSFV-specific neutralizing antibodies than control at 8 and 10 weeks old of age (Figure 5). At 10 weeks old, CSFV-neutralizing antibody responses in the pigs supplemented with ß-glucan at both concentrations were greater than the protective level ($5Log_2$) indicating the pigs were protected against CSFV infection. However, the levels of CSFV-specific neutralizing antibodies of the pigs in all experimental groups were declined, as observed at 13 weeks old. The results suggested that algae-derived ß-glucan supplementation possessed immunomodulatory role on humoral immune responses of the supplemented pigs.

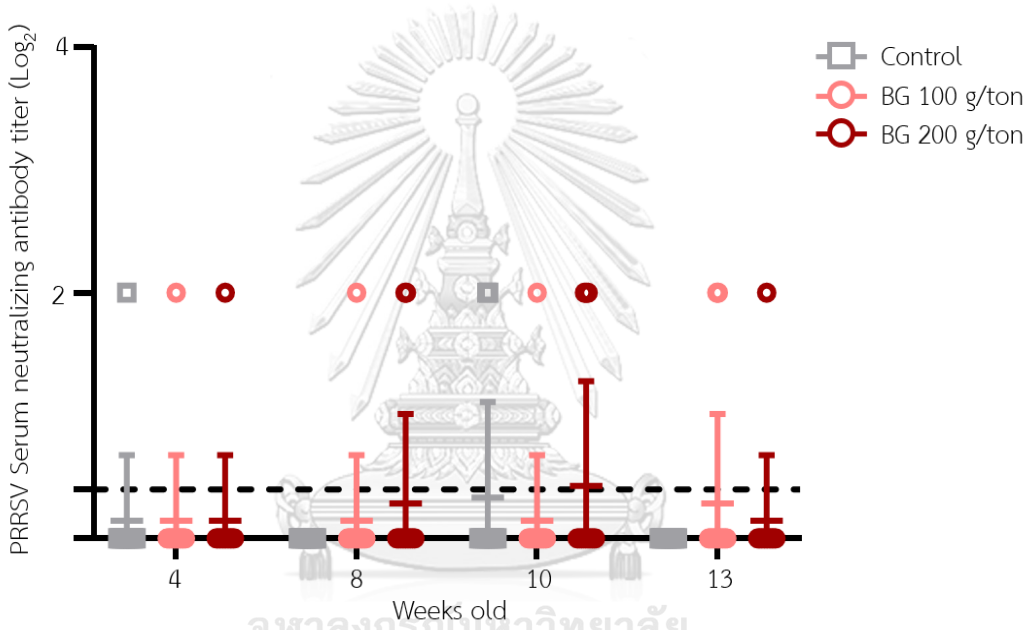


Figure 4 Mean PRRSV serum neutralizing antibody titers in each treatment group. All pigs were immunized PRRSV vaccine at 2nd weeks old. After supplementation with algae-derived ß-glucan, blood sample were collected at 4, 8, 10 and 13 weeks old for serum neutralizing test. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. The data were expressed as the mean ± S.D. of the numbers of pigs alive at the time of the sample collection. Dot line represent the protective level of PRRSV neutralizing antibody. a indicates significantly difference between control supplementation with algae-derived ß-glucan at 100 g/ton (BG 100g/ton). b indicates significantly difference between control and supplementation with algae-derived ß-glucan at 200 g/ton (BG 200g/ton).

