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for the Degree of Doctor of Philosophy

Effects of Bacteriophage and Choline as Feed Additives on Physiology and Productivity in Broilers and Pigs

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육계와 돼지의 생리와 생산성에 미치는 영향

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Overall Summary

Effects of Bacteriophage and Choline as Feed Additives on Physiology and Productivity in Broilers and Pigs

These experiments were performed to investigate 1) effects of bacteriophage on prevention of *Salmonella enteritidis* in broilers, 2) effects of bacteriophage on growth performance, fecal properties, blood profiles, and immune response in weaning pigs, and 3) effects of bacteriophage and choline supplementation on physiological responses, growth performance, microbial population, and blood profiles of lactating sows and piglets. Summarized results from each experiment are described as followings:

Experiment I. Effects of Bacteriophage on Prevention of *Salmonella enteritidis* in Broilers

The experiment 1 was conducted to investigate the effects of bacteriophage on prevention of *Salmonella enteritidis* in broilers. A total of 320 one day old male broilers (Ross 308) were allotted by randomized complete block (RCB) design in 8 replicates with 10 chicks per pen. The experimental diets were formulated for 2 phase feeding trial (phase I; 0-2nd wk, phase II; 3rd-5th wk), and 4 different levels (0%, 0.05%; 5×10^{8}pfu/g, 0.1%; 1×10^{9}pfu/g, and 0.2%; 2×10^{9}pfu/g, respectively) of *Salmonella enteritidis* targeted bacteriophage were supplemented in the basal diet. There were no differences in body weight (BW) gain, feed intake and feed conversion ratio (FCR) during the whole experimental period ($P>0.05$). Relative weights of liver, spleen, abdominal fat, and tissue muscle of breast obtained from each bacteriophage treatment were similar to control and those values tended to increase when 0.2% (2×10^{9}pfu/g) bacteriophage was supplemented. In addition, a numerical difference of glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and

low density lipoprotein (LDL) cholesterol level were observed when 0.2% (2×10^9pfu) of bacteriophage were provided even though blood profiles were not affected by supplemented levels of bacteriophage (P>0.05). In the result of a 14 d feeding after *Salmonella enteritidis* challenge to 160 birds from 4 previous treatments, mortality and *Salmonella enteritidis* concentration in the cecum were decreased with increasing bacteriophage level (P<0.05). This result demonstrated that supplementation of 0.2% (2×10^9pfu) *Salmonella enteritidis* targeted bacteriophage might not cause negative effect on growth, meat production, and it reduced mortality from *Salmonella enteritidis* challenge. Consequently, bacteriophage could be used as an alternative feed additive to antibiotics in broiler diets.

Experiment II. Effects of Bacteriophage on Growth Performance, Fecal Properties, Blood profiles and Immune Response in Weaning Pigs

The experiment 2 was performed to determine the effects of bacteriophage on growth performance, fecal properties, blood profiles and immune response in weaning pigs. A total of 160 pigs [(Yorkshire × Landrace) × Duroc] (BW = 6.78 ± 1.72 kg; weaned at day 24 ± 3) were allotted to 4 groups in a randomized complete block (RCB) design with 5 replication for 5 week growth trial. The experimental diets were formulated for 2 phase feeding trial (phase I; 0-2^{nd} wk, phase II; 3^{rd}-5^{th} wk), and 4 different levels (0%, 0.05%; 5×10^8pfu/g, 0.1%; 1×10^9pfu/g, and 0.2%; 2×10^9pfu/g, respectively) of 16 types of pathogen targeted bacteriophage were supplemented in the basal diet. During the whole experimental period, average daily gain, average daily feed intake, and gain:feed ratio were not affected by bacteriophage levels, resulting in similar BW among all treatments (P>0.05). With increasing bacteriophage level in the diets, fecal microbial population of pathogenic *Salmonella spp.* (linear, P<0.01; 2wk) and *Escherichia coli* (linear, P=0.053; 5wk) were decreased. However, the concentration of *Lactobacilli* was increased in feces when pigs were fed 0.2% of bacteriophage, showing linear response to

bacteriophage levels (linear, P<0.05, 2wk; linear, P<0.01, 5wk). The inclusion of bacteriophage in weaning pig diets resulted in decreasing the incidence of diarrhea (linear, P<0.01). In blood immune response, there was a linear decrease in IgA concentration as bacteriophage increased (linear, P<0.05). Although GOT and GPT levels were not affected by bacteriophage levels, total cholesterol (linear, P<0.01, quadratic, P<0.05, 2wk; linear, P<0.01, 5wk) and LDL cholesterol (linear, P<0.01, 2wk) levels were decreased by dietary bacteriophage. This experiment suggested that 16 types of pathogen targeted bacteriophage supplementation did not influence on growth performance. However, 0.2 % (2×10^9pfu/g) bacteriophage supplementation might have beneficial influences on microbial population, fecal status, immune response, and blood profiles in weaning pigs.

Experiment III. Evaluation of Bacteriophage and Choline Supplementation on Physiological Responses, Growth Performance, Microbial Population and Blood profiles of Lactating Sows and Piglets

The experiment 3 was conducted to investigate the effects of bacteriophage and choline supplementation on physiological responses, growth performance, microbial population and blood profiles of lactating sows and piglets. A total of 50 mixed-parity (average= 4.64) crossbred sows (F1, Yorkshire × Landrace; Darby, Korea) with an initial BW of 228.71 ± 15.81 kg were used in a 3 week lactation period and sows were allotted to one of five treatments based on BW and backfat thickness with 10 replicates by 1+2×2 factorial arrangement. The experimental treatments were divided by two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g) and choline chloride (0.05%; 250ppm or 0.1%; 500ppm) and NRC (1998) requirement is regarded as control treatment. The experimental diets were formulated based on corn-soybean meal diets, which contained 3,265 kcal of ME/kg, 16.8% crude protein, 1.08% lysine, respectively. There were no significant differences in BW, backfat thickness and feed intake of lactating sows by bacteriophage and choline supplementation. The BW changes were quadratically

decreased in lactation (day 0 to 21) as dietary choline increased (P<0.05). Supplementation of bacteriophage and choline to lactating diets did not influence on mortality, litter weight and piglet weight. However, numerically higher litter weight and piglets weight gain were observed in bacteriophage and choline treatment groups compared to control. No differences were found in estimation of milk production, dry matter, and energy content of milk in lactating sows during the whole lactational period. Bacteriophage and choline supplementation in diets did not alter the population of *Escherichia coli* and *Salmonella spp.* in feces of sows as well as piglets. However, the use of bacteriophage to lactation diets altered the concentrations of fecal *Lactobacilli* (P<0.001). In blood profiles, GOT, GPT, and non-esterified fatty acid (NEFA) levels of lactating sows and piglets were not affected by dietary treatment, while increasing bacteriophage levels tended to decrease GOT levels of lactating sows (linear, P=0.074). Inclusion of bacteriophage and choline did not influence on immunoglobulin concentration of sows at day 21 postpartum. This experiment suggested that choline supplementation in lactating diet showed an improvement of body reserves of lactating sows and increasing of fat contents in sow milks during lactation. But, bacteriophage had no effects on reproductive performance and physiological responses except of sow's fecal *Lactobacilli* population.

Three experiments demonstrated that positive responses were observed by bacteriophage supplementation in broilers and weaning pigs. However, sows did not show positive performance by dietary bacteriophage but body condition of sows was improved by choline supplementation.

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List of Abbreviation

ADFI	Average daily feed intake
ADG	Average daily gain
AOAC	Association of Official Agricultural Chemists
BW	Body weight
Ca	Calcium
CP	Crude protein
CRD	Completely randomized block
DM	Dry matter
E. coli	*Escherichia coli*
ETCT	Enterotoxigenic *Escherichia coli*
FCR	Feed conversion ratio
FI	Feed intake
G:F	Average daily gain per average daily feed intake
GLM	General linear model
GIT	Gastro-intestinal tract
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamic pyruvic transaminase
HDL	High density lipoprotein
Ig	Immunoglobulin
LB	Luria bertani
LDL	Low density lipoprotein
LSD	Least significant difference
ME	Metabolizable energy
MOI	Multiplicity of infection
MSY	Market pigs per sow per year
NRC	National research council
NEFA	Non-esterified fatty acid
PAF	Platelet activating factor
RCB	Randomized complete block
SAM	S-adenosylmethionine
SAS	Single attachment station
SBM	Soybean meal

SE	*Salmonella enteritidis*
TG	Triglyceride
TSA	Tryptic soy agar
VLDL	Very low density lipoprotein

Chapter I. General Introduction

The swine statistics in Korea showed that the number of market pigs per sow per year (MSY) in 2010 was 15.1 (Korea pork producers association, 2011), whereas those in Netherlands and Denmark were 26.0, and 25.5 respectively (Pig international, 2011). There were several reasons for lower productivity in Korea compared to advanced countries in swine industry, such as absence of standard manuals for pig production, and inadequate management technique without the verification experiment, resulting in high mortality from birth to slaughter pig production. In need, high mortality was happened in nursery and weaning period caused by problem of sanitary and malnutrition. Therefore, the strategies of practical nutritional management for decreasing mortality and maintaining of pig health is important to increase swine productivity in Korea.

Salmonellosis is a severe disease in humans caused by contamination of *Salmonella* bacteria in food. *Salmonella enteritidis* is a genetically homogeneous serotype of *Salmonella*, and it is well known as one of the most common types in food poisoning (Hudson et al., 2001; Chung et al., 2003; Gong et al., 2007). Chickens and eggs are frequently reported as the cause of outbreaks of Salmonellosis (St. Louis et al., 1988; Molbak and Neiman, 2002), and outbreaks of *Salmonella* bacteria are often detected in broiler flocks. From these aspects, prevention of *Salmonella enteritidis* infection is an important issue for maintaining food safety particularly in the poultry industry.

For improving productivity and health in animals, antibiotics were generally supplemented in the diet to prevent growth retardation and bacterial pathogens of diarrhea disease (Dierick et al., 1983; Cromwell, 2002). However, the use of antibiotics in the livestock industry revealed the risk of bacterial resistance in both livestock and human, creating public worries. As a reaction to that, those in European countries and Korea banned use of antibiotics for growth promotion

purpose and increased the need for researches on alternatives to antibiotics. As these materials are feed additives, animal producers are concerned about reducing feed cost with improvement of feed efficiency and animal growth. In this aspect, it may be noteworthy to focus on many ways and strategies for reduction in mortality and improving health status as well as increasing animal productivity for livestock industrial competitiveness in Korea.

Bacteriophage is a kind of virus that infects and multiplies in specific bacteria (McGrath et al., 2004). It commonly appears to have involved bacteria in nature and their characteristics was host specific and infect only one bacterial species (Ackerman, 1978). Numerous studies have indicated that use of bacteriophages in humans and animals was very safe and did not reported serious adverse effects (Weber-Dabrowska et al., 2000; Sulakvelidze et al., 2001; Summers, 2005; Kropinski, 2006; Parisien et al., 2008). Bacteriophage has two types of life cycle, lytic cycle and lysogenic cycle (Guttman et al., 2005). In lysogenic cycle, bacteriophages insert their DNA to host and come to be a prophage by DNA replication. In lytic cycle, bacteriophages however make some damage on DNA of host or host itself and break out by cell division. Due to physical mechanism of bacteriophage, lytic cycle of bacteriophages can be used for therapeutic use for bacterial infections. By administering a specific bacteriophage for pathogenic bacteria to a host, these bacteria can be completely destroyed. Their mechanism of recognition and destruction of bacteria will help reduce the transmission of bacterial resistance (Hanlon, 2007). When bacteriophage is applied in livestock for therapeutic use, also any bacterial resistance will not be arisen in humans and it helpful to feed cost because of the relatively easy production and cheap price.

Dietary choline are commonly used in animal feed industry as feed additives. It is essential materials for the formation of acetylcholine, which is released at the termination of parasympathetic nerves (Wauben and Wainwright, 1999). Another function is a source of labile methyl groups for formation of

methionine from homocysteine. Lastly, choline plays a major role for fat metabolism in the liver, as a lipotropic factor that affected on fat metabolism by decreasing of fat deposition. Fat metabolism with choline is most important for lactating animals because of choline levels within their milk are correlated with choline levels in maternal blood (Holmes McNarry et al., 1996; Ilcol et al., 2005). Choline reaches the maternal milk through a transporter from the maternal blood into mammary epithelial cells (Chao et al., 1988). In addition, liver triglycerides can be packaged into very low density lipoproteins (VLDL) and exported into blood. Choline may affect the synthesis of the apolipoprotein components of VLDL to increase triglycerides export from liver. Due to the fat metabolism of the mobilized fatty acids from adipose tissue to the mammary gland, choline can affect milk production. Therefore, enhancing the adequate milk supply to newborn piglets by supplying choline could be one of the nutritional manipulation to improve litter performance.

Consequently, three experiments were conducted to suggest proper application of bacteriophage and choline by examining 1) effects of bacteriophage on prevention of *Salmonella enteritidis* in broilers, 2) effects of bacteriophage on growth performance, fecal properties, blood profiles, and immune response in weaning pigs, and 3) effects of bacteriophage and choline supplementation on physiological responses, growth performance, microbial population, and blood profiles of lactating sows and piglets in this dissertation.

Chapter II. Literature Review

1. Bacteriophage

1.1 General Characteristic of Bacteriophage

In general, bacteriophage (informally, phage) in common that only infect and inject their DNA or RNA into host bacteria where new bacteriophage particles can be produced in their host cell, resulting in the destruction of the host. In 1917, bacteriophage were firstly discovered by Felix d 'Herelle (Beckerich and Hauduroy, 1923). Bacteriophages have been extensively studied and used for therapeutic purposes by kill bacteria in humans in the 20th century (Babalova et al., 1968; Zhukov-Verezhnikov et al., 1978; Slopek et al., 1987). They observed that use of bacteriophage as an alternative was effective materials for treat (Meladze et al., 1982).

Bacteriophages has many different sizes that range in size from 24-200 nm in length. Most of bacteriophage have a tail and head with a genome that consists of either ssDNA, ssRNA or dsRNA (Ackermann, 1988). Phenotype of bacteriophage differ greatly, whereas new bacteriophage virion is relatively similar and it consist of capsid there in genetic materials (Sulakvelidze, 2005; McGrath, 2007; Abeton, 2008).

Bacteriophage are widely spread in all natural environments and are related to locations of bacteria, such as soil, sea water and the intestine of animals. Indeed, they are most stable entities, and can survive on hostile environments.

With many similarities between bacteriophages and animal cell virus, their life cycle can be viewed as model systems for animal cell viruses. They are extremely specialized and attacks a different kind of bacteria. Due to bacteriophage infection of the obligate intracellular bacterial parasites, they are usually use of host biosynthetic machinery in the host. Therefore, information of the life cycle of bacteriophage is necessary to understand of mechanism bacterial genes transferred

from one host to another host.

1.2 Mode of Action of Bacteriophage

Bacteriophage have a two different major mode of action can take place in the host cell (Figure 1). Bacteriophage characteristics were divided into lytic cycle and lysogenic cycle. But, some type of viruses are capable of carrying out both. The decision for bacteriophage's lambda genes to enter the lytic or lysogenic cycle is determined by the concentration of the repressor and another protein (e.g. cro) in the host cell. Environmental conditions of the host cell, favoring the production of another protein will lead to the lytic cycle while favor the production of the repressor will lead to lysogenic cycle. Therefore, bacteriophage which develop both lytic and lysogenic cycles (Benett and Howe, 1998).

1.2.1 The Lysogenic Cycle of Bacteriophage

The lysogenic bacteriophage called such as temperate phage do not experience multiplication or lysis in the cytoplasm of the bacterium. When this cycle was started by infection, the phage genetic materials (e.g. viral DNA) may integrate into the bacterial DNA without causing lysis in the cytoplasm. Their bacteriophage (known as prophage) will be integrated into the host genome and hereby of genetic host materials will be transduced to a new bacterium. This step initiates the reproductive step, resulting in lysis of the host cell. In case of lysogenic cycle, bacteriophage allows the host cell enable to survive and reproduce. However, under certain circumstances, such as host condition, low energy level, heat shock and stress signals that the lytic cycle can occur (McGrath, 2007).

1.2.2 The Lytic Cycle of Bacteriophage

The lytic bacteriophage called virulent phage, get inside the bacterial cell and destroyed after immediate replication of the virion by lysing or by repturing the host cell wall (e. g. T, T2, T4, and T6 phages). Bacteriophage's lysis is usually regulated by two proteins; endolysin and holin (Ackermann, 1998). The initial lytic cycle step is beginning to infection and transcription of phage DNA. This step is needed for m-RNA's code for phage DNA synthesis and protein biosynthesis. Due to increasing the phage lysis protein and intracellular phage, intracellular bacteriophage begin to lysis and released into the host cell. Lytic cycle takes 20-60 minutes, resulting in the host cell lyses and the hundreds of new bacteriophage (virions) move on to new host. Due to those characteristics, lytic phages are suitable for bacteriophage therapy.

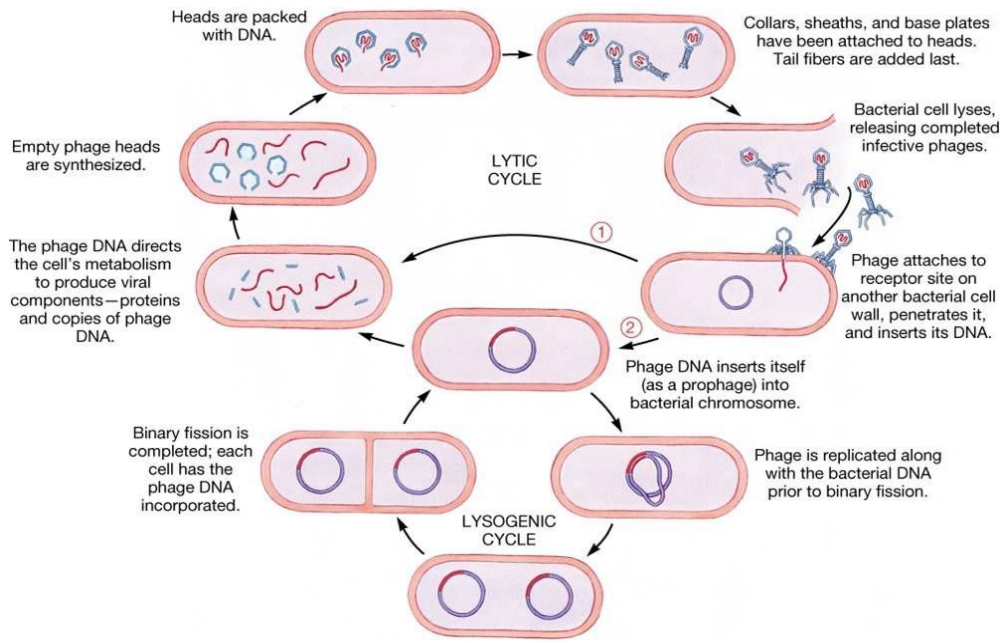

Figure 1. Lytic and lysogenic cycle of bacteriophage

(Adaptive from online source of Prof. Theresa Fischer:

http://faculty.ircc.edu/faculty/ tfischer/ micro%20resources.htm)

1.3 Bacteriophage Resistance Mechanism of Bacteria

With characteristics of symbiotic relationship of bacteria and bacteriophage over millions of years, bacteriophage acquired bacterium supplying the energy necessary to replicate and transferring virulence genes from bacteria. In addition, lytic bacteriophage can be harmful to bacteria and bacteria have developed in various ways to host evation which constructed the potential barriers against attacking of bacteriophage (Weinbauer, 2004; Hermoso et al., 2008). However, bacteriophage can be very effective in specific conditions and has some advantages compared to antibiotics. Although bacteria also develop resistance to bacteriophage, which is incomparably easier to develop new bacteriophage adjust to bacteria.

