A Thesis
for the Degree of Master of Science

Development of multi-species probiotics against calf diarrhea inducing pathogen and its validation of the effect on growth performance and intestinal microflora composition in Holstein Calves

송아지 설사병 유발 병원균 특이적 복합 미생물 생균제의 개발과 송아지에서의 성장 및 장내 균총 변화에 미치는 효과 검정

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농 학 석 사 학 위 논 문

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Summary

Calf diarrhea is the most common and severe disease in young calves in that major economic losses to dairy farms. During a pre-weaning period, young calves are susceptible to many infectious pathogens, especially *E.coli* K99 causing diarrhea and dysentery with blood and mucus in the feces. The use of antibiotics helps to alleviate diarrhea, lowers the calf mortality and decreases the protein requirement in the young calves. The abuse of antibiotics to animal, however, may lead antibiotics resistance to potential human pathogens. Because of these circumstances, feeding antibiotics have been prohibited since 2011 in Korea. Therefore, development of antibiotic alternatives is required for sustainable livestock production. Probiotics are live microorganisms, suggesting that it may have beneficial effects on a host gut ecosystem. I have chosen lactic acid bacteria (LAB) since LAB are the most common type of microbes producing lactic acid, bacteriocins and other metabolic products which protect pathogen colonization and modulate immune responses. Previously, two strains of lactic acid bacteria such as *Lactobacillus plantarum* genome shuffling 1 (LP-GS1) and *Pediococcus acidilactici* genome shuffling 4 (PA-GS4) with improved antimicrobial activity against *E.coli* K99 and *E.coli* O157 were achieved, respectively, by genome shuffling method.

In this study, the multi-species probiotics contained LP-GS1 and PA-GS4 were treated in Holstein calves. This newly developed multi-species probiotics include *Bacillus subtilis* T4 as a digestive enzyme source and *Saccharomyces boulardii* as an intestinal regulator and supplier of protein and mineral.

Total of 40 holstein male calves (age 5-18 days) were randomly assigned to four diet groups; Negative control (NC, no treatment), Positive control (PC, antibiotics treatment), Wild type LAB added probiotics mixture (WPM, mixture of *Pediococcus acidilactia* PA175, *Lactobacillus plantarum* LP177, *Saccharomyces boulardii* SB, and *Bacillus subtilis* T4), and Genome shuffled LAB added probiotics mixture (GPM, mixture of Genome shuffled *Pediococcus acidilactia* PA-GS4, Genome shuffled *Lactobacillus plantarum* LP-GS1, SB, and T4). Test and control groups were fed using a milk replacer and a calf starter with probiotics mixture (10^9 cfu each strain/d/head), with antibiotics (neomycin sulfate) or no treatment for 8 weeks. Growth effects of multi-species probiotics were tested in Holstein calves. GPM group showed same average daily gain as PC group. And there was 50% mortality in NC and WPM group while 90% of calves were survived in PC and GPM group. Furthermore, GPM showed good modulation effect of intestinal microflora. Potential pathogens such as *E.coli* and *Clostridium perfringens* were lower in GPM group than in NC group. In addition beneficial bacteria such as *Pediococcus acidilactici*, *Bacillus subtilis* and *Lactobacillus* spp were higher in GPM group than in NC group.

These results suggest that newly developed multi-species probiotics could use as promising antibiotic alternatives for making environment-friendly livestock products.

Key words: Multi-species probiotics, Calf diarrhea, Antibiotics, Fecal microflora, Lactic acid bacteria

Contents

List of Tables and Figures

Tables

Figures

List of Abbreviations

LAB : Lactic acid bacteria
GPM : Genome shuffled LAB added probiotics mixture
WPM : Wild-type LAB added probiotics mixture
NC : Negative control
PC : Positive control
LPW : *E.coli* K99 with wild-type *Lactobacillus plantarum*
LPG : *E.coli* K99 with GS-*Lactobacillus plantarum*
PAW : *E.coli* O157 with wild-type *Pediococcus acidilactici*
PAG : *E.coli* O157 with GS-*Pediococcus acidilactici*
ADG : Average daily gain
WT : Wild type
GS : Genome shuffling
MCK agar : MacConkey agar
PA : *Pediococcus acidilactici*
LP : *Lactobacillus plantarum*
T4 : *Bacillus subtilis*
SB : *Saccharomyces boulardii*
ELISA: Enzyme-linked immunosorbent assay
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction

I. Introduction

Calf diarrhea is a common and critical disease caused by the mortality in young calves. *E.coli* K99 is major pathogen which causes calf diarrhea from birth until 30 days of age. To prevent this problem, most of dairy farms have been used antibiotics specifically Neomycin sulfate in calves. However use of antibiotics imposes a selection pressure for bacteria that are resistant to antibiotics. Therefore use of antibiotics to animal has been prohibited since 2011 in Korea.

According to the banning of antibiotics in livestock, probiotics have been rising substitutes for antibiotics. Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). When administration probiotics to host animal, it may affect intestinal microflora by reducing harmful microorganism, enhance immune function and improve beneficial bacteria (Nahashon *et al.*, 1994; C. Ouwehand *et al.*, 2002).

For these reasons multi-species probiotics were designed containing GS-LAB, *Bacillus subtilis* T4 and *Saccharomyces boulardii*. GS-LAB which have been shown to improve an anti-microbial activity against pathogenic *E.coli* K99 (Seo, 2012) and *E.coli* O157 (Choi, 2011) and have better ability to eliminate pathogenic bacteria than wild-type LAB. Also, *Bacillus subtilis* produce digestible enzyme and *Saccharomyces boulardii* supply protein and minerals. (Figure 1)

Therefore, this study was conducted (1) to characterize LAB strains through determination of survivability in low pH, bile acid and low temperature, (2) to investigate the effects of

multi-species probiotics on the growth performance in Holstein calves compared to antibiotic supplement, (3) and to evaluate the effects of multi-species probiotics on the intestinal microflora composition.