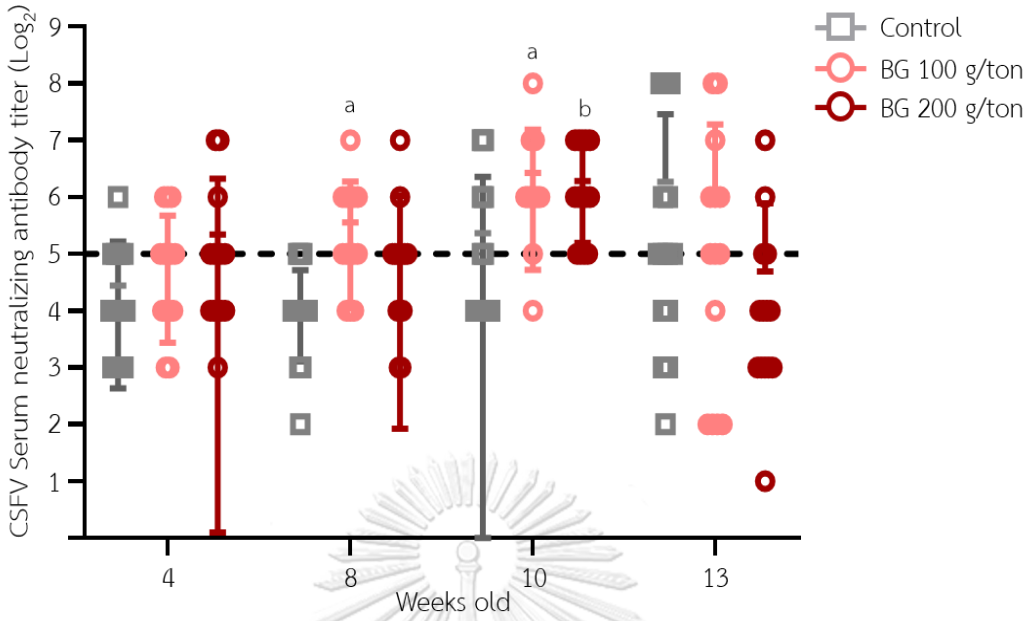


Figure 5. Mean CSFV serum neutralizing antibody titers in each treatment group. All pigs were immunized CSFV vaccine at 3rd and 5th weeks old. After supplementation with algae-derived ß-glucan, blood sample were collected at 4, 8, 10 and 13 weeks old for serum neutralizing test. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test. The data were expressed as the mean ± S.D. of the numbers of pigs alive at the time of the sample collection. Dot line represent the protective level of CSFV neutralizing antibody ($log_2 5$). a indicates significantly difference between control supplementation with algae-derived ß-glucan at 100 g/ton (BG 100g/ton). b indicates significantly difference between control and supplementation with algae-derived ß-glucan at 200 g/ton (BG 200g/ton).

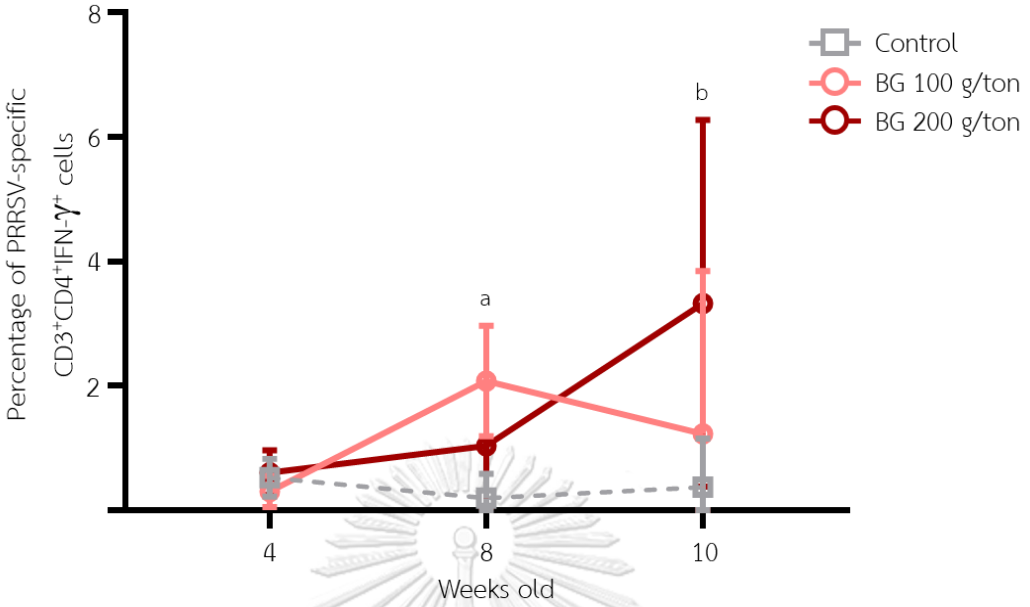
The immunomodulatory effects of algae-derived ß-glucan supplementation on PRRSV/CSFV-specific cell mediated immune responses

To investigate effects of algae-derived ß-glucan supplementation on specific T lymphocyte responses, the PBMCs obtained PRRSV/CSFV-immunized pigs were *in vitro* activated with PRRSV or CSFV, and subsequently subjected to immunofluorescence staining. At 8 weeks old, significantly increased numbers of PRRSV-specific IFN-γ^+-producing T helper (Th) lymphocytes ($CD3^+CD4^+$IFN-γ^+ cells)