To protect themselves against bacteriophage, bacteria can make a biofilm such as protective layer that called extracellular polymer substance. Moreover, extracellular polysaccharides and surface proteins was blocked. But, bacteriophage is recognizing about biofilm formation because phages possess enzyme which can digest or depolymerize (Ravenscroft et al., 1994; Doolittle et al., 1995). These sites are also known to phage recognition place and bacteriophage use extracellular polysaccharides to recognize, resulting in better environment were provide for phage proliferation (Rodriguez et al., 2008). After bacteriophage adhesion to bacteria, bacteria escape infection and mutations of the bacteriophage docking site (Koprivnjak et al., 2008). But, various type of bacteriophage that can infect a resistant bacteria which evade bacterial immune response and adapt to bacteria mutations (Brussow et al., 2004; Matsuzaki et al., 2005; Pepin et al., 2008). These observation were successfully used with enterotoxigenic *E. coli* (ETEC) diarrhea in calves (Smith et al., 1987), cecal *Salmonella enteritis* in broiler (Fiorentin et al., 2005), and *Salmonella* from poultry carcass rinses (Higgins et al., 2005).

Deoxyribonucleic acid-injection blocking can inhibit infection which associated with resistance to lysin in lactic acid bacteria (Marshall, 1997; Forde and

Fitzgerald, 1999). The bacteriophage DNA also be recognized and demolished by bacterial endonucleases. Finally, bacteriophage multiplication can be also avoided by inhibition of the bacteriophage processing in the bacteria (Forde and Fitzgerald, 1999). Therefore, bacteriophage resistance mechanism against bacteria will lead to destruction of bacteria, resulting in the preservation of the bacteriophage.

2. Therapeutic Application of Bacteriophage

2.1 Bacteriophage Utilization for Gastro-Intestinal Health

Gastrointestinal microflora is very complex and limited knowledge on their composition. Pigs and poultry had relatively small group of bacteria means that dominance of useful intestinal bacteria in gastro-intestinal tract (GIT) is important of their health and growth. Indeed, dominating bacterial groups will be ones which win the competition since they can uptake nutrients. Thus, bacteriophage therapy to harmful bacteria (such as *Salmonella ssp.,* and *E. coli*) will be maintained and improve the bacterial composition in GIT. As a result, optimizing gut integrity and health state helps immune function and increase nutrient absorption.

2.1.1 Salmonella ssp.

Salmonella enterica is well-known a gram-negative, rod-shaped, and flagellated bacterium that is causative agents of disease in humans and animals. *Salmonella typhimurium* is a common pathogens in poultry and commonly considered harmless in mature birds (Berry, 2001). Therefore, various studies have been conducted about bacteriophage therapy for reduction of *Salmonella. typhimurium* in broilers. *Salmonella gallinarum* is also pathogens in poultry. Fowl typhoid and pullorum disease are caused by *Salmonella gallinarum* that is affects fetal to young broilers. *Salmonella enteritidis* have been invasive in young broilers, resulting in high mortality in broiler flocks (Anon, 1988; Hinton et al., 1989). In contrast to interest about poultry *Salmonella* disease, porcine salmonellosis has been far less interest with regard to pigs, because of higher danger level of poultry transmission of *Salmonella* to humans compared to pork. However, rapid increase of *Salmonella* positive pigs becomes a great concern in the farm (Lee and Harris, 2002). S*almonella choleaesuis* and *typhisuis* can cause sepsis. These diseases are breaking out only on pigs. *Salmonella typhimurium* and *enteritidis* are also

causative agent of chronic digestive disorder in pigs. *Salmonella typhimurium* was infected in all species that bring about food poisoning. In spite of higher level of exposure of *Salmonella choleaesuis*, frequencies of disease is low in pigs.

2.1.2 *Escherichia coli*

Escheriachia coli (*E. coli*) are gram negative that are often found in the intestine of animals such as pigs and chicken. Most of *E. coli* strains are harmless but some type of serotype of *E. coli* are food-born pathogen of humans. Pathogenic *E. coli* are important cause of diarrhea in piglets and weaning pigs, resulting in high mortality and morbidity (Fairbrother et al., 2005). In contrast to pigs, *E. coli* can cause enteritis in poultry but, incidence rate is less abundant. Various researches were been reported bacteriophage with *E. coli* with regard to therapy (Chibani-Chennoufi et al., 2004; Xie et al., 2005; Raya et al., 2006). Smith and Huggins (1983) suggested that diarrhea was prevented with supplementation of bacteriophage after treating respiratory *E. coli* infection in piglets and chicken. Further, bacteriophage treatment in piglets indicated that reduction of colonization of pathogenic *E. coli* in the GIT and mortality in weaning pigs. Similarly, the intestinal *E. coli* concentration were reduced bacteriophage was orally administrated (Raya et al., 2006) and bacteriophage supplementation in the weaning diets showed lower *E. coli* concentration compared to control, resulting in provide a better ecosystem for development of *Lactobacillus* (Wang et al., 2013). According to the respiratory infection experiment, orally administered 10^8 PFU of bacteriophage showed that no mortality and increment of growth were observed in chicken. Moreover, incidence of death caused by bursal disease was three fold reduction compared antibiotic and control treatment (Xie et al., 2005). Barrow et al. (1998) observed that bacteriophage inoculated intramusculary in chickens after inoculated intramuscularly with *E. coli* H247 were effective in preventing and treating septicemia and cerebritis or meningitis in chickens. Therefore, supplementation of

against *E. coli* bacteriophage is effective to prevent *E. coli* infection in pigs and chickens.

2.2 Effects of Bacteriophage in Monogastric Animals

2.2.1 Poultry

Research work indicated that use of bacteriophage to treat and prevent bacterial infection. Barrow et al. (1988) suggested that use of lytic bacteriophage could effectively prevent and reduce *E. coli* septicemia and meningitis in chickens. A various trial on the use of bacteriophage to prevent pathogenic bacteria was published by Huff et al (2002). They reported that bacteriophage with *E. coli* prior to challenging the bird showed that *Colibacillosis* could be prevented (Huff et al., 2002). Also, they demonstrated that administration of bacteriophage as aerosol spray before challenging *E. coli* could be prevented for up to 3 days (Huff et al., 2002). Other types of therapeutic administration were positive effect in treatment of a *Colibacillosis*. Intramuscular injection with single or multiple of bacteriophage showed greater therapeutic effects (Huff et al., 2003). These researches indicated that bacteriophage could prevent and treat pathogenic bacteria for therapeutic treatment of poultry diseases.

But, little information is available for the effects of bacteriophage as feed additives. Lee (2010) indicated that anti-*Salmonella gallinarum* bacteriophage in broiler diets did not affect on growth performance with no infected *Salmonella gallinarum*. Moreover, after *Salmonella gallinarum* challenge, the mortality was decreased with bacteriophage supplementation. Another study reported the inclusion of bacteriophage could reduce the feed conversion ratio (FCR), *E. coli*, and *Salmonella* concentration (Wang et al., 2013). In laying hens with bacteriophage supplementation. They were beneficial effects on egg production and excreta microflora concentration in feces (Zhao, 2012). Therefore, use of bacteriophage for prevent or treat and pathogenic bacteria suggests that bacteriophage could be positive effects on poultry production.

2.2.2 Swine

Various experiments were conducted to reduce mortality and GIT pathogens with bacteriophage supplementation for swine (Jamalludeen et al., 2007; Cha et al., 2010; Gebru et al., 2010). Especially, *E. coli* targeted bacteriophage has already been extensively tested. Jamalludeen et al. (2007) indicated that ten bacteriophage against porcine post-weaning diarrhea relate to O149 ETEC *in vitro* tested after indicated that the nine bacteriophages are suitable for therapy of porcine post weaning diarrhea. After *in vivo* studies showed that ETEC challenge and bacteriophage administration resulted in significantly reduced diarrhea and increased growth performance compared to control.

In the state of low pH and lytic *E. coli* bacteriophage that functioned stably as phage titer in the stomach and intestine (Chibani-Chennoufi et al., 2004). Cha et al. (2010) suggested that bacteriophage administration after *Salmonella* injection showed lower lesions and clinical signs compared to no bacteriophage treatment in weaning pigs. Bacteriophage supplementation as feed additives showed improve swine performance. Gebru et al. (2010) has shown that supplementation of anti-*Salmonella enterica serotype Typhimurium* bacteriophage resulted in improve growth performance with lower fecal *Salmonella* contents during 28 days. Another result was reported that administering a bacteriophage by gavage to pigs before exposure bacteria contaminated environment can effectively reduce pathogenic bacteria concentration in naïve pigs (Wall et al., 2010). Moreover, Kim et al. (2011) reported the effects of bacteriophage against ETEC infection in piglets with bacteriophage supplementation treatment were lower villus atrophy and crypt hyperplasia.

Thus, phage therapy has the advantage of nontoxic, lytic against bacteria

and lower bacteriophage price could be a useful tool for *Salmonella* reduction materials prior to slaughter. It may be prevent of *Salmonella* contamination with fecal *Salmonella* contact with pork during processing (Morgan et al., 1987).

2.3 Actual Application in Bacteriophage

2.3.1 Feed additives

Recently, researchers have focused on bacteriophage therapy for animals and evaluated the therapeutic efficacy. However, published data about supplementation of bacteriophage as feed additives indicated that body condition and growth of animal were not affected because of bacteriophage host specificity. In the unsanitary situation, use of bacteriophage could be helpful to animals with improvement health status and their growth.

However, using of bacteriophage as feed additives must know about and understand the ecology of host pathogen and bacteriophage. All bacteriophage are not equally applicable for all pathogenic bacteria. Microflora can inhabit a wide range of environmental GIT conditions, including in pH ranging from 1 to 11 (Kobayashi et al., 2000). Furthermore, bacteriophage have different eco system with specificity, virulence, and lytic potential sensitivity which means that have very diverse survival conditions. However, bacteriophage are relatively susceptible to ultra violet light, heat, and a very high and low pH (Sulakvelidze, 2005).

After onset of diarrhea, connection of bacteriophage with ETCT was limited because most of ETEC associated with the epithelial lining of the small intestine (Moon et al., 1977; Kasman, 2005). At the same time, fast excretion of fluid GIT contents may affect a removal of the bacteriophage particles. Consideration of interactions between gastrointestinal response and diarrhea, bacteriophage dosage levels should be increased compared to the dosage level of approximately 10^8 to 10^9 used for bacteriophage therapy.

Based on these observations, developing of bacteriophage as feed additives must be confirm that survive residency in GIT, feed, and oral application route with

reduction of specific pathogens. In Korea, bacteriophage have been approved as feed additives for the first time since January 2010. Four types of bacteriophage (targeted *Salmonella garlinarum, Salmonella enteritidis, Salmonella thyphimurium,* and *Clostridium perpringens*) were approved in Control of Livestock and Fish Feed Act of Korea in recent years. Dietary specific pathogen-targeted bacteriophage as feed additives were presented in Table 1. The products of bacteriophage from CJ Cheiljedang (Seoul, South Korea) and CTCbio (Seoul, South Korea) were used in three experiment and their pathogen-targeted bacteriophage were listed in Table 1.

Table 1. Specific pathogen-targeted bacteriophage as feed additives

Pathogenic bacteria		Induction of disease symptom
Salmonella	*gallinarum*	Fowl typhoid
	thyphimurium	Diarrhea, paratyphoid fever
	Enteritidis	Diarrhea, food poisoning
	choleasuis	Diarrhea, sepsis
	derby	Diarrhea, food poisoning
Staphylococcus	*aureus*	Mastitis, secondary infection
	coli K88	Diarrhea
Eschericia	*coli* K99	Diarrhea
	coli F41	Diarrhea
	coli F18	Edema
	coli 987P	Diarrhea
Clostridium	*Perfringens* type A	Diarrhea, food poisoning
	Perfringens type B	Diarrhea, food poisoning
	Perfringens type C	Diarrhea, food poisoning
	Perfringens type D	Diarrhea, food poisoning
	Perfringens type E	Diarrhea, food poisoning

(From: CTCbio Co., Ltd., Seoul, South Korea)

2.3.2 Alternative to antibiotics

Bacteriophage therapy is an important alternative to antibiotics against to resistant pathogens. Previous studies also suggested that significant efficacy of phages against *E. coli, Acinetobacter spp., Pseudomonas spp.,* and *Staphylococcus aureus* (Kawano et al., 1983; Huff et al., 2002, 2003; Debarbieux et al., 2010; Turton et al, 2012). These results indicated that bacteriophage therapy may be proved as an important alternative to antibiotics for treating multidrug resistant pathogens.

However, not all bacteriophage would be suitable for therapy and some factors are affected their activity. Some bacteriophages are produce their progeny without destroying specific host, others have temporarily integrate their genome and potentially injects new traits or modifiers the expression to host cell. Animal's GIT environmental conditions were also affected limit their activity. Bacteriophage circulating in the blood stream, they can be attacked by antibodies, resulting in moved from circulation by the reticuloendothelial system. Also, gastrointestinal tract of pH, presence of enzyme and digestive compounds were limited bacterial activity. Most bacteriophage are able to survive between pH 5 to 8, some targeted *Enterobacter* and *E. coli* bacteriophage resistant to acid. In consideration of alternative to antibiotics, persistence of the bacteriophage in the host and optimum dosage level were determine the efficacy of bacteriophage therapy in animals. Despite of limited condition, bacteriophage still has a merit as antibacterial therapy. First, bacteriophages are observed here and by others (McLaughlin et al., 2006; O'Flynn et al., 2006). Second, bacteriophage activity is available in adequate condition (Hurley et al., 2008; El-Shibiny et al., 2009). Third, bacteriophage would be shipped in high initial concentration and a very short period time (~6 to 12 h)

was required for treat.

Consequently, antibiotic resistance concern on the food-born bacterial pathogen is widely transmitted to the human for public health. As one of the bacteriophage as alternative to antibiotics have been made to control the bacterial disease.

2.3.3 Bacteriophage display

Since bacteriophage displayed in 1985, it has been variously applied to immunology, cancer research, drug display, and infectious disease (Smith, 1985). It has been used to identify small peptide ligands and antibody inhibiting the function of targeted receptors for a wide range of applications. Bacteriophage display has become a powerful tool for binding of antibody against epitopes of the cell surface on intact live cell (Fong et al., 1994), and this technology is based on the construction of a peptide library connected to a bacteriophage coat protein. Both M13 and fd filamentous bacteriophage are most commonly used because of cell lysis occurs during their life cycle (Figure 2). Filamentous bacteriophages consist of a genome of ssDNA and a rod-shaped cylinder.

Using this bacteriophage display, therapeutic peptide have been isolated for pathogens such as fungal zoospore (Bishop-Hurley et al., 2002; Fang et al., 2006), bacteria (Bishop-Hurley et al., 2002; Sorokulova et al., 2005) and insects (Ghosh et al., 2002). After bacteriophage display development, it has shown great potential in the discovery of therapeutics (Christensen et al., 2001; Giuliani et al., 2007). In addition, bacteriophage display is playing an important role for the discovery of peptides and antibodies. These targets are generally divided into two categories; one is molecular targets such as replication/cell division enzymes and host-pathogen virulence factor, the other is whole bacteria cell (Huang et al., 2012).

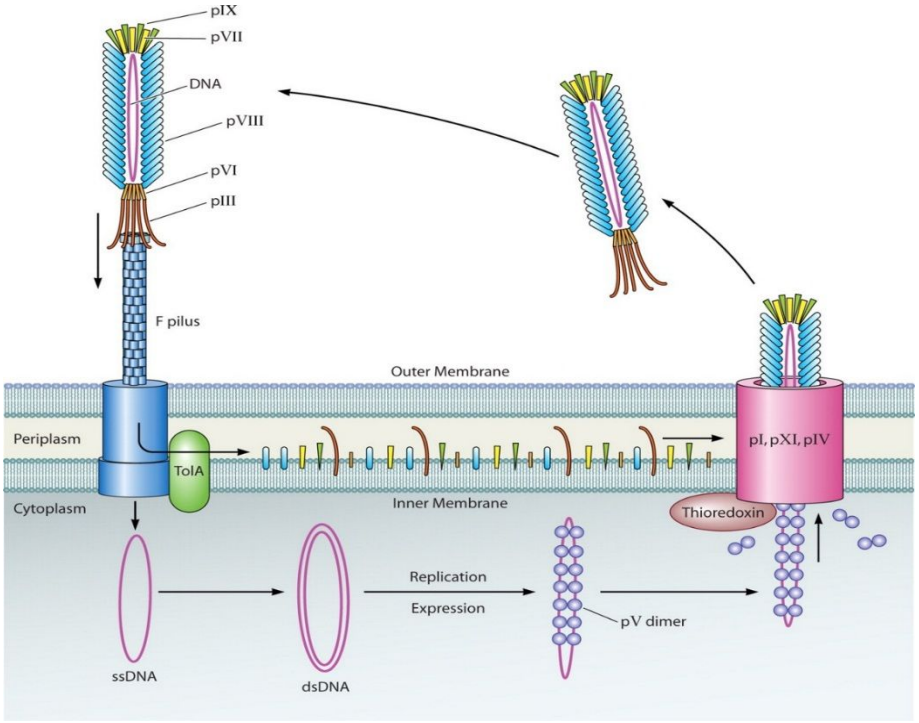

Figure 2. Life cycle of filamentous bacteriophage (phage display)

(Johnny et al., 2012)

3. Dietary Choline

3.1 General Characteristic of Choline

Choline is a water soluble essential nutrient and remains in the vitamin B category even though it does not entirely satisfy "trace organic nutrient" the strict definition of vitamin. Choline refers to the various quaternary ammonium salts which containing the N, N, N-trimethyl ethyl ammonium ion. The ion appears in the head groups of phosphatidylcholine and lecithin. Two types of phospholipid are abundant in all biological cell membranes. Choline is the precursor molecule for the neurotransmitter acetylcholine, which is involved in many functions including memory and muscle control.

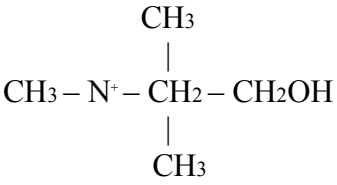

Figure 3. Structure of Choline

(McDowell, 1989)

Unlike B vitamins, it can be synthesized in the liver which greater amount required in the body and mostly function as a structural constituent rather than as a coenzyme. Choline is required to make essential the animal organism and choline with their compounds are utilized both as a building unit and as an essential component in regulation of certain body metabolism (Food and Nutrition Board, 1998; SH, 1999; Zeisel, 2000).

The role of choline nutrition was not known until 1930s and choline was observed to be active component of lecithin with prevent a fatty liver in rat. After the 1930s, lipotropic activity of choline related to transfer of methyl groups and sparing effects of choline on methionine, folacine and vitamin B_{12} were observed.

From 1940 to 1960, choline was prevent a perosis in turkey and spraddled-leg in pigs (Wintrobe et al., 1942; Johnson and James, 1948; Neumann et al., 1949). Recently research about choline reported that it was affected mobilization of liver lipid, other methyl donors on carcinogenesis and the neurotransmitter related syndromes.

3.2 Chemical Structure, Property and Metabolism of Choline

Characteristic of choline's chemical structure is its triplet of methyl group that would be allowed to serve as methyl donor. The chloride salt of this compound (e. g. choline chloride) is produced by chemical synthesis for use in the animal feed industry. Choline chloride exist as soluble white crystals that water and alcohol soluble. Choline is widely distributed in all plant and animal cells as a form of phospholipids phosphatidylcholine, lysophosphatidylcholine, choline plasmalogens, and sphingomyelin-essential components of all membranes (Zeisel, 1990). Lecithin is the predominant phospholipid in most animal membranes. Disaturated lecithin is the most active component of surface active agent in the lung (Brown, 1964). Choline is a precursor for the biosynthesis of the neurotransmitter acetylcholine. Glycerophosphocholine and phosphocholine are storage forms for choline within the cytosol and principal forms found in milk (Rohlfs et al., 1993).

Choline is present in the diet primary in the form of lecithin and as free base or sphingomyelin type also present. It is released from lecithin and sphingomyelin with hydrolysis by digestive enzyme in the intestinal rumen (Chan, 1991). Pancreatic secretion and intestinal mucosal cells contain enzymes were able to hydrolyzing lecithin in the diet. In need, digestion by pancreatic lipase is important process because most ingested lecithin absorption as a lysophosphatidylcholine (Zeisel, 1990). In the cells of the intestinal wall, lysophosphatidylcholine is able to deacylate to form glycerophosphocholine or reconstitute into lecithin.