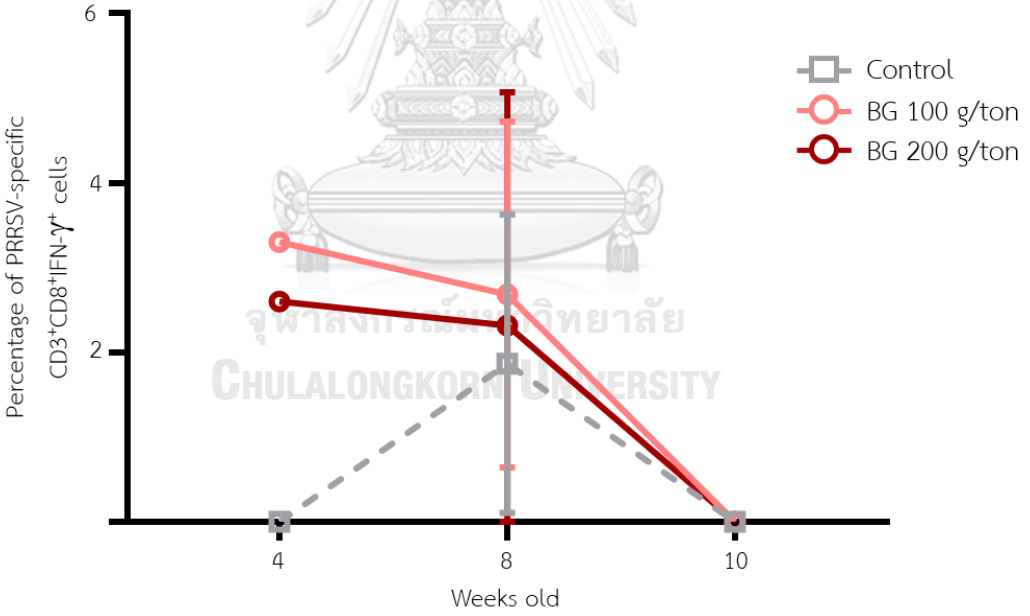
were observed in the 100 g/ton ß-glucan-supplemented pigs. In addition, supplementation with 200 g/ton ß-glucan in pig's diet significantly enhanced PRRSV-specific IFN-γ^+-producing Th lymphocytes at 10 weeks old (Figure 6a). At 8 weeks old, the data demonstrated that the numbers of putatively circulating PRRSV-specific memory T lymphocytes ($CD3^+CD4^+CD8^+$IFN-γ^+cells) were higher in the pigs supplemented with 100 g/ton and 200 g/ton ß-glucan, compared to the control pigs. However, the circulating PRRSV-specific memory cells in most of the experimental pigs were declined to based line at 10 weeks old (Figure 6c). In our observation, the numbers of PRRSV-specific IFN-γ^+-producing cytotoxic T lymphocytes (CTL, $CD3^+CD8^+$IFN-γ^+ cells) were comparable among control and 2 groups of pigs supplemented with ß-glucan (Figure 6b).

To further confirm the immunomodulatory effect of ß-glucan supplementation on specific cellular immune responses, we *ex vivo* quantitated the number of CSFV-specific T lymphocytes in those experimental pigs. At 8 weeks old, the supplementation with 200 g/ton ß-glucan significantly increased CSFV-specific IFN-γ^+-producing Th lymphocytes (Figure 7a). Although the numbers of CSFV-specific CTL among control and 2 groups of the ß-glucan-supplemented pigs were comparable throughout the observation period, we observed higher numbers of putatively circulating CSFV-specific memory T lymphocytes in the pigs supplemented with 100 g/ton and 200 g/ton ß-glucan at 8 weeks old (Figure 7c). Taken together, the findings indicated that supplementation of algae-derived ß-glucan could facilitate PRRSV/CSFV-specific cellular immune responses, as observed increasing in numbers of the IFN-γ^+-producing Th lymphocytes and the circulating memory cells.

a.



b.



c.

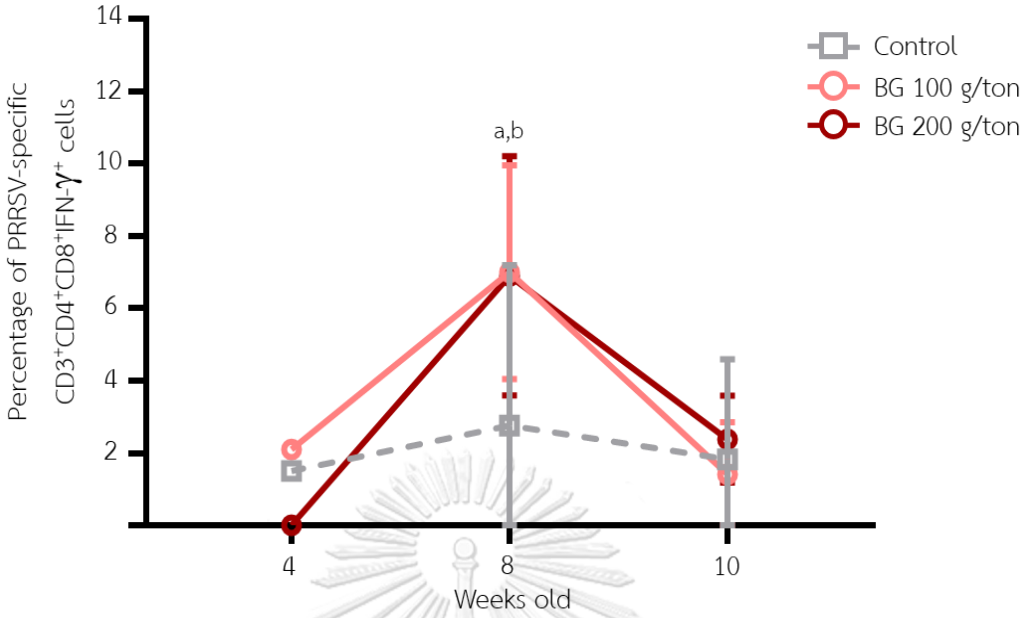
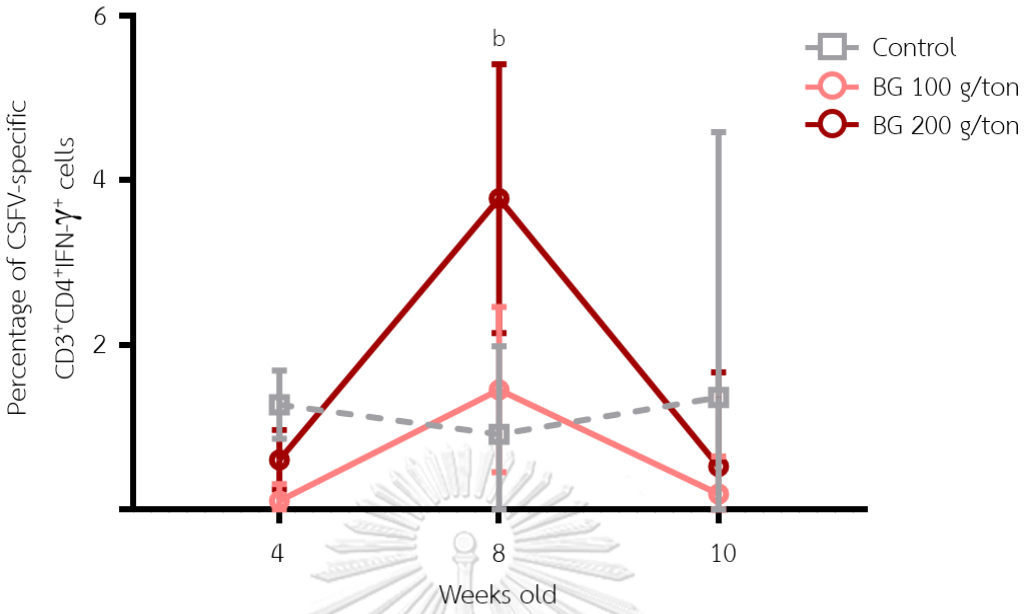