Choline is absorbed from the jejunum and ileum by the energy and sodium dependent of the several carrier-mediated mechanisms. In contrast to choline absorption, choline is released from lecithin in the upper part of small intestine. Also, only one-third of ingested choline absorbed intestinal tract while the two-thirds is metabolized by intestinal microorganism to trimethylamine (De La Huerga and Popper, 1952). Absorbed choline is transported into the lymphatic circulation with the form of lecithin bound to chylomicron, which transported into the tissue as phospholipids associated with the plasma lipoproteins.

Choline was accumulated in all tissues as an essential component of phospholipids in membranes. Especially, choline uptake sites in liver, mammary gland, kidney, placenta, and brain are important. Indeed, Uptake of choline by mammary cells are able to concentrate choline almost 70-fold versus maternal blood (Chao et al., 1988). Almost of species are able to synthesize choline by the methylation of phosphatidylethanolamine with phosphatidylethanolamine-N-methyltransferase. This process is due to use of S-adenosylmethionine (SAM) as the methyl donor for numerous biochemical reactions that is greatest in liver, but other tissues also occurred.

Choline may be oxidized in the body to form a metabolite called betaine in the liver and kidney (Mann et al., 1938; Weinhold and Sanders, 1973). They are released in free form in the tissues by phospholipase C activity, which lecithin to yield a diglyceride and phosphorylcholine. Firstly, free choline can be oxidized by the mitochondrial enzyme choline dehydrogenase to yield betaine aldehyde. Secondly, betaine aldehyde dehydrogenase to betaine by the cytosolic enzyme with the presence of NAD^+ (Tsuge et al., 1980). Choline dehydrogenase activity occurs in the mitochondria (Kaiser and Bygrave. 1968; Wilken et al., 1970; Streumer-Svobodova and Drahota. 1977). Betaine aldehyde is then oxidized to betaine by the NAD^+-dependent enzyme betaine aldehyde dehydrogenase both in mitochondria and cytosol (Dragolovich; 1994). Betaine is the actual source of methyl groups.

Although, small fraction of choline is acetylated, amount of neurotransmitter acetylcholine were provided.

3.3 Function in Body

The numerous function of choline and compounds derived from choline were extensively published (Zeisel, 1981, 2000; Food and Nutrition Board, 1998; Shils et al., 1999). This review focuses primarily on the choline function and physiologic roles in some categories in the animal body (Figure 3).

3.3.1 Structural integrity of cell membranes

Choline is used in the synthesis of the phospholipids, lecithin (phosphatidyl choline), plasmalogens, and sphingomyelin for building and maintaining animal cell structure. Choline is incorporated incorporation into cellular phospholipids by being converted to phosphoryl choline, and finally reacting with phosphatidic acid to lecithin. Phospholipids consist of the cell membrane bilayers and their primary role is to regulate cell membrane porosity by changing the ionic characteristics of the membrane. Lecithin is a part of animal cell membranes and transfer of the lipid in cell plasma membranes. It is also an essential component of very low density lipoprotein (VLDL), the blood transport molecule for triglyceride (Lombardi et al., 1966). Considering of symptom of perosis in animals, choline is required as a constituent of the phospholipids due to normal maturation of the cartilage matrix of the bone.

3.3.2 Lipid transport and metabolism

Choline plays an essential role in fat metabolism in the liver. Cell membrane structure characteristics of their choline, lack of choline is closely related phospholipid functions such as fatty liver, lesions of the kidney and lipoprotein metabolism. In addition, choline prevents abnormal accumulation of fat (e. g. fatty livers) by promoting its transport as lecithin or by increasing the utilization of fatty

acids in the liver. Jacobs et al. (2004) indicated that increasing of triglyceride in the liver and decreasing of serum triglyceride were observed when a choline deficient diet was fed to mice. Therefore, it's called lipotropic agent because of its function of acting on fat metabolism by control fat concentration in liver.

Choline also affect hepatic secretion of VLDL. The triacylglycerol produced by liver is delivered to other tissues mainly in the form of VLDL (Yao and Vance, 1988; 1989). Phosphatidylcholine is a required component of the VLDL particle, and in choline deficiency decreased in capacity of liver cells to synthesize phosphatidylcholine molecules, resulting in the intracellular accumulation of triglycerides. It has been indicated that accumulation of triglyceride in the liver was choline and methyl group deficient resulting from impairment in the synthesis of VLDL with their function of attachment of triglycerides to VLDL (Mookerjea, 1971).

3.3.3 Signal transduction

The choline containing phospholipids, phosphatidylcholine and sphingomyelin, are precursors for the agent released at the termination of the parasympathetic nerves (Wauben and Wain weight, 1999) and terminate the signaling process by generating inhibitory second messengers (Zeisel, 1993).

It is available for transmission of nerve impulses from presynaptic to postsynaptic fibers of the sympathetic and parasympathetic nervous systems. Apparently, brain tissue lacks the ability to synthesize sufficient choline (Ansell and Spanner, 1971) for neural function. However, apparently circulating choline is the major source of choline for acetylcholine synthesis.

Platelet activating factor (PAF) and sphingo-phosphorylcholine, are also known to be cell signaling molecules. The PAF affected through specific receptors located on cells and has a various biological functions, as the mediators of parturition, mediation of many processes of inflammation and allergy (Snyder, 1987;

Kumar and Hanahan, 1987 Prescott et al., 1990).

The sphingolipids are components of mammalian cells and are necessary for cellular survival and growth (Hanada, 1992). Their activation may constitute one of the signal transduction pathways for triggering differentiation (Kim et al., 1991; Dressler et al., 1992). After activated sphingomyelinase in cellular ceramide levels, a compound with potent biological activities including the triggering of apoptosis (Obeid et al., 1993). As a result of formation of sphingosine from ceramide may be critical for regulating PAF signal transduction.

3.3.4 Methyl donor

Choline, as a source of labile methyl groups for methionine and creatine, may be oxidized in the body to form a betaine (Figure 4).

Betaine is a source of methyl (CH_3) groups that required for methylation reactions. Methyl groups from betaine is able to convert homocysteine to methionine. Elevated homocysteine in the blood, resulting in increased risk of cardiovascular diseases (Gerhard and Duell, 1999). However, there were little choline-homocystine interrelationship with feeding animals because natural protein contains were very little of the metabolic intermediate homocystine (Ruiz et al., 1983).

Methyl groups are synthesis of purine and pyrimidine which are used in production of DNA. Methionine is converted to SAM in a reaction catalyzed by methionine adenosyl transferase. Methyl group metabolism with folacin, Vit. B_{12} in *de novo* choline synthesis are allowed these substances to substitute in some portion of choline. Severe folacin deficiency has been observed that liver choline deficiency in rats (Kim et al., 1994).

3.4 Choline Contents in Feed

In general, choline is found in the form of phosphatidylcholine or

sphingomyelin in most feed ingredients (Neill et al., 1978). Most cereal grains and fruits and vegetables with high starch content are low in choline content. Major mono-gastric animals feed ingredients of corn and corn by-products are very low in choline. Wheat, barley and oat containing approximately twice as much choline contents compared corn. On the contrary to cereal grains, oil seeds such as peanuts, cottonseed, and soybeans are good sources of choline (Engel, 1943) with their relatively high phospholipid contents.

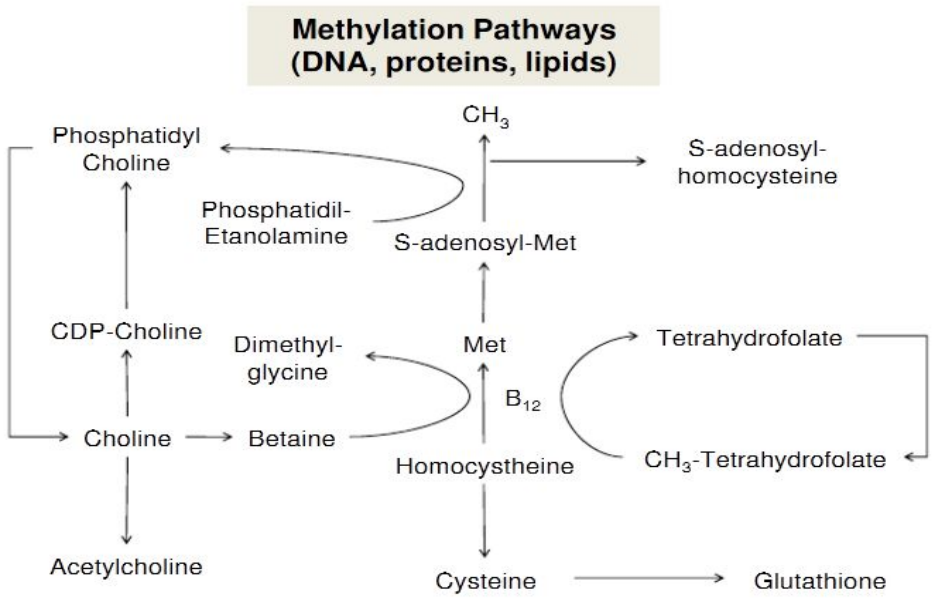

Figure 4. Metabolic pathways for choline and its relationships with folic acid and methionine (Krishnan Rajalekshmy, 2010)

4. Dietary Choline in Lactating Sows

4.1 Lactogenesis and Milk Yield in Lactating Sows

Lactogenesis is the onset of milk secretion and is generally divided into two stage process. It is the process of the functional differentiation of mammary tissue when change from a non-lactating to a lactating state. Lactogenesis Ⅰ occurs during pregnancy, the organelles associated with milk synthesis appear in mammary epithelial cells and there is gradual accumulation of pre-colostrum in the alveola lumina. Lactogenesis Ⅱ is the onset of copious milk secretion associated with parturition. It can be considered to be the period from the start of normal milk production until a steady production level is reached (Fleet et al., 1975; Tucker, 1988). Kengsinger et al. (1986) suggested that stage Ⅰ of lactogenesis occur between d 90 and 105 of pregnancy with stage Ⅱ occurring just prior to parturition in sows. However, definition of colostrum and transitional milk, traditionally used to describe the mammary secretion product during the first 4 days postpartum and from days 4 to 10 postpartum is not define clear-cut temporal changes in milk composition.

The piglets in the first two weeks after farrowing is important period and they are mainly dependent on the sow's milk for nutrition (King'ori, 2012). Approximately, 20-30% of early piglet mortality is due to lack of adequate nutrition with inadequate milk production by the sows (Fahmy and Benard, 1971). In general, Litter size (Quensel, 2011), birth weight (Smits et al., 1997), number of parity (Boyce et al., 1997), genetic (Eissen et al., 2000), endocrine status (Kveragas et al., 1986), during and after farrowing, and nutrition seem to influence sow's milk yield. To increase milk yield, various researches were conducted to supplementation of feed intake and fat levels (Coffey et al., 1982; Mullan and Williams, 1989). However, little information is available for the inclusion of another nutrition for improving milk yield in lactating sows.

4.2 Choline and Milk Production

Donkin (2011) reported that choline supplementation to improved the milk production on daily cow. Choline is important for assembly of VLDL to export triglyceride from liver. Especially, phosphatidylcholine is critical for VLDL formation. Increasing need for mobilize hepatic TG from the liver may increase the requirement for choline. Triglyceride which stored in adipose tissue release of non-esterified fatty acids (NEFA) into blood through lipolysis. The NEFA extracted by liver are either esterified to TG or partially oxidized to ketones to provide energy (adenosine triphosphate) for liver metabolism. Liver TG can be stored as droplets (fatty liver) or packaged into VLDL and exported into blood (Figure 4). Consequently, choline may affect the synthesis of the apolipoprotein components of VLDL to increase TG export from liver or the metabolism of ketones by peripheral tissues. Due to the fat metabolism of transport mobilized fatty acids from adipose tissue to the mammary gland, choline can affect milk production. However, there was lack of data on milk production and reproductive performance with choline supplementation in lactating sows.

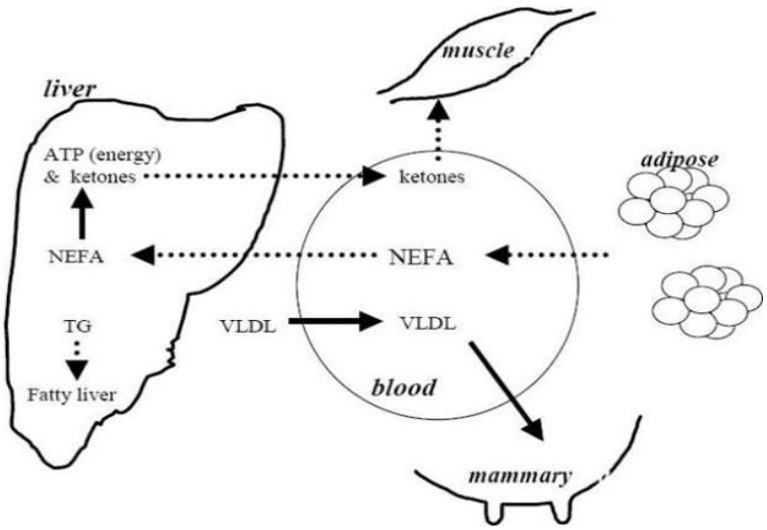

Figure 5. Mechanism of choline action in daily cow

(from Donkin, 2010)

4.3 Requirements of Choline for Lactating Sows

The NRC (2012) requirement for gestating and lactating sows were 1.25 g/kg and 1.00 g/kg, respectively, for maintenance, fetal growth, maternal tissue accretion and optimum reproductive performance. However, the requirement for choline may depend on the feed ingredient source. Barley (1,027 mg/kg) and wheat (778 mg/kg) is higher than in corn (440 mg/kg). It means that barley-wheat diets may not meet the requirement of choline for optimum reproduction in sows (Anonymous, 1980).

Many previous studies have showed that the level of methionine in the diet is important for determine the choline requirements. Pigs synthesize choline by methylating phosphatidyl ethanolamine in a three-step process involving methyl transfer from SAM. Thus, excess dietary methionine can eliminate the requirements for choline in pigs (Neumann et al., 1949; Nesheim et al., 1950; Kroening and Pond, 1967). Choline from soybean meal has been estimated to be 65 to 83 percent bioavailable compared to choline chloride as feed additives (Molitoris and Baker, 1976; Emmert and Baker, 1997). Because of high bioavailability in soy products, weaning to finishing pigs have not shown responses to supplemental choline when it was supplemented to corn–soy bean meal (SBM) or corn-isolated soy protein diets (Bryant et al., 1977; Russett et al., 1979b; North Central Region-42 Committee on Swine Nutrition, 1980).

4.4 Effects of Dietary Choline Levels of Sows

Feeding gestation gilts and sows fed grain-SBM diets with 434 to 880 mg of choline/kg supplementation has increased the number of live pigs at weaning. It may be noteworthy that choline supplementation would be helpful in gestation period (Kornegay and Meacham, 1973; Stockland and Blaylock, 1974; North Central Region-42 Committee on Swine Nutrition, 1976). Stockland and Blaylock (1974) also reported that corn-SBM diets with choline supplementation improved

conception rate. In addition, supplementation of choline in gestation diet can improve conception rate and the number of piglet born alive (Grandhi and Strain, 1980). Gilts fed choline supplementation during gestation farrowed heavier pigs (Luce et al., 1985). Cast et al. (1977) also observed an improvement in survival when sows fed lipid and additional choline during the last 5 day of pregnancy.

During lactation, lactating sows with choline supplementation showed the improvement of litter performance. A level of 1,000 mg of choline/kg of diet improved weight gain and prevented fat infiltration of the liver and kidneys in 2 day old pigs (Neumann et al., 1949). Kornegay and Meacham (1973) reported that sow fed choline supplemented diet farrowed more total pigs per litter and more weaning pigs per litter during the fifth and sixth parity. Cast et al. (1977) published that survival rate of piglets increased due to high level of choline. Supplementation of 260 mg of choline/kg to a diet consisting of 30 % vitamin-free casein, 37 % glucose, 26.6 % lard, and 2 % sulfathaladine, which contained 0.8 % methionine, showed prevention of choline deficiency in neonatal pigs (Johnson and James, 1948). However, choline supplementation of diets did not improve piglet survival or lipid mobilization when 500 ppm supplemental choline was included in the diet throughout lactation (Seerley et al., 1981). Moreover, a higher level of choline (824 mg/kg) diet did not improve the performance compared a lower level of choline (412 mg/kg) diet (Stockland and Blaylock, 1974). One possible reason for these observation can be explained by met the choline requirements of diet for reproduction in sows (Grandhi and Strain, 1981). In spite of the conflicting results, previous researches indicated that choline supplementation of sow diets may increase litter size at weaning, and may keep older sows in the producing swine farm longer. However, there is still lacking of published data for the effect of choline on sows which were conducted over the few decades. Recently, genetic improvement in lean yield and reproductive parameters such as litter size (CCSI, 2007) indicate that the choline effects and requirement for sows should be

reevaluated.

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Chapter III: Effects of Bacteriophage on Prevention of *Salmonella enteritidis* in Broilers

ABSTRACT: This study was conducted to investigate effects of bacteriophage on prevention of *Salmonella enteritidis* in broilers. A total of 320 one day old male broilers (Ross 308) were allotted by randomized complete block (RCB) design in 8 replicates with 10 chicks per pen. The experimental diets were formulated for 2 phase feeding trial (phase I; 0-2nd wk, phase II; 3rd-5th wk), and 4 different levels (0%, 0.05%; 5×10^{8}pfu/g, 0.1%; 1×10^{9}pfu/g, and 0.2%; 2×10^{9}pfu/g, respectively) of *Salmonella enteritidis* targeted bacteriophage were supplemented in the basal diet. There were no differences in body weight (BW) gain, feed intake and feed conversion ratio (FCR) during the whole experimental period (P>0.05). Relative weights of liver, spleen, abdominal fat, and tissue muscle of breast obtained from each bacteriophage treatment were similar to control and those values tended to increase when 0.2% (2×10^{9}pfu/g) bacteriophage was supplemented. In addition, a numerical difference of glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and low density lipoprotein (LDL) cholesterol level were observed when 0.2% (2×10^{9}pfu) of bacteriophage were provided even though blood profiles were not affected by supplemented levels of bacteriophage (P>0.05). In the result of a 14 d feeding after *Salmonella enteritidis* challenge to 160 birds from 4 previous treatments, mortality and *Salmonella enteritidis* concentration in the cecum were decreased with increasing bacteriophage level (P<0.05). This result demonstrated that supplementation of 0.2% (2×10^{9}pfu) *Salmonella enteritidis* targeted bacteriophage might not cause negative effect on growth, meat production, and it reduced mortality from *Salmonella enteritidis* challenge. Consequently, bacteriophage could be used as an alternative feed additive to antibiotics in broiler diets.

Key words: Bacteriophage, *Salmonella enteritidis*, Phage titer, Broilers

INTRODUCTION

In general, salmonellosis is a severe disease in humans caused by *Salmonella* bacteria in contaminated food. *Salmonella enteritidis* is a genetically homogeneous serotype of *Salmonella*, and it is well known as one of the most common types in food poisoning (Hudson et al., 2001; Chung et al., 2003; Gong et al., 2007). Chickens and eggs are frequently reported as the cause of outbreaks of Salmonellosis (St. Louis et al., 1988; Molbak and Neiman, 2002), and outbreaks of *Salmonella* bacteria are often detected in broiler flocks. In addition, once broilers are exposed to *Salmonella enteritidis*, the bacteria quickly spread to the entire poultry flock (Altekruse et al., 2006; Foley et al., 2008; Berrang et al., 2009). The feces from broilers infected with *Salmonella enteritidis* contaminated an entire chicken slaughterhouse (Shah et al., 2011). From these aspects, prevention of *Salmonella enteritidis* infection is an important issue in maintaining food safety and the poultry industry. In an attempt to reduce disease including *Salmonella enteritidis* in poultry, antibiotics were provided as a tool for effective prevention. However, in the recent years, poultry producers are becoming interested in alternative additives for replacing antibiotics in animal feed because of public concern of bacterial antibiotic resistance arising in products from animal origin (Huang et al., 2007; Li et al., 2008).

A bacteriophage or phage is a kind of virus that infects only bacteria (Carlton, 1999). They can be observed widely in nature and isolated from sewage and many places. Bacteriophage can attach to bacterial cells with their tail fibers and inject the required amount of components for bacteriophage replication into the bacterium. Several studies indicated that *Salmonella* could be controlled by the use of bacteriophage (Barrow et al., 1987; Berchieri et al., 1991; Sklar and Joerger, 2001). In Korea the use of antibiotics in animal feed was prohibited from July, 2011, consequently many poultry producers were interested in bacteriophage as an alternative feed additive to dietary antibiotics for controlling *Salmonella* infection.

A wide variety of methods for bacteriophage application such as air sac inoculation, drinking supply, aerosol spray, and their combination have been reported (Huff et al., 2003). Few experiments on poultry have been conducted with bacteriophage as feed additives to antibiotics.

Therefore, this experiment was conducted to investigate effects of anti-*Salmonella enteritidis* bacteriophage on growth performance and protection against induced *Salmonella enteritidis* infection in broilers.

MATERIALS AND METHODS

Bacteriophage Isolation and Amplication

Bacteriophage screening samples were collected from slaughter houses and sewage nearby disposal plants. Samples were centrifuged at 4,000 rpm for 10 min and the supernatants were filtered through a 0.45 μm filter. A mixture of 18 ml of filtered samples and log-phase *Salmonella enteritidis* in 2 ml of ten times strength Luria-Bertani (LB) medium (tryptone 10 g; yeast extract 5 g; NaCl 10 g; in a final volume of 1 L) was incubated at 37 °C for 18 h and centrifuged at 4,000 rpm for 10 min. The supernatants were filtered through a 0.2 μm filter and then mixed with fresh log-phase *Salmonella enteritidis* and 3 ml of 0.7 % agar. The fluid was poured into tryptic soy agar (TSA) plates and incubated overnight at 37 °C. One clear plaque was selected and subsequently plated three times to purify the isolate, the purified plaque was suspended in SM buffer (20 mM Tris-HCl, 2 mM $MgCl_2$ pH 7.0), and stored at 4 °C until use. The selected bacteriophage was shaken in *Salmonella enteritidis* cultured broth for amplification. After an aliquot of 1.5×10^{10} pfu/g was centrifuged at 4,000 rpm, the pellet was re-suspended in 4 ml of a sterile solution. The suspension was inoculated 7.5×10^{7} pfu/g of the bacteriophage at an MOI (multiplicity of infection) of 0.005 and incubated at 37 °C. After 20 min of incubation, this solution was inoculated on LB media in a flask and cultured at 37 °C for 5 h. The solution was centrifuged at 4,000 rpm and then passed through a

0.2 μm filter. The bacteriophage was quantified by making serial dilutions and spreading on soft agar overlay plates. The final bacteriophage culture was collected and prepared as a powder. The plaque forming unit of 10^9 pfu/g was used as feed additives in the dietary treatments.

Salmonella enteritidis Challenge Culture

Salmonella enteritidis strain used in current study provided by the *Salmonella* Genetic Stock Center (Calgary, AB, Canada). The isolate *Salmonella enteritidis* strain was grown overnight in LB broth (Difco, Franklin Lakes, USA) at 37 °C. For the challenge trial, the *Salmonella enteritidis* culture was serially diluted in sterile phosphate buffered saline and marked clarify, and the cfu was determined to achieve the final concentration.

Experimental Design, Diets, and Animal Managements

Three hundred and twenty one-day-old male broilers (Ross 308), with an average body weight of 41.4 g were used for a 5 wk growth trial. Broilers were randomly allocated to one of four treatments in a randomized complete block (RCB) design and 8 replicates with 10 broilers per pen. The birds were raised on rice hull bedding in an environmentally controlled house at a research unit of Seoul National University, South Korea. All broilers had free access to feeders and automatic waterers. The ambient temperature was maintained at 35 °C for 2 d, 31 °C in the first week and then gradually reduced to 22 °C by the end of the experiment.

Two basal diets were formulated for the broilers during phase I and II of a 5 week feeding trial. Phase I diet was fed during 0 to 2 wks and phase II diet was provided thereafter until week 5. Basal diets were based on corn-soy bean meal (SBM) and 4 levels of bacteriophage (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) used as feed additive was included at the

expense of corn to the basal diet. The basal diet contained 3,050 and 3,100 kcal of metabolizable energy (ME)/kg and total lysine contents were 1.19 and 1.05 % for the phase I and II, respectively. All other nutrients were formulated to meet or exceed the Korean Feeding Standard for Poultry (2007). Experimental diets were provided by a mash type of feed and nutrients contents in the diets were analyzed via the method of the AOAC (1995). The ingredient composition and calculated nutrients are shown in Table 1. Body weight (BW) and feed intake (FI) were recorded at the end of phase I and II periods to calculate the BW gain and feed conversion ratio (FCR).

Relative Weights of Tissue and Blood Sampling

After 5 week of growth trial, eight chickens from each treatment were randomly selected for sacrifice. The liver, spleen, abdominal fat, breast muscle and leg muscle were removed and weighed immediately for a relative weight calculation. Blood samples from 32 broilers that represented the 4 treatments were taken from the carotid artery end of growth trial. The samples were centrifuged for 5 min at 3,000 rpm at 18 °C to collect serum and then carefully transferred to plastic tubes and stored at -20°C until analysis. Glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, total cholesterol, HDL cholesterol, and LDL cholesterol in blood were analyzed according to Association of Official Agricultural Chemists (AOAC, 1995).

Phage Titer Analysis

After 5 week of growth trial, broilers were sacrificed to take sample of feed, feces and samples of breast and leg muscle to detect phage titer. Five grams of each samples were set in 10 ml SM buffer and homogenized for 1 min. 100 ml of the homogenizing sample was serially diluted (10-fold dilution) in SM buffer and

plated on LB plate (Difco, BD science, USA). After 24 hrs of incubation at 37 °C the plaques were measured.

Mortality and Cecal Microflora after Salmonella enteritidis Challenge

One hundred and sixty, five-week-old male broilers (Ross 308), with average BW of 1,805.5 ± 28.0 g were allotted from the previous treatments to the challenged by *Salmonella enteritidis* trial with 4 replicates and 10 birds per pen according to completely randomized design (CRD). The *Salmonella enteritidis* isolate was confirmed serotyped and the final concentration confirmed. For bacterial challenge, all broilers received 1×10^9 cfu of *Salmonella enteritidis* by oral inoculation and chicks were daily observed for mortality for 14 days. Feeding and environmental control were managed the same as the previous growth trial. Also, for the quantitative analysis of microflora, eight broilers were randomly selected from each treatment and sacrificed, and the cecal content were collected. For enumeration of bacterial populations, 1 g of cecal content was diluted in saline solution and re-suspended by vortex mixing, then serially diluted in saline solution. A volume of 100 um volumes of each dilution was spread overlaid on the surface of an agar plate by spread plate method. This procedure was performed in duplicate over *Salmonella shigella* agar (supplemented 100 um of chloramphenicol), MacConkey agar, and *Lactobacilli* agar (Difco, BD science, USA). Plates were incubated at 37 °C. After 24 h of incubation, each plate was spotted for cecal microflora count and examined for the presence of each bacterial growth. The protocol for the experiment was approved by the Institutional Animal Care and Use Committee of the Seoul National University.

Statistical Analysis

The experimental data were analyzed as a completely randomized design

using the least significant difference (LSD) procedure of SAS (SAS Inst., Inc., Cary, NC, USA) and in randomized complete block design, the general linear model (GLM) procedure of SAS was performed. A pen was considered as the experimental unit for data on growth performance and mortality. The individual broiler was used as the unit for data in the experiment on blood profiles relative weight of organ and muscle, and cecal microflora. Linear and quadratic effects for equally spaced treatments were assessed by orthogonal polynomial contrast to determine the effect of supplementation level of bacteriophage on all measurements. Difference were declared significant at $P<0.05$ and highly significant at $P<0.01$.

RESULTS AND DISCUSSION

Growth Performance

Salmonella enteritidis pathogen free status was maintained before and after feeding trial, so that none of experimental broilers were infected. Growth performance of broilers fed the different levels of bacteriophage in the diet during 5 weeks is shown in Table 2. Body weight (BW) and BW gain were not affected by dietary bacteriophage supplementation at d 14 and d 35 of the experimental period ($P>0.05$). When the dietary bacteriophage level was increased, feed intake (FI) and feed conversion ratio (FCR) were not affected by supplementation level of bacteriophage. Based on the result of growth performance, bacteriophage supplementation as a feed additive had no detrimental effect on feed consumption or broiler growth. As growth performance is one of the important parameters in poultry production, lower BW equates with increase cost for broiler meat production. Bacteriophage appeared with bacteria that infected one serotype species (Ackerman et al., 1978). Characteristics of their bacteriophage, it can't attack and infect other type of species pathogenic bacteria. Several studies on the effect of bacteriophage (Barrow et al., 1998; Carlton, 1999; Huff et al., 2003) demonstrated that the pathogenic bacterium, *Salmonella enteritidis*, was clearly decreased by various

methods of pathogenic bacteria infection. When bacteriophage is utilized as a feed additive instead of antibiotics in animal feed then its inhibitive effect on harmful bacteria replication in the gastrointestinal tract can be expected (Yongsheng et al., 2008). Lim et al. (2010) demonstrated 10^8 pfu/g of bacteriophage was excreted via feces when bacteriophage was supplemented to broilers diet. However, their study indicated that supplementation of *Salmonella enteritidis* targeted bacteriophage as feed additives did not affect growth performance because of only *Salmonella enteritidis targeted bacteriophage were supplement* without *Salmonella enteritidis* infection in broilers.

Relative Weights of Organ and Muscle

The effects of the inclusion levels of bacteriophage on relative organ and muscle weight are presented in Table 3. There were no effects of supplementation of bacteriophage on relative weight of organs at 35 d of the experiment (P>0.05). The relative weight of organs and muscle showed similar values to commercially produced broilers. However, the relative weights of leg muscle in the bacteriophage treatments were numerically higher than that of the control treatment. This result implies that the addition of bacteriophage would not have a detrimental effect on poultry meat production. Additionally, broilers maintained a normal growth and morphological response in liver and spleen when bacteriophage was supplemented. Merril (2008) reported that bacteriophage DNA was observed in the cells of Peyer's patches of the gastrointestinal tract, peripheral white blood cells, and the cells of the liver and spleen after feeding of phage M13 DNA. Similarly, Molenaar et al. (2002) reported that M13 bacteriophage was found in liver, spleen, lung, and kidney after oral administration of M13 bacteriophage by systemic circulation. Current and previous studies suggest that the accumulation of bacteriophage in the liver and spleen is not affect immune function in broilers.

Blood profiles

Data for the blood profiles are presented in Table 4. In the presence of bacteriophage supplemented in the diet, the responses of GOT and GPT were not different significantly (P>0.05) among treatments, even though the lower GOT concentration in blood was observed when broilers were fed bacteriophage compared to control treatment. Serum GOT is a kind of cytosolic enzyme specially in liver, consequently it can represent the status of liver damage when high level of GOT activity is observed in the blood. In the current study, the bacteriophage supplementation in broilers diet did not induce damage to cells in the liver. There were no significance differences in total cholesterol, HDL, and LDL between the bacteriophage levels (P>0.05). However, supplementation of bacteriophage in broilers diet tended to reduce the concentration of serum LDL cholesterol while numerically increased the serum HDL cholesterol concentration. Barrett-Connor and Suarez (1982) reported that serum GOT and HDL cholesterol have a negative relationship in humans. In the current study, the increased level of HDL cholesterol may be related to the reduction of GOT concentration. In this respect, supplementation of bacteriophage as a feed additive would not damage hepatic or liver tissues of broilers and consequently it can be safely supplemented in broilers diets.

Phage Titer

A greater phage titer in feed and feces was observed as the bacteriophage level increased (Table 5). Moreover, phage titer was not detected from leg and breast muscle in any treatment. When broilers were fed bacteriophage, bacteriophage passed through the gastrointestinal tract and was excreted in the feces, consequently dietary bacteriophage was not accumulated into the muscles. The inclusion of bacteriophage in the diet could reduce intestinal *Salmonella enteritidis* level because high levels of bacteriophage retarded their growth in the

gastrointestinal tract. Additionally, the bacteriophage can be used to reduce environmental contamination by *Salmonella enteritidis* when high levels of fecal bacteriophage are excreted. The stability of bacteriophage under various conditions is an important parameter. Consequently, an evaluation of the stability of bacteriophage is necessary prior to considering bacteriophage as an alternative to antibiotics (Mathur et al., 2003).

Mortality after Salmonella enteritidis Challenge

Mortality of broilers fed different levels of bacteriophage after *Salmonella enteritidis* challenge is shown in Table 6. Mortality after inoculation by *Salmonella enteritidis* was significantly decreased ($P<0.05$) as bacteriophage level increased. It indicates that bacteriophage parasitize *Salmonella enteritidis* bacteria, resulting in a reduction of mortality. In general, infection by *Salmonella enteritidis* has been observed in both young and adult chickens. However, symptoms can be different for each broiler. Young chickens often develop a systemic disease with different mortalities (Suzuki, 1994; Duchet-Suchaux et al., 1995; Velge et al., 2005). In contrast, adult hens, typically remained asymptomatic after infection with *Salmonella enteritidis* (Lister, 1988; Velge et al., 2005; Golden et al., 2008). These various responses in mortality of chickens challenged with *Salmonella enteritidis* might depend on *Salmonella enteritidis* phage types. Since the various strains of *Salmonella enteritidis* phage types are found in poultry, many researchers have observed that *Salmonella enteritidis* phage types are related to mortality in broilers. Dhillon et al. (1999) used six strains of *Salmonella enteritidis* to infect one day old broilers by crop gavage and reported that the mortality range was from 0 to 23%. In addition, Barrow (1991) concluded that the mortality ranged from 20 to 96% between six strains of *Salmonella enteritidis*.

Cecal Microflora after Salmonella enteritidis Challenge

The quantitative total of the microbes, *Lactobacilli*, *E. coli*, and *Salmonella*

enteritidis in the cecal microflora were measured in the study having the effectiveness of bacteriophage in *Salmonella enteritidis* challenged broilers (Table 7). The different inclusion levels to broiler diet did not alter the population of bacteria (*Lactobacilli* and *E. coli*) after *Salmonella enteritidis* inoculation. However, the number of *Salmonella enteritidis* in cecal digsta from broilers fed all bacteriophage diet was reduced compared to control in a linear response to supplementation levels ($P<0.05$). In general, bacteriophages have a high level of host specificity for certain species of bacteria (Ackermann et al., 1978; Joerger, 2003). Bacteriophage attaches to specific receptors of their bacterial host and it use the host's intracellular machinery to translate their own genetic code. Hence, bacteriophage is harmless to the normal intestinal microflora (Oliveira et al., 2009). There are many studies regarding the utilization of bacteriophage to prevent infection with harmful bacteria (Huff et al., 2005; Sulakvelidze and Barrow, 2005; Sulakvelidze and Kutter, 2005). In the study reported by Borie (2008), inclusion of bacteriophage by spray, drinking water and aerosol methods reduced the *Salmonella enteritidis* colonization in infected chickens. Data from the current study suggested that bacteriophage showed therapeutic effects against *Salmonella enteritidis* colonization in the cecum.

IMPLICATION

Since the EU banned the use of antibiotics in animal feed, many countries have tried to prohibit the supplementation of antibiotics to farm animal feed and searched for the alternatives particularly in the feed industry. As *Salmonella enteritidis* infection is still an important issue to maintain food safety, dietary bacteriophage has been suggested as a feed additive instead of antibiotics. This study demonstrated that dietary *Salmonella enteritidis* targeted bacteriophage from 0.05 to 0.2 % of total diet can be useful tool to prevent *Salmonella enteritidis*

infection and subsequently reduce mortality without any detrimental influences on broiler productivity. With a consideration of their safety, stability, and therapeutic efficacy in broiler production, the use of bacteriophage in broiler diets can be an alternative feed additive to antibiotics.

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Table 1. Composition of experimental basal diet (as-fed basis)

Ingredients[1]	Phase Ⅰ (d 0 to 14)	Phase Ⅱ (d 14 to 35)
Ground corn	52.14	54.15
Soybean meal, 46% CP	31.90	28.01
Wheat bran	1.43	3.87
Corn gluten meal	6.76	5.73
Fish meal	0.10	0.04
Tallow	3.74	4.69
Dicalcium phosphate	1.87	1.61
Limestone	1.37	1.28
Salt	0.22	0.22
L-lysine·HCl	0.04	0.00
DL-methionine	0.13	0.10
Choline chloride (50%)	0.10	0.10
Anticoccidials	0.05	0.05
Vitamin-mineral premix[2]	0.15	0.15
SUM	100.00	100.00
Chemical composition[3] (%)		
Metabolizable energy (kcal/kg)	3,056.71	3,106.72
Crude protein (%)	23.01	21.01
Lysine (%)	1.19	1.05
Methionine (%)	0.52	0.46
Calcium (%)	1.00	0.90
Avail. Phosphorus (%)	0.45	0.40

[1] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g and, 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented as fed basis.

[2] Provided the following quantities of vitamin-mineral mixture per kg of complete diet: vitamin A, 18,000 IU; vitamin D_3, 3,750 IU; vitamin E, 30 mg; vitamin K_3, 2.7 mg; vitamin B_1, 3 mg; vitamin B_2, 9 mg; vitamin B_6, 4.5 mg; vitamin B_{12}, 30 mcg; Cal-Pan, 15 mg; Niacin, 37.5 mg; Folic acid, 1.5 mg; Biotin, 75 mcg; Mn, 97.5 mg; Zn, 97.5 mg, Fe, 75 mg; Cu, 7.5 mg; Co, 375 mcg; I, 1.5 mg; Se, 225 mcg, Antioxidant, 9 mg.

[3] Calculated value.

Table 2. Effect of bacteriophage on growth performance in broilers[1,2]

Criteria	Bacteriophage[3], %				SEM[4]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
Body weight, g/bird							
Initial	41.4	41.4	41.4	41.4	0.03	-	-
2 week	382.1	369.6	379.6	375.6	2.68	0.689	0.414
5 week	1,988.4	1,959.1	1,990.0	1,991.1	10.64	0.623	0.644
BW gain, g/bird							
0-2 week	340.7	328.2	338.3	334.3	2.69	0.689	0.414
2-5 week	1,606.3	1,589.5	1,620.3	1,615.5	9.28	0.515	0.762
Overall	1,947.0	1,917.7	1,958.6	1,949.7	10.64	0.623	0.644
Feed intake, g/bird							
0-2 week	483.7	471.7	476.4	481.9	2.73	0.975	0.119
2-5 week	2,727.3	2,740.7	2,789.6	2,728.9	20.35	0.745	0.322
Overall	3,210.9	3,212.4	3,266.0	3,210.7	21.69	0.763	0.473
FCR, feed/gain ratio							
0-2 week	1.42	1.44	1.41	1.45	0.01	0.728	0.681
2-5 week	1.70	1.73	1.72	1.69	0.01	0.771	0.219
Overall	1.65	1.68	1.67	1.65	0.01	0.857	0.256

[1] A total of 320 broilers were fed from average initial body weight 41.4 ± 0.0 g and the average of final weight was 1,982.2 g.

[2] Least squares means for eight pens/treatment with ten broilers/pen.

[3] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented.

[4] Standard error of means.

Table 3. Effect of bacteriophage on the relative weight of organ and muscles in broilers[1,2]

Criteria	Bacteriophage[3], %				SEM[4]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
Relative weight of organ, g/100g BW							
Liver	1.89	1.94	1.77	1.80	0.05	0.374	0.927
Spleen	0.08	0.08	0.08	0.07	0.01	0.522	0.310
Abdominal fat	2.23	2.22	2.31	2.15	0.08	0.804	0.597
Breast muscle	7.80	7.64	7.57	7.81	0.09	0.967	0.300
Leg muscle	9.31	9.43	9.44	9.47	0.06	0.339	0.724

[1] A total of 32 broilers were used at 5 week-old of age and the average body weight was 1,996.3 ± 8.75g.

[2] Least squares means for eight broilers per treatment.

[3] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented.

[4] Standard error of means.

Table 4. Effect of bacteriophage on blood profiles in broilers[1,2]

Criteria	Bacteriophage[3], %				SEM[4]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
Blood profiles							
GOT, U/L	287.0	273.0	279.8	278.8	4.6	0.774	0.644
GPT, U/L	6.9	7.1	6.6	6.9	0.1	0.674	1.000
Total cholesterol, mg/dL	124.9	122.5	126.3	126.5	2.5	0.617	0.733
HDL cholesterol, mg/dL	94.9	94.3	97.9	99.3	2.1	0.292	0.776
LDL cholesterol, mg/dL	15.5	13.6	15.0	13.3	1.0	0.164	0.941

[1] A total of 32 broilers were used at 5 week-old of age and the average body weight was 1,996.3 ± 8.75g.
[2] Values are means for eight broilers per treatment.
[3] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented.
[3] Standard error of means.