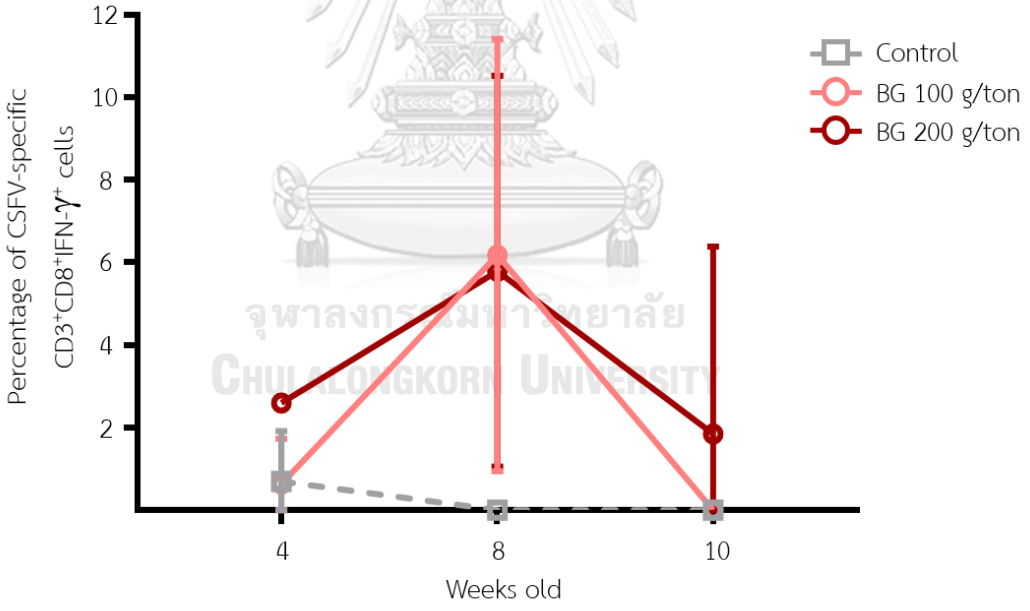
Figure 6 Percentages of PRRSV-specific $CD3^+CD4^+IFN\text{-}\gamma^+$ subpopulation (a.), percentages of PRRSV-specific $CD3^+CD8^+IFN\text{-}\gamma^+$ subpopulation (b.) and percentages of PRRSV-specific $CD3^+CD4^+CD8^+$ IFN-γ+ subpopulation (c.) of the experimental pigs. PBMCs obtained from experimental pigs were in vitro reactivated with 0.1 m.o.i. of type 2 PRRSV or mock-infected MARC-145 cell lysate (mock) for 48 h.

Statistical significances were analyzed using ANOVA following by Tukey's multiple comparison test. $P < 0.05$ indicates significant difference between experimental groups. a indicates significantly difference between control supplementation with algae-derived ß-glucan at 100 g/ton (BG 100g/ton). b indicates significantly difference between control and supplementation with algae-derived ß-glucan at 200 g/ton (BG 200g/ton).

a.



b.



c.

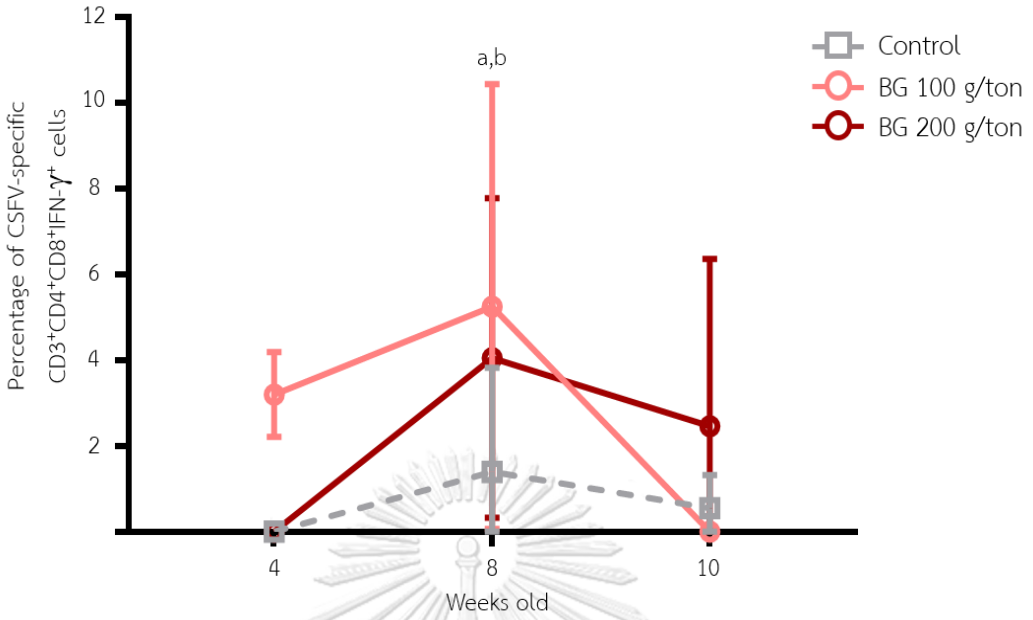


Figure 7 Percentages of PRRSV-specific $CD3^+CD4^+IFN\text{-}\gamma^+$ subpopulation (a.), percentages of PRRSV-specific $CD3^+CD8^+IFN\text{-}\gamma^+$ subpopulation (b.) and percentages of PRRSV-specific $CD3^+CD4^+CD8^+IFN\text{-}\gamma^+$ subpopulation (c.) of the experimental pigs.
PBMCs obtained from experimental pigs were in vitro reactivated with 0.1 m.o.i. of CSFV or mock-infected SK-6 cell lysate (mock) for 48 h. Statistical significances were analyzed using ANOVA following by Tukey's multiple comparison test. $P < 0.05$ indicates significant difference between experimental groups. a indicates significantly difference between control supplementation with algae-derived ß-glucan at 100 g/ton (BG 100g/ton). b indicates significantly difference between control and supplementation with algae-derived ß-glucan at 200 g/ton (BG 200g/ton).

CHAPTER V

DISCUSSION

Due to various types of stressors and the window of susceptibility during post-

weaning period, nursery pigs are more susceptible to the diseases comparing to other production phases. Additionally, using of antibiotic in sub-therapeutic dose as a growth promoter in animal feed was prohibited (Cromwell, 2002). In this study, we found that algae derived ß-glucan could be an alternative feed additive using in swine production. The supplementation with algae-derived ß-glucan demonstrated positive effect on both humoral and adaptive immune responses in nursery to growing pigs.

The obtained data suggests that ß-glucan extracted from algae might have a potential immunomodulatory effect enhancing the efficacy of the PRRSV and CSFV modified lived virus (MLV) vaccines through enhancing CSFV-specific neutralizing antibody and CSFV/PRRSV-specific T lymphocyte responses. Similar observation was reported in a mice model, as oral ingestion of ß-glucans significantly increased influenza antibody titers following influenza virus inoculation (Vetvicka and Vetvickova, 2015). Supplementation of algae derived ß-glucan in broilers enhanced frequency of NDV positive animals up to 86% of the herd after vaccination (Horst et al., 2019). In response to viral infection, IFN-γ^+ production from lymphocytes is a hallmark of antiviral activity (Chesler and Reiss, 2002). Helper ($CD4^+$) T lymphocytes is an essential component of the adaptive immune system, by which various signals as cytokines, chemokines, and other mediators are used to activate and modulate other immune cells. Porcine memory/effector ($CD4^+CD8^+$) T lymphocytes exhibit properties of mature antigen-experienced cells that are activated by the recall antigens (Summerfield and Ruggli, 2015). Here, we also demonstrated that increasing in numbers of CSFV/PRRSV-specific porcine T lymphocytes and memory T lymphocytes were observed in the pigs supplemented with ß-glucan. In consistent with the previous study, pigs supplemented with ß-glucan from non-algae source significantly increased both CSFV-specific T lymphocytes and blocking antibodies (Wang et al., 2008). In context of PRRSV infection, ß-glucan supplementation might provides synergistic effect with vaccination in order to control PRRSV viremia at 8 weeks old, as most of pigs showed low level of PRRSV viral load at that time. Higher numbers of PRRSV-specific T helper and antigen-experienced T helper lymphocytes at 8 weeks old of ß-glucan-supplemented pigs might concurrently contribute to PRRSV control. The supporting evidence is that

treatment of influenza-infected mice with ß-glucan showed remarkably increasing in number of IFN-γ^+ producing cells, which were well correlated with protection rate of influenza infection (Vetvicka and Vetvickova, 2015). Here, the neutralizing titers of PRRSV were not detected in our observed. The possible explanations could be that ß-glucan had no immunomodulatory effect on level of neutralizing antibody against PRRSV Hiss and Sauerwein (2003) and immunosuppressive properties of PRRSV might override and delay the production of neutralizing antibody (Lopez and Osorio, 2004). However, the immunomodulatory mechanism of ß-glucan from *E.gracilis* is still inconclusive. One of possible mechanism has been reported, as ß-glucan plays a role as a pathogen-associated molecular patterns (PAMPs) that can be recognized by various pattern recognition receptors (PRRs) resulting immune activation (Barsanti et al., 2011).