Table 5. Effect of bacteriophage on phage titer in broilers

Criteria	Bacteriophage[1], %				SEM[2]
	0	0.05	0.1	0.2	
	---------- Log_{10}pfu/kg content----------				
Feed	ND[3]	7.30	7.56	7.83	-
Floor (rice hull)	ND	5.93	6.31	6.25	0.81
Breast muscle	ND	ND	ND	ND	-

[1] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented.
[2] Standard error of means.
[3] Not detected (under 2×10^2 pfu/kg).

Table 6. Effect of bacteriophage on mortality after *SE* challenge in broilers[1,2]

Criteria	Bacteriophage[3], %				SEM[4]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
Mortality, %	30.0[a]	17.5[ab]	12.5[b]	10.0[b]	3.10	0.020	0.110

[1] A total of 160 broilers were used at 5 week-old of age.

[2] Least square means for 4 pens/treatment with ten broilers/pen.

[3] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented.

[4] Standard error of means.

[ab] Means with different superscripts in the same row significantly differ ($P<0.05$).

Table 7. Effect of bacteriophage on cecal microflora after *SE* challenge in broilers[1]

Criteria	Bacteriophage[2], %				SEM[3]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
	---------- Log_{10}cfu/g content----------						
Lactobacilli	6.25	6.25	6.16	6.27	0.09	0.946	0.672
E. coli	4.92	5.01	4.78	4.91	0.22	0.752	0.906
Salmonella enteritidis	6.31^{A}	4.32^{B}	4.27^{B}	4.23^{B}	0.21	0.001	0.202

[1] Values are means for eight broilers per treatment.

[2] Four different levels (0%, 0.05%; 5×10^{8} pfu/g, 0.1%; 1×10^{9} pfu/g, and 0.2%; 2×10^{9} pfu/g, respectively) of bacteriophage were supplemented.

[3] Standard error of means.

[AB] Means with different superscripts in the same row significantly differ ($P<0.01$).

Chapter IV: Effects of Bacteriophage on Growth Performance, Fecal Properties, Blood profiles and Immune Response in Weaning Pigs

ABSTRACT: This current study was conducted to investigate the effects of bacteriophage supplementation on weaning pigs. A total of 160 pigs [(Yorkshire × Landrace) × Duroc] (BW=6.78 ± 1.72 kg; weaned at day 24 ± 3) were allotted to 4 groups in a randomized complete block (RCB) design with 5 replication for 5 week growth trial. The experimental diets were formulated for 2 phase feeding trial (phase I; 0-2nd wk, phase II; 3rd-5th wk), and 4 different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) of 16 types of pathogen targeted bacteriophage were supplemented in the basal diet. The basal diet was regarded as the control treatment. All weaning pigs were allowed to access diet and water *ad libitum*. For the whole experimental period, ADG, ADFI, and G:F ratio were not affected by bacteriophage levels, resulting in no different BW among all treatments (P>0.05). With increasing bacteriophage level in the diets, fecal microbial population of pathogenic *Salmonella spp.* (linear, P=0.010; 2wk) and *Escherichia coli* (linear, P=0.053; 5wk) were reduced. However, *Lactobacilli* concentration was increased in feces when pigs were fed 0.2% of bacteriophage, showing linear response to bacteriophage levels (linear, P=0.019, 2wk; linear, P=0.001, 5wk). The inclusion level of bacteriophage in weaning pig diets resulted in decreasing the incidence of diarrhea (linear, P=0.001), and linear (P=0.002) and quadratic (P=0.008) responses to the bacteriophage levels were also observed in fecal moisture content. In blood immune response, there was a linear declines in IgA concentration as bacteriophage increased (linear, P=0.043). Based on the blood profiles results, GOT and GPT levels were not affected by bacteriophage levels, whereas total cholesterol (linear, P=0.001, quadratic,

P=0.028, 2wk; linear, *P*=0.002, 5wk) and LDL cholesterol (linear, *P*=0.002, 2wk) levels were decreased by the level of bacteriophage in the diets. Results from this experiment suggested that 16 types of pathogen targeted bacteriophage supplementation did not influence on growth performance in the minimal disease status of the experimental facility. However, 0.2 % (2×10^9 pfu/g) bacteriophage supplementation might have beneficial influences on microbial population, fecal status, immune response, and blood profiles in weaning pigs.

Key words: Bacteriophage, Growth performance, Fecal properties, Immune response, Weaning pigs

INTRODUCTION

Generally, *Salmonella*, and *E. coli* are pathogenic bacteria that cause acute infections such as sepsis and gastroenteritis in the intestine. These diseases reduce growth performance and productivity because of diarrhea after weaning (Foley and Lynne, 2008). Moreover, they are zoonotic pathogens (Delpech et al., 1998). In Korea, prohibiting antibiotics in animal feeds since July 2011 has caused swine ileitis (*Lawsonia intracellularis*) and growth retardation after weaning. To prevent this and other diseases, there have been various studies on decreasing pathogens in swine and on alternatives to antibiotics for swine growth and treats (Kodama et al., 2008; Jung et al., 2010). Indeed, bacteriophage therapy against pathogenic bacteria has been one area of those studies (Lee and Harris, 2001; García et al., 2008; Cairns et al., 2009; Jamalludeen et al., 2009).

Bacteriophages are viruses which are parasites of pathogenic bacteria (McGrath et al., 2004) and classified by their lytic and lysogenic cycles during their life cycle. In the lysogenic cycle, bacteriophages insert their DNA into the host and become prophages through DNA replication. In the lytic cycle, bacteriophages damage the DNA of the host or the host itself and are released by cell lysis. Therefore, the lytic cycle of bacteriophage can be used for therapy. Bacteriophage supplementation can cure diarrhea caused by *E. coli* in calves, piglets, and sheep (Smith and Huggins 1982, 1983; Smith et al., 1987). Toro et al. (2005) showed a decrease in *Salmonella* by bacteriophage therapy in the intestine and fecal samples of broilers. Moreover, bacteriophage injections have the effects of decreasing diarrhea rate and damage of the villus (Kim et al., 2011). Wall et al. (2009) also observed a decrease in *Salmonella* in the tongue, ileum and cecum of finishing pigs by bacteriophage supplementation after injecting *Salmonella typhimurium*. Moreover, bacteriophage can be positive effects of the population of intestinal microflora, which are necessary for the development of gut and immune system (Wang et al., 2013). These results indicated that bacteriophage can effectively treat

and decrease host pathogens and develop the immune system. However, there is still a lack of published data on effects of bacteriophage as feed additives for weaning pigs.

Therefore, this experiment was conducted to investigate the effects of bacteriophage supplementation on growth performance, microorganism composition of the feces, blood profiles and immune responses in weaning pigs.

MATERIALS AND METHODS

Bacteriophage Preparation

Bacteriophage had been used against diarrheal disease caused by *Salmonella gallinarum, Salmonella thyphimurium*, *Salmonella enteritidis*, *Salmonella durby*, *Salmonella cholerasuis*, *Staphylococcus aureus*, *Escherichia coli* K88 · K99 · K41 987P, and *Clostridium perfringens* type A · B · C · D · E. These pathogenic bacteria against bacteriophage provided by a local company (CTCbio Co., Ltd., Seoul, South Korea) was type of powder. The plaque forming unit of 10^9 pfu/g was used as feed additives in the dietary treatments.

Experimental Design, Diets, and Animal Managements

A total of 160 crossbred pigs [(Yorkshire × Landrace) × Duroc; Seoul National University experimental facility, Muan, South Korea] with an average initial BW of 6.78 ± 1.72 kg were used for 5-week growth experiment. Immediately on being weaned from sows at 24 ± 3 days of age, piglets were transported to the Seoul National University experimental facility and allotted to 4 treatments in 5 replicates by randomized complete block (RCB) design with 8 pigs (4 barrows and 4 gilts) per pen. Animals were housed in a 1.2 × 3.6 m^2 plastic floor, equipped with a feeder and a nipple drinker to allow free access to feed and water during the experimental period. The ambient temperature in the weaning house was maintained at 31 ℃ and then gradually fallen to 27 ℃ at the end of the experiment. Individual

BW and pen feed intake were recorded during the whole experimental period. Body weight and feed consumption were recorded at 0, 2 and 5 weeks to calculate average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F ratio). Two basal diets were formulated for the weaning pigs during phase I and II of a 5 week feeding trial. Phase I diet was fed during 0 to 2 wks and phase II diet was provided thereafter until week 5. Basal diets were based on corn-soy bean meal (SBM) and 4 levels of bacteriophage (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) used as feed additive was included at the expense of corn to the basal diet. The basal diet contained 3,265 kcal of ME/kg and total lysine contents were 1.35 and 1.15 % for the phase I and II, respectively. All other nutrients were formulated to meet or exceed the National Research Council (NRC, 1998). Experimental diets were provided by a mash type of feed and nutrients contents in the diets were analyzed via the method of the AOAC (1995). The ingredient composition and calculated nutrients are shown in Table 1.

Blood Sampling and Profiles

Blood samples were collected from anterior vena cava of six pigs per treatment, a total of 28 pigs at initial, 2 wk and 5 wk for analyses of serum blood urea nitrogen and glucose. Blood samples were collected into vacuum tube were centrifuged for 5 min at 3,000 rpm at 18°C after clotting during 30 minute at room temperature. The serum was carefully removed to specific vials and stored at 4 °C until blood profiles analysis and was kept at -20 °C for serum immunoglobulins (IgG and A) analyses. The enzyme-linked immunosorbent assay accessory starter kit (E101; Bethyl Laboratories Inc., USA) and quantitation kit (E100; BETHYL) were used for this analysis. The enzyme-linked immunosorbent assay (ELISA) was performed in duplicate, using the infinite M200 pro ELISA reader (TECAN, Inc., Switzerland) and analysis results were expressed as milligrams of IgG and A per milliliter of serum. Glutamic-oxaloacetic transaminase, glutamic-pyruvic

transaminase, total cholesterol, HDL cholesterol, and LDL cholesterol in blood were analyzed according to Association of Official Agricultural Chemists (AOAC, 1995).

Fecal Microbiology

Fecal *E. coli, Salmonella spp.*, and *Lactobacilli* counts were proceed at initial, 2 wk and the end of feeding trial (5 wk). Fecal samples were collected from 5 weaning pigs per treatment (average body weight 25.56 ± 1.44 kg). For enumeration of bacterial populations, 1 g of fecal content was diluted in saline solution and re-suspended by vortex mixing, then serially diluted in saline solution. A 100 mL volumes of each dilution was spread overlaid on the surface of an agar plate with spread plate method. This procedure was performed in duplicate over MacConkey agar, *Salmonella shigella*, agar and *Lactobacilli* agar (Difco, BD science, USA). Plates were incubated at 37 °C. After 24 h of incubation, each plate was spotted for fecal microflora count and examined for the presence of each bacterial growth.

Incidence of Diarrhea and Fecal Moisture Contents

The incidence of diarrhea was recorded during whole experimental period and diarrhea incidence was scored into 5 numbers by counting weaning pigs showing evidence of diarrhea (Diarrhea incidence score: 1=hard feces; 2=slightly soft feces; 3=soft, partially formed feces; 4=loose, semiliquid feces and 5=watery, mucus-like feces) by Sherman et al. (1983) method. Fecal samples were collected from 8 pigs per treatment with 24 hours by rectal dilatation. Analysis of fecal moisture by the AOAC (1995) procedure 967.03 was applied that 1 g of sample and dried in 3 hours at 105 °C drying oven. After drying, samples were discharged in desiccator for 30 min.

Moisture contents (%) = (1 g sample weight - dried 1g sample weight) × 100 / 1 g sample weight

Statistical Analysis

The experimental data were analyzed the least significant difference (LSD) procedure of SAS (SAS Inst., Inc., Cary, NC, USA) and in randomized complete block design, the general linear model (GLM) procedure of SAS was performed. A pen was considered as the experimental unit for data on growth performance and incidence of diarrhea. The individual weaning pig was used as the unit for data in the experiment on fecal microbiology, fecal moisture contents, blood profiles and serum immunoglobulin. Linear and quadratic effects for equally spaced treatments were assessed by orthogonal polynomial contrast to determine the effect of supplementation levels of bacteriophage on all measurements. Statistically significant and trend between treatments were considered at $P<0.05$ and $P<0.10$, respectively.

RESULTS AND DISCUSSION

Growth Performance

During the whole growth trial, no enteric diseases were recorded, reflecting the minimal disease status of the experimental facility. Moreover, *Salmonella spp.* and *E. coli* were not observed before growth trial. Data for the growth performance were summarized in Table 2. The inclusion of bacteriophage did not affect the BW on days 14 and 35 of the experimental period. No significant differences and tendency were observed in the ADG, ADFI, and G:F ratio during the first 2 weeks, last 3 weeks and during the whole experimental period ($P>0.05$). In the present study, bacteriophage supplementation in weaning pigs was conducted without pathogenic bacteria challenge (under normal physiological conditions). Data from this experiment showed that the inclusion of bacteriophage resulted in no differences in pig growth, feed intake, and feed utilization during the whole

experimental period. These observations are in agreement with the results from Huff et al. (2002) and Kim et al. (2013) who observed that the growth rate of broilers was not affected by bacteriophage supplementation without pathogenic bacteria challenge. Because of the characteristic of bacteriophage attaching to a specific receptor of the bacteria, they do not attach and kill other pathogenic bacteria. Moreover, this experiment was investigated in a clean facility, which may lack the substrate for bacteriophage activity. These observations may suggest that it can work better at high levels of against harmful bacteria in the digestive system or at a facility. In another bacteriophage study, FCR was affected by bacteriophage supplementation during the starter phase in broilers (Wang et al., 2013). This result indicated that broilers were more susceptible to pathogenic bacteria and sensitive to feed additives, resulting in improved FCR. Consequently, data from the growth performance suggest that bacteriophage supplementation without pathogenic bacteria challenge showed no detrimental effects on their growth in weaning pigs.

Fecal Microbiology

Data for the fecal *Salmonella spp.*, *E. coli,* and *Lactobacilli* concentrations from weaning pigs are summarized in Table 3. In phase I, no significant difference was observed in the fecal *Salmonella spp.* concentration ($P>0.05$), however as the inclusion of bacteriophage increased, the concentration of fecal *E. coli* linearly decreased ($P=0.053$). In phase II, fecal *Salmonella spp*. was linearly decreased as the bacteriophage levels increased ($P=0.010$), but there was no significant difference on the fecal *E. coli* concentration. In contrast, the fecal *Lactobacilli* concentration incereased linearly as the bacteriophage levels increased in both phase I and II ($P=0.002$, $P=0.001$, respectively). The results of this study suggest that bacteriophage supplementation in weaning pigs diets decrease the fecal *Salmonella spp.* and *E. coli* concentrations. Wall et al. (2010) reported that treatment with an anti-*Salmonella* phage cocktail which specifically reacted to

Salmonella enteridis and *Salmonella typhimurium* significantly reduced the cecal *Salmonella* concentrations, while also numerically reducing the ileal *Salmonella* concentrations. It also agreed with the reports of Lee and Harris (2001) that weaning pigs infected with *Salmonella* followed by a *Salmonella*-specific lytic bacteriophage treatment reduced *Salmonella* colonization in the cecum and tonsils but not in the intestine. There were several reports on the effects of bacteriophage in broilers on the microbial population. Atterbury et al. (2007) demonstrated that the inclusion of bacteriophage could decrease cecal *Salmonella* colonization in broiler. In addition, Huff et al. (2004) suggested that bacteriophage supplementation in broiler diets could reduce *E. coli* colonization. In addition, Wang et al. (2013) concluded that bacteriophage supplementation reduce *E. coli* and *Salmonella* colonization of broilers presumably by improving the microbial ecosystem. These results support our current experiment that bacteriophage. Supplementation of bacteriophage in livestock has a positive effect because it provides a better microbial ecosystem in the GIT. In general, *Lactobacilli* is known as friendly bacteria that normally live in our digestive, urinary, and genital systems without causing GIT diseases, especially diarrhea. Greube et al. (2010) and Yan et al. (2012) observed that bacteriophage supplementation reduced pathogenic bacteria and promoted the proliferation of useful bacteria resulted in improved intestinal microflora. The results of the present study agreed with those of previous findings that the *Lactobacilli* concentration was increased and *E. coli* and *Salmonella spp.* concentrations decreased with bacteriophage supplementation. It seems that bacteriophage have a positive effect on the domination of *Lactobacilli* in the GIT by reducing the *E. coli* and *Salmonella spp.* concentrations.

Incidence of Diarrhea and Fecal Moisture Contents

Data for the incidence of diarrhea and fecal moisture contents in each treatment are shown in Table 4. In phase I, incidence of diarrhea score decreased

linearly as the bacteriophage inclusion levels increased (P=0.001), as well as linear (P=0.002) and quadratic (P=0.008) responses were observed in the fecal moisture contents. However, no difference was observed for the incidence of diarrhea and the fecal moisture contents in phase II (P>0.05). At weaning, the GIT changed over 1-2 weeks mainly because of challenges to the piglets diet and environment. The villus height decreased, and the crypt depth increased within 24 hours of weaning, and consequently, a reduction in the absorptive capacity occurred because of the low activity of the digestive enzymes. A lack of nutrient absorption in the small intestine is often associated with the proliferation of enterotoxigenic bacteria or with fermentation of less digestible nutrients in the large intestine (McCracken and Kelly, 1993). Either way, this could lead to diarrhea. Such diseases could be associated with the colonization and overgrowth of bacteria, viruses or intestinal parasites. Diarrhea occurs as a result of inflammation of the intestinal tract, as well as disorders of intestinal motility. Diarrhea manifests as an increase in the water content of the feces, and increased digesta passage rate. Diarrhea is associated with damage to epithelium and loss of its function and brush border tend to be acidic and bulky. In this study, it is found that bacteriophage supplementation significantly decreased the colonization of fecal *E. coli* in phase I, as well as reduced the concentration of fecal *Salmonella spp.* in phase II. Therefore, it could be hypothesized that the beneficial effect of bacteriophage supplementation is attributed to the reduced number of enteropathogenic *coliform bacillus*. Our results on the incidence of diarrhea and fecal moisture contents also support this hypothesis.

Immune Response

The serum immunoglobulin of the weaning pigs is presented in Table 5. During phase I, bacteriophage supplementation showed no difference in the IgA and IgG concentrations (P>0.05). However, during phase II, the IgA concentration was linearly increased by bacteriophage level increased (P<0.05). Immunoglobulin

G has the highest concentration in the blood and for this reason, plays a major role in antibody mediated defense mechanisms. Because it is the smallest of the immunoglobulin molecules, IgG can escape from the vessels easier than the other immunoglobulin molecules. This is especially important in inflamed tissues, where an increase in vascular permeability readily allows IgG to participate in the defense of tissues and body surface (Sasaki et al., 1987; Moon and Bunn, 1993; Demicheli and Jefferson, 1996). Passive transfer of immunity via the colostrum is important in piglets because the epitheliochorial nature of the placenta prevents the transfer of immunoglobulin across the placenta. New-born piglet are therefore reliant on IgG absorbed from the colostrum for humoral immune protection until its own immune system has sufficiently matured to produce antibodies against foreign antigens (Rooke and Bland, 2002). Therefore, the plasma IgG concentrations in piglets shortly after birth are positively correlated with survival (Klobasa et al., 1981; Drew and Owens, 1988). IgA, which is the second most abundant immune molecule in the colostrum, inhibits the mucosal colonization of microorganisms, neutralizes viruses and hampers the penetration of soluble antigens. Importantly, because of its stability, IgA can retain its antibody activity for remarkably prolonged periods in a hostile environment such as the gut lumen and the oral cavity (Haneberg, 1974). This immune exclusion function is most likely reinforced by the relatively high levels of cross-reacting IgA antibodies present in external secretions.

Kim (2011) reported that piglets administrated bacteriophage and challenged with enterotoxigenic *E. coli* K88 showed increased IgA concentrations. In agreement with this finding, we found that bacteriophage supplementation decreased the IgA concentration. Decreased IgA production could be due to the inhibition of enterotoxigenic *E. coli* K88 colonization (Bosi et al., 2004). On the other hand, there were no treatment effects on the IgG concentration. One possible reason is because of no oral challenge with enterotoxigenic *E. coli* K88, so that no effect from the bacteriophage was observed. Consequently, bacteriophage

supplementation showed the possibility of potentially improving the immune development of weaning pigs.

Blood profiles

Data for the GOT, GPT, total cholesterol, LDL, and HDL cholesterol of weaning pigs are summarized in Table 6. There were no treatment effects on the concentrations of GOT and GPT (P>0.05). However, total cholesterol (linear, P=0.001, quadratic, P=0.028, 2wk; linear, P=0.002, 5wk) and LDL cholesterol (linear, P=0.002, 2wk) linearly decreased as the inclusion levels of the bacteriophage increased, while no difference was observed on HDL cholesterol (P>0.05). The liver is largest metabolism and immune organ of the animals, it is affect the gastrointestinal system and its associated lymphoid function (Insoft et al., 1996). Therefore, parameters for the function of the liver are including GOT, GPT, and cholesterol (Jerry Kaneko et al., 2008; Velkova et al., 2014). Serum GOT is a kind of cytosolic enzyme, consequently it can represent the status of liver damage when a high level of GOT activity is observed in the blood. In the current study, although there were no significant differences in the serum GOT levels, a numerical decline was observed in bacteriophage supplementation at week 5. The GPT is normally present in liver and heart cells. It is released into blood when the liver or heart is damaged. The blood GPT levels are thus elevated with liver damage or with an insult to the heart. Considering the characteristics of bacteriophage which are prone to accumulate in various parts of the organs, it can be explained that the bacteriophage supplemented treatments reduced the serum GPT numerically in phase II. Consequently, bacteriophage supplementation did not show any detrimental effect on the serum GPT levels during the whole period of the experiments. Cholesterol is used by many organisms as a structural element in membranes and as the starting material for synthesizing bile salts and steroid hormones. Although most cells seem to synthesize most of the cholesterol that they

need, cells that make cholesterol-based hormones have an increased tendency to acquire cholesterol from the bloodstream. LDL-cholesterol, at a high plasma concentration, is known as a risk factor for coronary heart disease (Baker et al., 1984). Adversely, HDL-cholesterol is known as a preventive factor of coronary artery disease. In this process, apoprotein-A plays a key role in restraining the consumption of LDL-cholesterol (Leon et al., 1979; Kannel et al., 1979; Goldberg et al., 1985). In this study, the total and LDL cholesterol linearly decreased as the bacteriophage level increased. This can be explained that bacteriophage have a positive effect on the colonization and overgrowth of bacteria, viruses or intestinal parasites, and consequently, impact indirectly the cholesterol concentration. It has been found that adding bacteriophage suppresses pathogenic bacteria and increases the fostering of probiotics in the current study, resulting in liberate bile salt through the probiotics and increasing bile by de novo synthesis in the liver by reducing the recirculation of bile acids from the increased excretion. Consequently, it appears the induced effect of reducing cholesterol in the blood is according to the suppression of bile reabsorption (Gilliland et al., 1985; Klaver and van der Meer, 1993; Noh et al., 1997; Lin and Chen, 2000).

IMPLICATION

The results of the current study indicated that bacteriophage did not influence growth performance in weaning pigs. However, there were positive relation of population of *Lactobacilli* in GIT and a decrease of fecal moisture contents. In addition, 0.2% (2×10^9 pfu/g) bacteriophage supplementation can be beneficial to immune response without any detrimental effects on immune response. Therefore, the bacteriophage might be beneficial to weaning pigs via non-nutritional effects.

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Table 1. Composition of experimental basal diet (as-fed basis)

Ingredients[1,2]	Phase Ⅰ (d 0 to 14)	Phase Ⅱ (d 14 to 35)
Ground corn	32.68	46.14
Soybean meal, 44% CP	23.58	22.09
Whey powder	3.00	0.00
Lactose	8.00	4.00
Barley	19.01	19.92
Soytide	8.00	2.00
Wheat bran	1.00	1.00
Soy oil	1.50	1.00
Dicalcium phosphate	1.43	1.10
Limestone	0.77	0.93
L-lysine·HCl	0.38	0.39
DL-methionine	0.05	0.03
Vitamin mix[3]	0.10	0.10
Mineral mix[4]	0.10	0.10
Salt	0.20	0.20
Choline chloride (50%)	0.10	0.10
Zinc oxide	0.10	0.10
Sum	100.00	100.00
Chemical composition[5] (%)		
Metabolizable energy (kcal/kg)	3,265.00	3,265.00
Crude protein (%)	21.00	18.00
Lysine (%)	1.35	1.15
Methionine (%)	0.35	0.30
Calcium (%)	0.80	0.70
Phosphorus (%)	0.65	0.60

[1] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g and, 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented as fed basis.

[2] Bacteriophage against *Salmonella thyphimurium, Salmonella enteritidis, Salmonella derby, Salmonella cholerasuis, Salmonella galinarum, Staphylococcus aureus, E. coli* K88, K99, F41, F18, 987P*, Clostridium perfringens* type A, B, C, D and E. Bacteriophage provided by a local company (CTCbio Co., Ltd., Seoul, South Korea).

[3] Provided the following quantities of vitamins per kg of complete diet: vitamin A, 8,000 IU; vitamin D_3, 1,600 IU; vitamin E, 32 IU; D-biotin, 64g; riboflavin, 3.2 mg; calcium pantothenic acid, 8 mg; niacin, 16 mg; vitamin B_{12}, 12g; vitamin K, 2.4 mg.

[4] Provided the following quantities of minerals per kg of complete diet: Se, 0.1 mg; I, 0.3 mg; Mn, 24.8 mg; Cu·SO_4, 54.1mg; Fe, 127.3 mg; Zn, 84.7 mg; Co, 0.3 mg.

[5] Calculated value.

Table 2. Effect of bacteriophage supplementation on growth performance in weaning pigs[1,2]

Criteria	Bacteriophage[3], %				SEM[4]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
Body weight, kg							
Initial	6.76	6.81	6.77	6.78	-	-	-
2 week	9.21	9.32	9.36	9.46	0.286	0.294	0.828
5 week	17.72	17.91	17.84	18.02	0.206	0.475	0.950
Average daily gain, g							
0-2 week	175	179	185	191	6.969	0.322	0.897
2-5 week	406	409	404	408	8.385	0.960	0.951
0-5 week	313	317	316	321	5.307	0.497	0.985
Average daily feed intake, g							
0-2 week	319	311	318	316	7.812	0.992	0.724
2-5 week	729	715	720	714	3.513	0.302	0.553
0-5 week	565	553	559	555	4.632	0.662	0.754
Gain:feed ratio							
0-2 week	0.550	0.581	0.592	0.609	0.027	0.406	0.780
2-5 week	0.556	0.572	0.561	0.571	0.121	0.616	0.879
0-5 week	0.554	0.573	0.567	0.579	0.010	0.239	0.741

[1] A total of 160 crossbred pigs was fed from average initial body weight 6.76 ± 1.72 g and the average of final weight was 17.87 ± 1.93 kg.
[2] Least squares means for five pens/treatment with eight pigs/pen.
[3] Four different levels (0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, and 0.2%; 2×10^9 pfu/g, respectively) of bacteriophage were supplemented.
[3] Standard error of means.

Table 3. Effect of bacteriophage supplementation on fecal microflora in weaning pigs[1]

Criteria	Bacteriophage[2], %				SEM[3]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
***Salmonella spp.*, Log10cfu/g**							
Initial	3.81	3.81	3.81	3.81	-	-	-
2 week	3.92	3.63	3.61	3.68	0.104	0.435	0.277
5 week	4.67^{a}	4.06^{ab}	3.17^{b}	3.29^{b}	0.200	0.010	0.085
***E. coli*, Log10cfu/g**							
Initial	4.64	4.64	4.64	4.64	-	-	-
2 week	4.77	4.57	4.44	4.36	0.069	0.053	0.421
5 week	4.76	4.50	4.45	4.32	0.139	0.382	0.746
***Lactobacilli*, Log10cfu/g**							
Initial	5.76	5.76	5.76	5.76	-	-	-
2 week	5.29^{b}	5.35^{a}	5.36^{a}	5.38^{a}	0.016	0.019	1.157
5 week	5.29^{B}	5.32^{B}	5.34^{B}	5.50^{A}	0.025	0.001	0.144

[1] Least squares means for five pigs per treatment.
[2] Four different levels (0%, 0.05%; 5×10^{8} pfu/g, 0.1%; 1×10^{9} pfu/g, and 0.2%; 2×10^{9} pfu/g, respectively) of bacteriophage were supplemented.
[3] Standard error of means.
[ab] Means with different superscripts in the same row significantly differ ($P<0.05$).
[AB] Means with different superscripts in the same row significantly differ ($P<0.01$).

Table 4. Effect of bacteriophage supplementation on incidence of diarrhea and fecal moisture contents in weaning pigs

Criteria	Bacteriophage[1], %				SEM[2]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
Incidence of diarrhea[3,4]							
2 week	2.82^{A}	2.73^{B}	2.66^{B}	2.57^{C}	0.054	0.001	0.323
5 week	2.30	2.26	2.29	2.24	0.022	0.439	0.974
Fecal moisture contents[5], %							
2 week	75.67^{A}	75.03^{A}	73.12^{B}	73.87^{B}	0.303	0.002	0.008
5 week	75.61	74.82	75.61	75.97	0.233	0.346	0.421

[1] Four different levels (0%, 0.05%; 5×10^{8} pfu/g, 0.1%; 1×10^{9} pfu/g, and 0.2%; 2×10^{9} pfu/g, respectively) of bacteriophage were supplemented.
[2] Standard error of means.
[3] Incidence of diarrhea: 1=hard feces; 2=slightly soft feces; 3=soft, partially formed feces; 4=loose, semiliquid feces and 5=watery, mucois-like feces.
[4] Least squares means for five pens/treatment with eight pigs/pen.
[5] Least squares means for eight pigs per treatment.
[AB] Means with different superscripts in the same row significantly differ ($P<0.01$).
[ABC] Means with different superscripts in the same row significantly differ ($P<0.01$).

Table 5. Effect of bacteriophage supplementation on immune response in weaning pigs[1]

Criteria	Bacteriophage[2], %				SEM[3]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
Serum IgG, mg/ml							
Initial	0.52	0.52	0.52	0.52	-	-	-
2 week	0.68	1.09	0.97	0.70	0.082	0.721	0.105
5 week	1.93	2.50	2.90	2.82	0.208	0.173	0.849
Serum IgA, mg/ml							
Initial	0.70	0.70	0.70	0.70	-	-	-
2 week	0.69	0.58	0.71	0.70	0.029	0.542	0.683
5 week	0.64^{a}	0.53^{ab}	0.42^{ab}	0.39^{b}	0.040	0.043	0.805

[1] Least squares means for six pigs per treatment.
[2] Four different levels (0%, 0.05%; 5×10^{8}pfu/g, 0.1%; 1×10^{9}pfu/g, and 0.2%; 2×10^{9} pfu/g, respectively) of bacteriophage were supplemented.
[3] Standard error of means.
[ab] Means with different superscripts in the same row significantly differ ($P<0.05$).

Table 6. Effect of bacteriophage supplementation on blood profiles in weaning pigs[1]

Criteria	Bacteriophage, %				SEM[2]	*P-value*	
	0	0.05	0.1	0.2		Lin-	Quad-
GOT, U/L							
Initial	109.1	109.1	109.1	109.1	-	-	-
2 week	79.3	79.7	81.5	81.3	3.32	0.834	0.928
5 week	230.7	222.8	208.3	200.5	8.35	0.192	0.780
GPT, U/L							
Initial	32.9	32.9	32.9	32.9	-	-	-
2 week	41.5	37.0	36.0	39.8	1.68	0.858	0.202
5 week	44.5	41.7	42.0	42.3	1.13	0.650	0.513
Total cholesterol, mg/dL							
Initial	132.5	132.5	132.5	132.5	-	-	-
2 week	82.0^{A}	83.2^{A}	65.5^{B}	67.7^{B}	2.01	0.001	0.028
5 week	116.8^{A}	119.3^{A}	86.7^{B}	84.2^{B}	4.72	0.002	0.478
LDL cholesterol, mg/dL							
Initial	91.0	91.0	91.0	91.0	-	-	-
2 week	51.3^{A}	50.7^{A}	41.0^{B}	38.7^{B}	4.32	0.002	0.312
5 week	52.0	43.7	49.0	46.0	3.25	0.694	0.749
HDL cholesterol, mg/dL							
Initial	46.1	46.1	46.1	46.1	-	-	-
2 week	24.0	28.0	22.5	23.2	0.65	0.110	0.539
5 week	25.2	23.3	22.3	24.2	0.74	0.739	0.221

[1] Least squares means for six pigs per treatment.
[2] Four different levels (0%, 0.05%; 5×10^{8} pfu/g, 0.1%; 1×10^{9} pfu/g, and 0.2%; 2×10^{9} pfu/g, respectively) of bacteriophage were supplemented.
[2] Standard error of means.
[AB] Means with different superscripts in the same row significantly differ ($P<0.01$).

Chapter V: Evaluation of Bacteriophage and Choline Supplementation on Physiological Responses, Growth Performance, Microbial Population and Blood profiles of Lactating Sows and Piglets

ABSTRACT: This study was conducted to investigate the effects of bacteriophage and choline supplementation on physiological responses, growth performance, microbial population, and blood profiles of lactating sows and piglets. A total of 50 mixed-parity (average= 4.64) crossbred sows (F1, Yorkshire × Landrace; Darby, Korea) with an initial BW of 228.71 ± 15.81 kg were used in a 3 week lactation period and sows were allotted to one of five treatments based on BW and backfat thickness with 10 replicates by 1+2×2 factorial arrangement. The experimental treatments were divided by two levels of bacteriophage (0.05%; 0.5×10^{8} pfu/g, or 0.1%; 1×10^{9} pfu/g) and choline chloride (0.05%; 250ppm or 0.1%; 500ppm) and NRC (1998) requirement is regarded as control treatment. The experimental diets were formulated based on corn-soybean meal diets, which contained 3,265 kcal of ME/kg, 16.8% crude protein, 1.08% lysine, respectively. There were no significant differences in BW, backfat thickness and feed intake of lactating sows by bacteriophage and choline supplementation. The BW changes were quadratically decreased in lactation (day 0 to 21) as dietary choline increased (P<0.05). Supplementation of bacteriophage and choline to lactating diets did not influence on mortality, litter weight and piglet weight. However, numerically higher litter weight and piglets weight gain were observed in bacteriophage and choline treatment groups compared to control. No differences were found in estimation of milk production, dry matter, and energy content of milk in lactating sows during the whole lactational period. Bacteriophage and choline supplementation in diets did not alter the population of *Escherichia coli* and *Salmonella spp.* in feces of sows as well as piglets. However, the use of bacteriophage to lactation diets altered the

concentrations of fecal *Lactobacilli* ($P<0.001$). In blood profiles, GOT, GPT, and non-esterified fatty acid (NEFA) levels of lactating sows and piglets were not affected by dietary treatment, while increasing bacteriophage levels tended to decrease GOT levels of lactating sows (linear, $P=0.074$). Inclusion of bacteriophage and choline did not influence on immunoglobulin concentration of sows at day 21 postpartum. This experiment suggested that choline supplementation in lactating diet showed an improvement of body reserves of lactating sows and increasing of fat contents in sow milks during lactation. But, bacteriophage had no effects on reproductive performance and physiological responses except of sow's fecal *Lactobacilli* population.

Key words: Bacteriophage, Choline, Reproductive performance, Piglets growth, Microbial population

INTRODUCTION

The aim of swine farms is to increase litter sizes with healthier and heavier piglets in sow productivity (Beaulieu et al., 2010). However, according to the annual Korean swine farm report (2012, Korean Pork Producers Association), the piglet mortality rate during suckling was 9.5%, which was as high as 31.9% before growing period. Therefore, increasing sow milk and reducing piglet mortality are important parts for sow productivity. Piglets diarrhea is one of the major problems during the neonatal and weaning periods. It is usually due to contact with sow feces and poor quality of sow milk leading to viral and bacterial diarrhea.

Bacteriophage is known as obligate intracellular parasites and viruses that have a specific host cell for their replication (Carlton, 1999; Mayer, 2005). Bacteriophage attaches to the surface of host cells with their tail fiber and subsequently injects nucleic acid into the bacteria. After replication, bacteriophage cling to the cellular walls, destroys them and finally spreads to other bacteria. Several studies indicated that by using the bactericidal function of bacteriophage, it can reduce the propagation of bacteria such as *E. coli* (Huff at al., 2005; Borie et al., 2008; Kim et al., 2011) and *Salmonella* (Barrow et al., 1987; Berchieri et al., 1991; Sklar and Joerger, 2001). Moreover, bacteriophage supplementation had no deleterious effects on the liver and kidneys in a normal feeding trial (Lee et al., 2010; Kim et al., 2013).

Choline can be supplied by dietary supplementation and synthesized from methionine in the pig's body (Greenberg, 1963). Methionine is a component of the cell membrane and a methyl donor for various physiological effects. Donkin (2011) reported that choline supplementation improved milk production in daily cows. Liver triglycerides can be packaged into very low density lipoproteins (VLDL) and exported into blood. Choline may affect the synthesis of the apolipoprotein components of VLDL to increase the export of triglycerides from the liver. Because of fat metabolism of transport, mobilized fatty acids from adipose tissue to the

mammary gland, choline can affect milk production. Supplementation with choline in a gestation diet can improve the conception rate and the number of piglets born alive (Kornegay and Meacham, 1973; NRC, 1976; Grandhi and Strain, 1981). However, there is little data on milk production and reproductive performance with choline supplementation in lactating sows. Previous studies have shown that piglets eating a sow diet develop gastrointestinal digestive organs (Owsley et al., 1986; Wallenbeck et al., 2005; Heo, 2013). Supplementation with bacteriophage and choline could affect sow productivity including milk and healthy piglet production with the reduction in pathogenic bacteria and with improving in immune function. Therefore, this study was conducted to investigate the effects of bacteriophage and choline supplementation on physiological responses, growth performance, microbial population and blood profiles of lactating sows and piglets.

MATERIALS AND METHODS

Bacteriophage and Choline Preparation

Bacteriophage had been used against diarrheal disease caused by *Salmonella thyphimurium*, *Salmonella enteritidis*, *Salmonella derby*, *Salmonella cholerasuis*, *Salmonella galinarum*, *Staphylococcus aureus*, *E. coli* K88, *E. coli* K99, *E. coli* F41, *E. coli* F18, *E. coli* 987P, *Clostridium perfringens* type A, B, C, D and E. Bacteriophage provided by a local company (CTCbio Co., Ltd., Seoul, South Korea) was type of powder. The plaque forming unit of 10^9 pfu/g was used as feed additives in the dietary treatments. Choline provided by a local company (KOFAVET special, Inc., Seoul, South Korea) was type choline chloride 50% powder (500 ppm).

Experimental Animals, Design and Diets

A total of 50 crossbred sows (Yorkshire × Landrace; Darby Genetics Inc.,

Anseong, South Korea) with an average body weight 228.71 ± 15.81 kg and a parity of 4.64 ± 1.14 were used in a 3 wk trial at research farm located in Eum-seong, South Korea. Sows were allotted to one of five treatments considering their body weight and backfat thickness in completely randomized design (CRD). The five diets were based on corn-soy bean meal (SBM) in a 1+2×2 factorial arrangement, with 2 levels of bacteriophage (0.05%; 0.5× 10^8 pfu/g, or 0.1%; 1× 10^9 pfu/g), 2 levels of choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) supplemented as feed additive was included at the expense of corn to the basal diet. For the lactation period, experimental diets composition was presented in Table 1. A lactation diets based on corn-SBM was formulated to contain 3,265 kcal of ME/kg, 16.80 % crude protein and 1.09 % total lysine, respectively. All other nutrients were met or exceeded requirements of NRC (1998). Experimental diets were provided by a mash type of feed and nutrients contents in the diets were analyzed via the method of the AOAC (1995). All lactation sows were fed the same diets and water was provided *ad libitum* during 3 wk lactation period.