In the other hand, ß-glucan also act as a prebiotic, as ß-glucan can be fermented by gut microbiota producing the short chain fatty acid, which provides indirect effect of improving gut environment, nutrient absorption and health of the animals (Jayachandran et al., 2018). Thus, additional of ß-glucan in pig's diet might promote the production performance of the fed pigs. Unfortunately, in our observation, supplementation of algae derived ß-glucan did not show any effect on the fecal score, mortality rate, ADG, feed gain, ADFI and FCR. Similar to previous report, there were no significant effects of algae-derived ß-glucan supplementation in term of enhancing production performances, feed efficiency and intestinal microbiota population of the weaning pigs (Yamamoto et al., 2018; Kim et al., 2019). Consistent to the data in in broilers, Cheng et al. (2004) pointed out that 6 weeks supplementation of ß-glucan in broilers did not show any effects on weight gain or feed efficiency.

On the contrary, there are some reports demonstrating that the supplementation of ß-glucan showed significantly increased ADG, FCR and nutrient digestibility in the growing to finishing pigs (Li et al., 2006; Park et al., 2018; Wu et al., 2018; Luo et al., 2019b; Luo et al., 2019a). The discrepancy might be due to a variety of ß-glucan used in the studies or other confounding factors in the animal experiment.

It should be noted that differences in sources, structure, molecular weight and purity of ß-glucan possess distinct biological activities and physiological functions (Brown and Gordon, 2005). In addition, different outcomes after ß-glucan supplementation might be from various hygienic status, pathogen load and housing system (Hiss and Sauerwein, 2003). As the unbranched ß, (1,3) structure, ß-glucan from *E. gracilis* have high binding affinity to the ß-glucan receptors, this might affect the magnitude of immune activation leading to enhancing down-stream immunological responses. It has been reported that activatory effect of ß-glucan derived from *E. gracilis* on immune responses is shown to be dose dependent (Sonck et al., 2010). Moreover, the particulate ß-glucan originated from algae source could enhance lymphocyte proliferation. As algae-derived ß-glucan strongly induced cytokine secretion from antigen presenting cells such as macrophages (Baert et al., 2015), these cells may travel and trigger immune activation in the secondary lymphoid organs resulting in induction of specific lymphocyte responses (Barsanti et al., 2011; Teng and Kim, 2018). Taken together, these issues suggest that ß-glucan derived from algae might provide greater immunomodulatory characteristic, as compared to other sources of ß-glucan.

For future study, dose expansion of ß-glucan supplementation should be conducted to observe further efficacy and safety in the pig through measurement of production performances and specific immunological responses following routine vaccination. To the best of our knowledge, this is the first study of beneficial effect of linear ß-(1,3) glucan from algae extracts on production performances and viral specific humoral and adaptive immune responses in nursery to growing pigs. Although the results of production performance still be inexplicit, algae-derived ß-glucan demonstrated the immunopotentiator properties on both viral-specific humoral and cellular immunity, which may lead to increasing disease resistance in the affected pigs. Accordingly, algae-derived ß-glucan is one of the promising feed additives for nursery to growing pigs.

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APPENDIX

1. DNA extraction

-	Proteinase K	25	µl
-	B3 buffer	200	µl
-	Ethanol	210	µl
-	BW buffer	500	µl
-	B5 buffer	600	µl
-	RNA free water	50	µl

2. RNA extraction

-	DL buffer	200	µl
-	Proteinase K	5	µl
-	Ethanol	200	µl
-	MDB	350	µl
-	rDNase	95	µl
-	RB2 buffer	200	µl
-	RB3 buffer	850	µl
-	RNA free water	50	µl

3. qPCR assay for PCV2 detection (volume/reaction)

-	Lunar universal qPCR master mix	10	µl
-	PCV2 probe	0.4	µl
-	PCV2 ORF2 forward primer	0.8	µl
-	PCV2 ORF2 reverse primer	0.8	µl
-	DNase free water	4	µl

4. qPCR assay for PRRSV detection (volume/reaction)

-	Lunar universal probe one-step RT-qPCR master mix	10	µl
-	Lunar WarmStart® RT enzyme mix	1	µl
-	PRRSV probe	0.4	µl
-	PRRSV ORF7 forward primer	0.8	µl
-	PRRSV ORF7 reverse primer	0.8	µl
-	DNase free water	4	µl

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