Animal Managements

Lactating sows were moved into environmentally controlled farrowing house after washing with an antiseptic solution and allotted to farrowing crate (2.5 × 1.8 m) at 110 d of gestation. They were provided a feeder and nipple waterer supplier for each sow and a heat lamp for nursery piglets. Body weight of individual piglets as well as BW and backfat thickness of sow were recorded within 12 h after farrowing and iron injection (Fe-dextran, 150ppm), clipping needle teeth, tail docking, ear notching were done to nursery piglets. Male piglets were castrated at 3 d of age. Cross-fostering was performed based on average number of piglets in litter, BW and gender ratio. Piglets had no access to creep feed during suckling. Lactating sows were weighed at farrowing and d 21 postpartum. Simultaneously, backfat thickness of lactating sows also measured at each side of P_2 position using a Lean-

Meater (Renco Crop., Minneapolis, MN). Feed intake of lactating sows was recorded when lactating sows were weighted. Energy and fat mass of primiparous sows were calculated using the equations of Dourmad et al. (1996):

Energy (kg) = 257 + 3.267 × (live weight, kg) + 10.99 × (backfat, mm)

Fat (kg) = -26.40 + 0.221 × (live weight, kg) + 1.333 × (backfat, mm)

Milk Composition and Estimation of Sow Milk Production

Colostrum and milk were collected after 5 IU of the oxytocin (Komi oxytocin inj. Komipharm international Co., Ltd., Korea) injection at within 12 h after farrowing and d 21 of lactation period. After collection, the frozen milk samples were stored in a freezer (-20 °C) until subsequent analyzed for fat, CP, lactose, total solid-not-fat contents by using a milk composition autoanalyzer (Milkoscan FT20, Foss Electronic Co., Denmark). Estimation of sow milk production was calculated using the equations of Noblet and Etienne (1989)

Milk production (g) = 2.50 × piglets ADG (g) + 80.2 × initial piglets BW (kg) + 7

Milk DM (g) = 0.401 × piglets ADG (g) + 12.9 × initial piglets BW (kg) + 19

Milk energy (Kcal) = 2.54 × piglets ADG (g) + 78.7 × initial piglets BW (kg) + 153

Blood profiles and Immunoglobulins

Blood was collected in EDTA tube from 6 sows and piglets per treatment through jugular vein within 12 h after farrowing and d 21 of lactation period. After blood sample was collected into vacuum tube, samples were centrifuged for 5 min at 3,000 rpm and 18 °C after clotting during 30 minute at room temperature. The serum was carefully removed to specific vials and stored at 4 °C until blood profiles analysis and was kept at -20 °C for blood profiles and immunoglobulins analysis. The GOT, GPT, non-esterified fatty acid (NEFA) in blood were analyzed according

to AOAC (1990). The ELISA was used to quantitatively measure levels of IgG and IgA in serum. The ELISA accrssory starter kit (E101; Bethyl Laboratories Inc., USA) and quantitation kit (E100; BETHYL) were used for this analysis.

Fecal Microbiology

Fecal *E. coli, Salmonella spp.*, and *Lactobacilli* count were measured at the end of lactation period. Fecal samples were collected from 6 sows and piglets per treatment. For enumeration of bacterial populations, 1 g of fecal content was diluted in saline solution and re-suspended by vortex mixing, then serially diluted in saline solution. A 100 mL volumes of each dilution was spread overlaid on the surface of an agar plate. This procedure was performed in duplicate over MacConkey agar, *Salmonella shigella* agar and *Lactobacilli* agar (Difco, BD science, USA). Plates were incubated at 37 °C. After 24 h of incubation, each plate was spotted for fecal microflora count and examined for the presence of each bacterial growth.

Statistical Analysis

Considering sources and levels of bacteriophage and choline as factor, the data were analyzed by factorial arrangement. All of collected data were carried out by least square mean and were evaluated using PDIFF option in the General Linear Model (GLM) procedure of SAS (SAS Institute, 2004). Each individual sows and their litters were used as an experimental unit. Differences were considered at $P<0.05$ and highly significant at $P<0.01$. Orthogonal polynomial contrasts were also used to determine linear and quadratic effects of increasing levels of bacteriophage and choline.

RESULTS AND DISCUSSION

Performance of Lactating Sows

The effect of bacteriophage and choline supplementation on body weight,

backfat thickness gain, feed intake, and estimated energy and protein accumulation was presented in Tables 2 and 3. There were no effects of bacteriophage and choline levels on BW, backfat thickness, and feed intake except for d of 21 lactation of BW changes, resulting in a quadratic decrease as the dietary choline increased (P<0.05). In addition, estimated energy and fat accumulation of lactating sows was not significant different. But, numerically increased estimation of energy and fat accumulation by choline supplementation. Results from the present study showed that changes in BW and backfat thickness were not affected in lactating sows (0-21 d). Although the performance of lactating sows did not differ, BW change on d 21 of lactation was affected by the increased choline levels. It is well known that a sow's BW at weaning is lower than that of farrowing, for which the dietary nutrient intake is not sufficient for milk production regardless of the amount of voluntary feed intake during lactation (Mullan and Williams, 1984; Yang et al., 1989). However, these BW changes did not agree with the results of the current study. One possible reason for the conflicting observations could be explained by choline effect with lower BW changes in lactating sow. Donkin (2011) reported that choline supplementation improved the milk production on daily cow, which is related to fat metabolism. Moreover, dietary methionine and choline functioning as methyl donors can affect each other and their metabolic pathways (Russett et al., 1979). The current experimental basal diets contained enough methionine to meet its requirement. Therefore, dietary choline treatments were affected sow body condition and more concentrated on fat metabolism and milk production than that of basal diet treatment. Generally, after farrowing, lactating sows are both in an anabolic and a catabolic status because they mobilize body reserve to produce milk when the lack of nutrient intake during lactation (Mullan and Williams, 1990). Moreover, depletion of maternal reserves may finally compromise the current lactation and subsequent reproduction in lactating sows (Clowes et al., 1998; 203). In this experiment, there were significant differences in estimated energy and fat

gain at lactation through the calculation Dourmad et al. (1997). However, choline supplementation showed increased positive body weight gain during lactation and numerically increased estimation of energy and fat accumulation by choline supplementation. It meant that choline supplementation in lactation diet increased the fat metabolism, resulted in a sufficient body reserves of lactation sows during lactation. Very little information is available for the effects of dietary choline and bacteriophage on feed intake in lactating sows. Boyd et al. (1982) reported that 220 or 770 mg/kg dietary choline did not affect feed intake in lactating sows. Similarly, Stockland (1974) indicated that the feed intake of lactating sow was not influenced by the presence of dietary choline (1,500, 2,250 or 3,000 mg/day), and these changes were agreed with the results of this study. In contrast to choline, there is no information on the effects of bacteriophage in the diets for of sows. Data from this experiment showed that the bacteriophage in the lactation diet did not alter the BW changes, back-fat thickness, feed intake, and estimated energy and fat accumulation revealing that bacteriophage might not affect the physiological responses of lactating sows.

Piglet Growth

The effect of bacteriophage and choline supplementation on piglet growth was shown in Table 4. Bacteriophage and choline supplementation during lactation did not affect the mortality of piglets, litter weight and piglet weight. However, numerically higher litter weight and piglet weight gain were observed in bacteriophage and choline supplementation groups compared to the control treatment. Data from this experiment suggested that piglet growth was not significantly influenced by the bacteriophage and choline supplementation. These observations were in agreement with previous studies on choline supplementation in lactation diets (Stockland and Blayock, 1974; Seerley et al., 1981). Those previous studies showed that there were no beneficial effects on piglet survival and growth performance with choline supplementation in the lactation diets of sows. In addition,

Kornegay and Meacham (1973) concluded that the number and weight of pigs weaned per litter had no significant differences with choline supplementation. The reason for this observation can be explained by the choline contents of corn-SBM basal diet was adequate for their litter growth, which does not affect piglet growth. The current data suggested that bacteriophage levels up to 0.1% during lactation did not show adverse or beneficial effects on litter weight and piglet growth. In this study, bacteriophage were supplemented in the sow diets and creep feed was not provided to the piglets. Therefore, the piglets had restricted access to bacteriophage, resulting in no bacteriophage effect on piglet performance was observed in this experiment. Moreover, the experimental facility maintained a clean status during lactation, indicating that bacteriophage activity and its effect were lower than low hygiene status of swine farm.

Milk Characteristics and Estimation of Sow Milk Production

Data for the composition and estimation of sow milk production from sows were summarized in Tables 5 and 6. Milk fat contents for d of 21 lactation of BW changes, resulting in a quadratic increase as the dietary choline increased ($P<0.05$). However, there were no significant differences in the composition of protein, solid-not-fat, Total solid, and lactose (Table 5). Moreover, estimation of sow milk production, dry matter and energy were not influenced by the treatment (Table 6). During the lactating period, choline supplementation was related to milk production (Janovick Guertzky et al., 2006). A negative energy balance with inadequate feed intake results in stored triglycerides mobilizing to the NEFA in blood. The NEFA is used by the mammary gland for milk synthesis and esterified to triglyceride (TG) in the liver (Drackly, 1999). Esterified TG can be exported as VLDL (Grummer, 1993), which can affect phosphatidylcholine production. Therefore, the lack of a choline supply, results in an increased requirement for methionine in milk synthesis. Moreover, choline deficiency leads to an increased rate of TG export from the liver

and decreased milk production. Therefore, increased fat composition of milk contents of sows fed diet containing choline in this study might be associated with fat metabolism with choline. However, in the present study, choline supplementation did not affect the milk composition and production except of milk fat contents. One possible reason could be that dietary methionine and choline directly affect each other and their metabolic pathway. They function as methyl donor in animal metabolism. It meant that choline requirement is affected by methionine content in the diets. The current experimental diets contained adequate amount of methionine to meet the dietary methionine requirement, resulting in no effect on the estimated sow milk production.

Fecal Microflora and Moisture Contents

The effect of bacteriophage and choline supplementation on fecal microflora and moisture contents was presented in Table 7 and Figure 1. In the fecal microflora of sows, the use of the bacteriophage supplement in the sow diet did alter the population of *Lactobacilli* ($P<0.001$), showing a linear response to the bacteriophage levels ($P<0.05$). However, no effect from the dietary choline supplement in sow diets was observed on the populations of *E. coli*, *Salmonella spp.,* and *Lactobacilli*. In the fecal microflora of piglets, no significant differences and no tendencies were noted the treatments with respect to the *E. coli*, *Salmonella spp.,* and *Lactobacilli* contents during lactation. The fecal moisture content of the piglet feces decreased by supplementation of bacteriophage and linear response were observed (bacteriophage effect, $P<0.05$; linear response, $P<0.05$; Figure 1). This result showed that there were no significant differences and trends among the treatments except for the sow fecal *Lactobacilli* population. Moreover, the piglet fecal microbial population was not affected by the bacteriophage and choline levels. In this study, bacteriophage against 17 types of pathogenic bacteria supplementation resulted in no detectable differences of fecal microbial population. One possible

reason for these unexpected observations could be due to the clean environment and optimum temperature and humidity of the location where the experiment were carried out, which can hinder bacteriophage activity. Interestingly, bacteriophage attaches to specific receptors on their hosts so they can cause harm to the normal intestinal microflora (Oliveira et al., 2009). Therefore, the inclusion of bacteriophage numerically reduced the *E. coli* and *Salmonella spp.* concentration, resulting in a positive relationship with a higher concentration of *Lactobacilli* in the GIT. It has been suggested that the GIT microflora played important roles in animal growth (Huang et al., 2004), showing that the sow GIT status could affect the intestinal environment of piglet by preventing the growth of harmful bacteria and subsequently improving their health status. Although the piglets fecal microflora population was not affected by the bacteriophage and choline levels, the fecal moisture contents suggested that bacteriophage could enhance the development of intestinal function reducing diarrhea and mortality in piglets. In contrast to bacteriophage, choline supplementation did not affect the fecal microbial population and moisture contents in the feces of sows and piglets.

Blood profiles

The effect of bacteriophage and choline supplementation on blood profiles was presented in Table 8. No significant differences were observed in the GOT, GPT and NEFA levels of sows and piglets. However, increasing bacteriophage and choline levels showed the trend for decreased (linear, P=0.074) the GOT levels in sows. Generally, serum GOT and GPT are cytosolic enzymes specially in liver, consequently they can represent liver damage when high levels of GOT and GPT activity are observed in the blood (Lumeiji, 1997). Hung et al. (2011) reported that bacteriophage supplementation did not elevate GOT and GPT levels in mice with intragastric inoculation of 2×10^8 *K. pneumonia*. In addition, Lee et al. (2010) observed that there were no significant differences between blood profiles and

bacteriophage in broilers. This study observed no changes in GOT and GPT levels by any treatment. These results indicated that bacteriophage supplementation in sow diets was not harmful to liver. Generally, adipose tissue stores TG, resulting in the release of NEFA with VLDL into blood. Choline could also affect the synthesis of the apolipoprotein components of VLDL to increase TG export from liver. Therefore, the NEFA concentration is an indicator of fat metabolism and milk production. However, in this study, the NEFA concentration in blood was not affected by the choline and bacteriophage levels. These results suggested that dietary choline supplementation affects milk production, and it is possible that sufficient choline contents are present in the lactating diets.

Immune Response

The effect of bacteriophage and choline supplementation on immunoglobulin was presented in Table 9. Serum immunoglobulin was not affected by bacteriophage and choline supplementation, and no detectable differences were observed at day 21 postpartum. The results of this study showed that bacteriophage and choline supplementation did not improve the immune response. The reason for this observation is not clear, but it may be associated with clean status of the experimental facility and the reproductive performance of the sows. Moreover, because of the lactating sows' sufficient ability to nursing, increased piglet weight gain and a high number of weaning pigs were observed in all treatments. Wang et al. (2013) demonstrated that bacteriophage supplementation did not affect the percentage of red blood cells, white blood cells and lymphocytes under normal physiological conditions. Recently published research showed that bacteriophages adhered to the mucus providing a non-host derived immunity (Barr et al., 2013). Mucosal surfaces are important entry sites for pathogens and the principal sites of defense against infection. Both bacteria and bacteriophage are associated with this mucus. In the current study, the mode of action for bacteriophage supplementation

might be located on the mucosal surfaces of the GIT, which affected pathogenic bacteria, resulting in the modulation of the immune status in lactating sows and piglets.

IMPLICATION

The results of the current study indicated that the use of bacteriophage up to 0.1 % (1×10^9 pfu/g) in lactating diets showed no influence of reproductive performance. However, bacteriophage influenced population of non-pathogenic bacteria in feces of lactating sows. Therefore, bacteriophage might be beneficial to lactating sows via non-nutritional effects. In contrast to the results of bacteriophage, body condition of sows and milk fat contents were improved by choline supplementation.

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Table 1. Composition of experimental basal diet (as-fed basis)[1,2]

Ingredients	Lactation
Ground corn	67.47
Soybean meal 45%	25.51
Sugar molasse	1.02
Wheat bran	0.12
Soy-oil	1.29
L-lysine·HCl	0.60
Dicalcium phosphate	2.30
Limestone	0.85
Vitamin mix[2]	0.20
Mineral mix[3]	0.10
Salt	0.42
SUM	100.00
Chemical composition	
Metabolizable energy, Kcal/Kg	3,265.00
Crude protein, %	16.80
Lysine, %	1.09
Methionine, %	0.25
Calcium, %	0.20
Sodium, %	0.90
Total phosphorus, %	0.70

[1] Sows had free access to lactation diet throughout 3 weeks of lactation period.

[2] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[3] Provided per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 1,500IU; vitamin E, 35IU; vitamin K, 3mg; vitamin B_2, 4mg; vitamin B_6, 3mg; vitamin B_{12}, 15μg; pantothenic acid, 10 mg; biotin, 50 μg; niacin, 20 mg; folic acid 500 μg.

[4] Provided per kg of diet: Fe, 75 mg; Mn, 20 mg; Zn, 30 mg; Cu, 55 mg; Se, 100 μg; Co, 250 mg; I, 250 mg.

Table 2. Effects of bacteriophage and choline supplementation on body weight, backfat thickness and feed intake in lactating sows[1]

Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
No. of Sows	10	10	10	10	10				
Body weight, kg									
24 hrs postpartum	238.6	233.5	226.9	231.7	232.9	5.99	0.280	0.881	0.408
d 21 of lactation	238.6	236.7	230.2	239.9	236.9	5.56	0.541	0.083	0.328
Changes (d 0-21)*	0.0	3.2	3.4	8.2	4.0	3.52	0.701	0.962	0.534
Backfat thickness, mm									
24 hrs postpartum	19.0	18.6	19.3	19.6	19.1	0.44	0.701	0.962	0.534
d 21 of lactation	18.4	19.2	19.1	19.7	18.3	0.46	0.915	0.539	0.567
Changes (d 0-21)	-0.6	0.6	-0.2	0.1	-0.8	0.12	0.441	0.259	0.971
Feed intake, kg	6.46	6.80	6.40	6.58	6.57	0.091	0.679	0.679	0.220

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2]Standard error of means.

[3]Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

* Quadric response by the supplementation of choline (P<0.05).

Table 3. Effects of bacteriophage and choline supplementation on estimated energy and fat accumulation

Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
Estimated energy mass, Mcal[4]									
24hrs postpartum	961.9	940.9	926.8	946.5	878.5	13.93	0.461	0.162	0.353
D 21 of lactation	983.1	985.5	963.9	1,000.2	911.5	17.37	0.588	0.099	0.303
Changes (d 0-21)	21.2	44.7	37.1	55.5	33.0	11.74	0.837	0.365	0.652
Estimated fat mass, kg[5]									
24hrs postpartum	51.6	50.8	49.4	51.0	46.0	1.08	0.610	0.253	0.356
D 21 of lactation	50.8	51.4	49.9	52.8	45.9	1.14	0.639	0.130	0.318
Changes (d 0-21)	-0.9	1.4	0.5	1.9	-0.1	0.70	0.952	0.295	0.719

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2]Standard error of means.

[3]Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

[4]Prediction equation from Dourmad et al. (1997) : 257+3.267×(live weight, kg) +10.99×(backfat, mm).

[5]Prediction equation from Dourmad et al. (1997) : -26.40+0.221×(live weight, kg) +1.333×(backfat, mm).

Table 4. Effects of bacteriophage and choline supplementation on piglets performance

Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
No. of Sows	10	10	10	10	10				
After cross-fostering	11.10	11.90	11.70	11.40	11.20	0.119	0.710	0.459	1.000
Death	0.50	0.20	0.56	0.30	0.30	0.102	0.795	0.437	0.437
Weaning pigs	10.60	11.70	11.20	11.10	10.90	0.155	0.145	0.253	0.621
Litter weight, kg									
After cross-fostering	16.04	16.12	16.15	15.74	15.85	0.440	0.719	0.941	0.968
d 21	63.72	67.69	68.96	68.34	66.98	1.554	0.592	0.762	0.827
Litter weight gain	47.67	51.57	52.82	52.60	51.13	1.409	0.663	0.726	0.803
Piglets weight, kg									
After cross-fostering	1.44	1.36	1.38	1.38	1.42	0.037	0.659	0.631	0.900
d 21	5.97	5.78	6.18	6.15	6.14	0.106	0.574	0.498	0.469
Litter weight gain	4.52	4.42	4.79	4.77	4.72	0.099	0.706	0.644	0.459

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2]Standard error of means.

[3]Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

Table 5. Effects of bacteriophage and choline supplementation on milk composition

Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
Fat, %									
Colostrum	7.18	7.18	7.18	7.18	7.18	-	-	-	-
d 21 of lactation*	5.59	6.50	5.88	6.77	6.83	0.242	0.296	0.624	0.551
Protein, %									
Colostrum	6.39	6.39	6.39	6.39	6.39	-	-	-	-
d 21 of lactation	4.62	4.51	4.63	4.56	4.68	0.076	0.761	0.498	0.995
Lactose, %									
Colostrum	4.39	4.39	4.39	4.39	4.39	-	-	-	-
d 21 of lactation	5.74	5.97	5.86	6.22	5.81	0.086	0.459	0.080	0.286
Solid-not-fat, %									
Colostrum	10.55	10.55	10.55	10.55	10.55	-	-	-	-
d 21 of lactation	10.84	10.86	10.72	10.59	10.67	0.075	0.293	0.843	0.460
Total solid, %									
Colostrum	20.09	20.09	20.09	20.09	20.09	-	-	-	-
d 21 of lactation	17.72	18.02	17.68	18.46	18.01	0.217	0.430	0.411	0.912

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2]Standard error of means.

[3]Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

* Quadric response by the supplementation of choline ($P<0.05$).

Table 6. Effects of bacteriophage and choline supplementation on estimation of milk production in lactation

Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
Milk production, g/piglets, day[4]									
	661	656	689	686	683	12.19	0.408	0.351	0.296
Milk dry matter, g/piglets, day[5]									
	124	123	128	128	128	1.97	0.420	0.323	0.299
Milk energy, Kcal/piglets, day[6]									
	814	809	842	839	836	12.37	0.411	0.355	0.296

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2]Standard error of means.

[3]Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

[4]Milk production (g/piglets, d) = 2.50 × piglets ADG (g) + 80.2 × initial piglets BW (kg) + 7

[5]Milk dry matter (g/piglets, d) = 0.401 × piglets ADG (g) + 12.9 × initial piglets BW (kg) + 19

[6]Milk energy (Kcal/piglets, dl) = 2.54 × piglets ADG (g) + 78.7 × initial piglets BW (kg) + 153

Table 7. Effects of bacteriophage and choline supplementation on fecal microflora of lactating sows and piglets

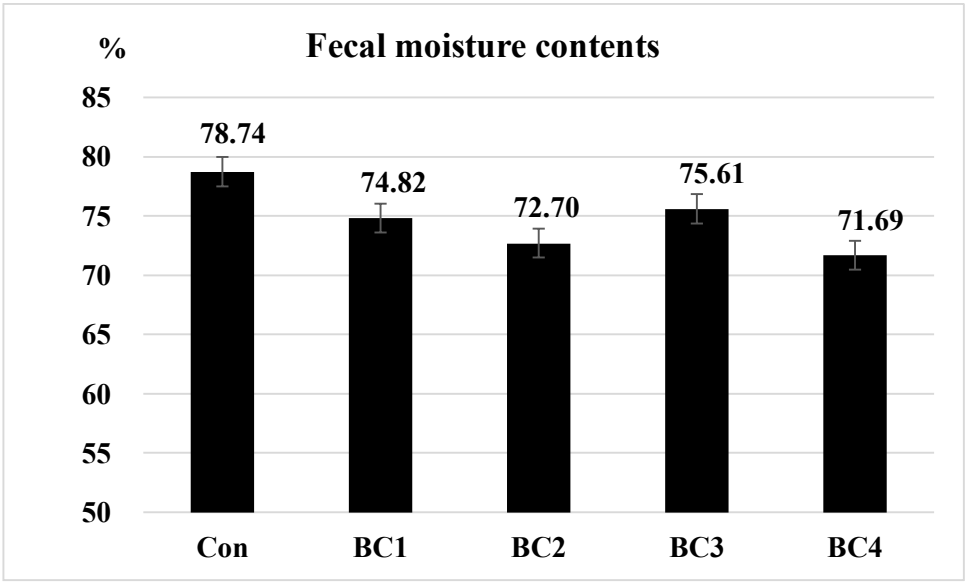
Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
Sows, d 21 of lactation (Log10cfu/g)									
E. coli									
initial	5.82	5.82	5.82	5.82	5.82	-	-	-	-
d 21 of lactation	5.66	5.31	5.43	5.33	5.24	0.064	0.603	0.907	0.506
Salmonella spp.									
initial	5.38	5.38	5.38	5.38	5.38	-	-	-	-
d 21 of lactation	5.32	5.21	5.03	4.98	5.07	0.064	0.588	0.778	0.438
Lactobacilli									
initial	5.56	5.56	5.56	5.56	5.56	-	-	-	-
d 21 of lactation*	5.72	5.83	5.78	6.06	6.07	0.038	0.001	0.769	0.594
Piglets, d 21 of lactation (Log10cfu/g)									
E. coli	5.07	5.20	4.83	4.74	4.83	0.185	0.158	0.382	0.171
Salmonella spp.	4.39	3.75	3.78	3.94	3.80	0.135	0.404	0.676	0.515
Lactobacilli	5.24	5.50	5.39	5.38	5.36	0.188	0.467	0.527	0.660

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2]Standard error of means.

[3]Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

* Linear response by the supplementation of bacteriophage ($P<0.05$).



(Bacteriophage effect, $P<0.05$; Linear response, $P<0.05$)

Figure 1. Effects of supplementation of bacteriophage and choline on fecal moisture contents of piglets

Table 8. Effects of supplementation of bacteriophage and choline on blood profiles of lactating sows and piglets

Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
Sows, d 21 of lactation									
GOT, U/L	76.2	60.3	69.5	60.2	46.3	4.38	0.170	0.777	0.176
GPT, U/L	39.0	39.2	41.5	40.0	37.7	1.82	0.747	1.000	0.617
NEFA, μU/mL	141.3	135.2	152.8	153.5	143.8	8.97	0.816	0.875	0.556
Piglets, d 21 of lactation									
GOT, U/L	79.0	77.8	78.2	76.3	81.5	4.05	0.921	0.766	0.794
GPT, U/L	37.8	35.3	38.7	35.5	38.5	1.48	1.000	0.311	0.957
NEFA, μU/mL	403.5	402.7	416.8	395.8	419.5	24.98	0.968	0.718	0.928

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), and choline chloride (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2] Standard error of means.

[3] Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

Table 9. Effects of supplementation of bacteriophage and choline on immune response of lactating sows and piglets

Criteria[1]	Con	Bacteriophage 0.05		Bacteriophage 0.1		SEM[2]	*P-value*[3]		
		C0.05	C0.1	C0.05	C0.1		Bac-	Cho-	B×C
Sows, d 21 of lactation, mg/ml									
IgA	1.46	1.53	1.42	1.50	1.60	0.085	0.689	0.981	0.581
IgG	0.71	0.85	0.87	0.74	0.68	0.069	0.231	0.771	0.518
Piglets, d 21 of lactation, mg/ml									
IgA	1.23	1.23	1.12	1.33	1.35	0.052	0.130	0.663	0.540
IgG	0.64	0.68	0.55	0.58	0.64	0.034	0.884	0.650	0.196

[1] Two levels of bacteriophage (0.05%; 0.5×10^8 pfu/g, or 0.1%; 1×10^9 pfu/g), 2 levels of choline (0.05%; 250ppm, or 0.1%; 500ppm) were supplemented.

[2] Standard error of means.

[3] Abbreviation: Bac. (bacteriophage level), Cho. (choline level), and B×C (interaction between bacteriophage and choline level).

Chapter VI. Overall Conclusion

Regulation in Korea has banned the use of antibiotics in animal feed in July 2011, but there is no perfectly alternative sources for antibiotics available yet. Bacteriophage can be a replacement of antibiotics with no resistant bacteria. Moreover, choline is helpful in fat metabolism of lactating sow, resulting in improvement of piglets growth. However, limited information is available for alternative materials and their mechanism as a feed additives. Therefore, three experiments were conducted to investigate 1) Effects of bacteriophage on prevention of *Salmonella enteritidis* in broilers, 2) Effects of bacteriophage on growth performance, fecal properties, blood profiles and immune response in weaning pigs, and 3) Effects of bacteriophage and choline supplementation on physiological responses, growth performance, microbial population and blood profiles of lactating sows and piglets.

In the first study, supplementing against *Salmonella enteritidis* bacteriophage to diets had no effect on growth performance of broilers in the clean state. Relative weight of organ and tissue muscle of breast and leg were not affected by inclusion level of bacteriophage as well as broiler growth. In addition, broilers fed bacteriophage diets is not associated with GOT, GPT and cholesterol levels of blood. In the results of *Salmonella enteritidis* challenge experiment, mortality was linearly decreased with increasing bacteriophage level (linear, $P<0.05$) and *Salmonella enteritidis* concentration in the cecum was decreased with increasing levels of bacteriophage (linear, $P<0.05$).

In the second study, the supplementation of bacteriophage against diarrheal disease to weaning diets had no significant effect on growth performance of weaning pigs under normal physiological state. When bacteriophage was provided to weaning pigs, the *Salmonella spp.* (linear, $P<0.05$, 2wk) and *E. coli* (linear, $P<0.1$, 5wk) concentration of feces were clearly reduced and concentration of *Lactobacilli* was increased (linear, $P<0.05$, 2wk; linear, $P<0.05$, 5wk). Incidence of diarrhea

(linear, $P<0.01$, 2wk) and fecal moisture contents (linear, quadratic, $P<0.01$, 2wk) were also decreased by dietary bacteriophage levels. Bacteriophage in weaning pigs induced lower IgA production as bacteriophage level increased (linear, $P<0.05$, 5wk). Then, the linear decline of total cholesterol (linear, quadratic, $P<0.05$, 2wk; linear, $P<0.01$, 5wk) and LDL cholesterol (linear, $P<0.01$, 2wk) concentration were observed by the level of bacteriophage in the diets.

On the third experiment, bacteriophage and choline are not significantly associated with body weight, backfat thickness and voluntary feed intake at day 21 postpartum, whereas the BW changes in lactation were changed by the supplementation of choline, resulting in quadratic response ($P<0.05$). In the milk composition, fat content of milk was increased by choline supplementation level (quadratic, $P<0.05$). However, other milk composition including milk lactose, protein, total solid, and solid-not-fat were not significantly different among dietary treatments and no differences were observed estimation of milk production of lactating sows. Supplementing bacteriophage and choline to the lactating sow diet had no effects on body weight and weight of gain of nursery pigs. The lactating sows fed diets contacting bacteriophage showed linear effects of population of *Lactobacilli* ($P<0.05$) and the presence of bacteriophage decreased fecal moisture contents of piglets (linear, $P<0.05$). In blood profiles, GOT, GPT, and NEFA levels were not affected by supplementation bacteriophage and choline levels. Then, immune response was not shown by bacteriophage and choline supplementation.

Consequently, bacteriophage as a feed additive may not cause any detrimental effect on growth, productivity and immune function of organs of GIT. Further, it has some beneficial effects on gut heath and disease when animals were exposed to harmful environmental condition. However, investigation is further needed to understand the milk production mechanism and choline in lactating sows.

Chapter VII. Summary in Korea

본 학위논문에서는 사료첨가제로서 박테리오파지 및 콜린의 첨가가 육계와 돼지의 생리 및 생산성에 미치는 영향에 대해 조사하기 위하여 총 3개의 실험이 진행되었다. 첫째로는, 육계에서 *Salmonella enteritidis* 방지를 위한 박테리오파지 첨가가 육계에 미치는 영향을, 둘째로는 박테리오파지의 첨가가 이유자돈의 성장, 미생물 성상 및 면역성상에 미치는 영향에 대해 연구를 수행하였다. 마지막으로 포유모돈 사료 내 박테리오파지와 콜린의 첨가가 포유모돈의 번식성적, 면역성상, 미생물성상 및 포유자돈의 성장성적에 미치는 영향에 대하여 조사를 실시하였다.

실험 1: 육계에서 *Salmonella enteritidis* 방지를 위한 박테리오파지의 이용검증

총 320수의 1일령 육용 수평아리를 공시하여 4처리 8반복, 반복당 10수로 완전임의 배치하였다. 기간별로 2단계의 실험사료에 *Salmonella enteritidis*에 특이적으로 반응하는 박테리오파지를 각각 0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, 및 0.2%; 2×10^9 pfu/g 첨가하였으며, 각 단계 내에서 모든 처리구의 사료 내 영양소 함량은 동등하도록 하였다. 사양시설은 왕겨 바닥의 평사로 pen별로 사료 급이기와 급수기를 동일하게 설치하였으며, 사료와 물은 자유채식 및 음수 시켰다. 사양실험 종료 후 160두를 공시하여 *Salmonella enteritidis* 공격접종을 실시하였다. 총 5주 동안의 사양실험에서 체중, 증체량, 사료섭취량, 사료효율 등의 성장성적에는 유의적 차이가 나타나지 않았다 (P>0.05). 상대적 장기 중량(간,

비장, 복강지방, 가슴육, 다리육)에서 비록 유의적인 차이가 없었으나 (P>0.05), 박테리오파지 0.2%를 첨가한 처리구에서 수치상 높은 가슴육과 다리육 함량을 보였다. 혈액성상 (GOT, GPT, 콜레스테롤)에서 처리구간 유의적인 차이가 관찰되지 않았으나 (P>0.05), 0.2% 박테리오파지 처리구가 대조구에 비해 낮은 GOT, LDL cholesterol 농도와 높은 HDL cholesterol 함량을 나타내었다. 사양실험 종료 후 160수의 *Salmonella enteritidis* 공격접종 이후 폐사율에서는 박테리오파지 첨가수준이 증가함에 따라 폐사율이 유의적으로 감소되었으며 (P<0.05), 접종 후 2주차 장내 미생물 성상에서 *Salmonella enteritidis*는 박테리오파지 첨가수준이 증가함에 따라 유의적으로 감소하였으며 (P<0.05), linear하게 감소되는 경향을 나타내었다 (P<0.05). 사료, 분변 및 계육을 대상으로 한 phage titer 분석 결과에서 사료와 분변 내 박테리오파지 농도가 첨가수준에 맞게 안정적으로 유지되는 것이 확인되었으며, 계육에는 박테리오파지가 축적되지 않는 것으로 나타났다. 이러한 결과를 볼 때, 사료 내 0.2% (2×10^9 pfu/g)의 박테리오파지의 첨가 급여가 육계의 성장이나 계육 생산에 부정적 영향을 주지는 않았으며, *Salmonella enteritidis* 감염 시 육계 폐사율을 감소시키는 것으로 사료된다. 결론적으로 사료첨가제로서의 박테리오파지는 육계에서 안전성이 확인되었고, 사료와 소화기 내에서 안정적으로 작용하여 *Salmonella enteritidis*에 효과를 보이는 것으로 검증되었으며, 이는 육계사료 내 항생제를 대체할 수 있는 가능성을 나타내었다.

실험 2: 박테리오파지의 첨가가 이유자돈의 성장성적, 분변성상, 혈액성상 및 면역성상에 미치는 영향

24 ± 3일령에 이유한 160두의 3원 교잡종 ([Yorkshire × Landrace] × Duroc) 이유자돈이 공시되었다. 기간별로 2단계의 실험사료에 16종류의 소화기 질병에 특이적으로 반응하는 박테리오파지를 0%, 0.05%; 5×10^8 pfu/g, 0.1%; 1×10^9 pfu/g, 및 0.2%; 2×10^9 pfu/g 첨가하였으며, 각 단계 내에서 모든 처리구의 사료 내 영양소 함량은 NRC (1998) 권장량보다 같거나 높도록 배합하였다. 사양실험 전 기간 사료와 물은 자유채식토록 (*ad libitum*) 하였다. 각 단계 별, 또는 전체 5주간의 사양실험 기간 동안 사료 내 박테리오파지 첨가에 따른 일당증체량, 일당 사료섭취량, 사료효율이 처리구에 따른 영향이 관찰되지 않았으며, 결과적으로 사양실험 종료 시 체중에 있어서 차이가 발견되지 않았다 (P>0.05). 미생물 배지를 통해 측정된 분변 내 미생물 조성에 있어서는 병원성 미생물인 *E. coli*의 경우 5주차 (linear, P=0.010), *Salmonella spp.*의 경우 2주차 (linear, P=0.053)에 박테리오파지 첨가 수준이 증가함에 따라 유의적으로 감소되었다. 반면 병원성 미생물의 감소에 따라 유익균인 *Lactobacilli*의 경우 박테리오파지 첨가구들이 높은 농도를 보이는 것으로 관찰되었다 (linear, P=0.019, 2wk; linear, P=0.001, 5wk). 박테리오파지 첨가수준이 증가함에 따라, 설사 및 분변 상태에서는 2주차에 분변점도가 linear하게 감소되어 분변상태가 개선됨을 보였으며 (P=0.001), 분변점도에 있어서도 수분의 함량이 linear (P=0.002), quadratic (P=0.008) 하게 감소되었다. 박테리오파지의 사료 내 수준별 첨가는 5주차 혈청 내 IgA 농도를 유의적으로 감소시켰으나 (linear, P=0.043), IgG 농도는 처리구에 의한 영향을 받지 않았던 것으로 관

찰되었다. 혈액성상에서 glutamic oxaloacetic transaminase (GOT)와 glutamic pyuvic transaminase (GPT)는 박테리오파지 첨가에 따른 영향을 받지 않았으나, total cholesterol (linear, *P*=0.001, quadratic, *P*=0.028, 2wk; linear, *P*=0.002, 5wk) 및 LDL cholesterol (linear, *P*=0.002, 2wk)의 경우 박테리오파지 첨가수준이 증가함에 따라 감소하였다. 본 실험에서의 결과를 통해 볼 때, 특정 병원균에 감염되지 않은 사양조건에서의 이유자돈 사료 내 박테리오파지는 성장능력을 개선시키지는 못하였다. 하지만 0.2% 박테리오파지의 첨가는 유해균을 억제하고 유익균을 증가시켜 장내 미생물 균총에 긍정적인 영향을 미치고 분변성상 개선에 따른 설사 저감과 동시에 면역개선 효과를 나타내었다.

실험 3: 박테리오파지 및 콜린의 첨가가 포유 모돈의 번식성적, 포유자돈 성장 미생물 성상 및 혈액성상 미치는 영향

포유모돈 사료에 박테리오파지와 콜린 첨가가 포유모돈 및 그 자돈에 미치는 영향을 조사하기 위해 총 50두의 Yorkshire × Landrace 교잡종 F1 경산 모돈이 공시되었다. 처리구는 1+2×2 요인배치 (factorial arrangement)로 박테리오파지와 콜린을 첨가하지 않은 대조구와 요인 1은 박테리오파지 0.05%; 5×10^8 pfu/g 와 0.1%; 1×10^9 pfu/g 첨가수준과 요인 2는 염화콜린 0.05% (250 ppm)와 0.1 % (500 ppm) 첨가수준이였다. 3 주간의 포유기동안 모든 처리구의 모돈은 동일한 포유모돈 사료를 자유채식하도록 하였다. 실험결과 포유모돈의 체중 및 등지방 그리고 사료섭취량의 경우 처리구에 따른 유의적인 차이가 관찰되지 않았다. 반

면에 포유기 체중 변화량의 경우, 콜린 첨가구들의 수준이 증가할수록 포유기 동안 체중이 증가하는 것으로 나타났다 (linear, $P<0.05$). 포유자돈의 성장성적의 경우 박테리오파지와 콜린 첨가에 따른 유의적인 차이나 경향이 나타나지 않았으나, 대조구와 비교 시 수치상 높은 성장성적을 보였다. 또한 포유모돈 추정 유생산량, 모유 내 건물 및 에너지 함량 역시 박테리오파지 및 콜린 첨가에 따른 효과가 관찰되지 않았다. 분변 내 병원성 미생물인 *E. coli* 와 *Salmonella spp.*의 경우 처리구간 유의적인 차이가 나타나지 않았으나 박테리오파지의 첨가는 모돈 분변 내 *Lactobacilli*의 함량을 유의적으로 증가시켰으며 ($P<0.001$), 자돈 분변 내 수분함량이 대조구에 비해 낮게 나타났다($P<0.05$). 혈액성상에 있어서는 처리구에 따른 혈중 GOT, GOT 및 NEFA 농도에 영향을 미치지 않았다. 하지만 박테리오파지의 첨가수준이 증가할수록 GOT 농도가 낮아지는 경향이 나타났다 (linear, $P=0.074$). 면역성상에 있어서는 박테리오파지 및 콜린의 첨가에 따른 모돈과 포유자돈의 혈청 면역글로불린 차이가 관찰되지 않았다. 본 실험결과, 포유기 사료 내 콜린의 첨가는 모돈의 포유기 체중 및 돈유 내 지방함량을 증가시키는 것으로 나타났지만 포유자돈의 포유성적 및 유생산량에 영향을 미치지는 못하였다. 박테리오파지의 첨가는 모돈의 분변 내 유익균인 *Lactobacilli* 함량을 증가시켰으나 모돈의 생리 및 번식성적, 그리고 포유자돈의 성장성적에 특별한 영향을 미치지 않는 것으로 나타났다.