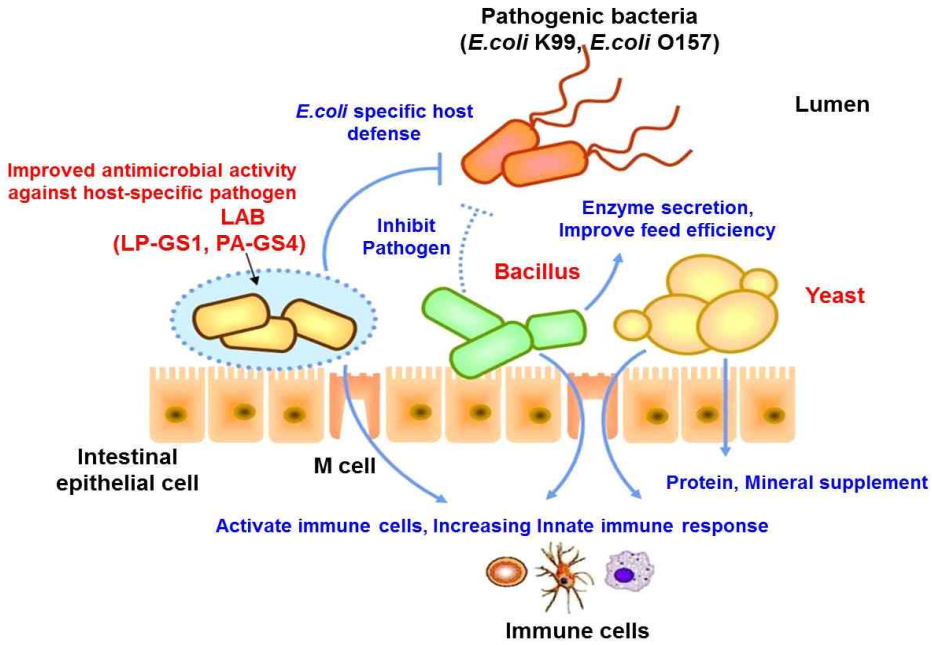


Figure 1. Schematic diagram of multi-species probiotics. LP-GS1 and PA-GS4 improved anti-microbial activity against pathogenic *E.coli* K99 and *E.coli* O157 respectively. Bacillus produce digestible enzyme and Yeast supply protein and minerals.

II. Review of Literature

1. Neonatal calf diarrhea

Calf diarrhea is the main reason of mortality in dairy calves (Gardner, 1990; Virtala, 1996). It is mainly occurred by enterotoxigenic *E.coli*, *Salmonella*, rotavirus, and coronavirus (Cho, 2010). Also, decreased colostral transfer of passive immunity imposes more chance to infection by these pathogens.

Numerous studies found that diarrhea calves have overgrowth coliform bacteria in the intestine (Carpenter, 1924; Gay, 1965; Constable, 2004) (Figure 2). Colonization of *E.coli* is associated with altered small intestinal function, changed epithelial cell morphology, and increased susceptibility to bacteremia.

Generally, antibiotics have been used for preventing neonatal calf diarrhea (Constable, 2004; Sawant, 2005). Administration of antibiotics reduces intestinal microbes including pathogenic bacteria in calves with diarrhea (Rusoff, 1953; Quigley III, 1997). Inhibition of the growth of pathogenic bacteria results in lowered mortality, prevented changing intestinal epithelial cell morphology, and ameliorated digestion (Sissons, 1989; Mack, 1999). However overuse of antibiotics impose a selection pressure for bacteria that are resistance to antibiotics and also deposit some residue in product (Tajick, 2006). Thus using antibiotics in animal feed has been prohibited since 2011 in Korea suggesting that substitutional antibiotics are needed.

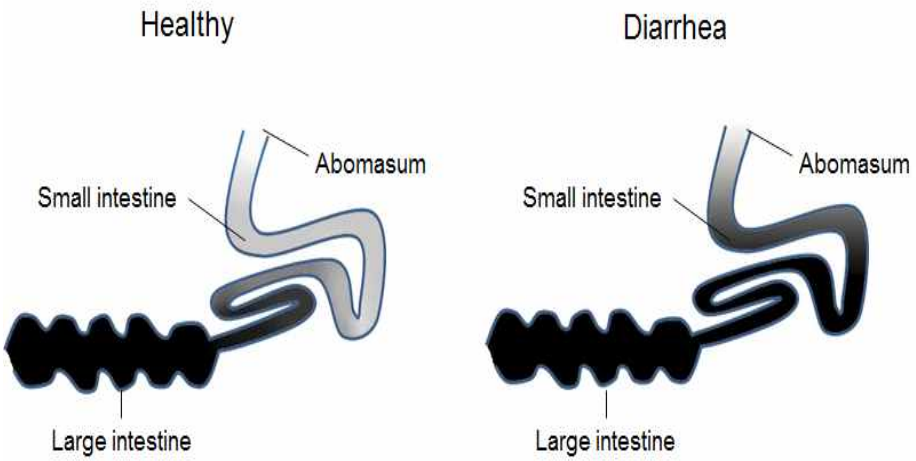


Figure 2. Schematic diagram of *Escherichia coli* concentration in the intestinal tract of a calf with diarrhea. The number of *E.coli* in the large intestine is similar between healthy and Diarrhea. However diarrhea calf increased *E.coli* in their small intestine.

2. Probiotics

1) Concept of probiotics

The 'probiotic' is derived from the Greek meaning 'for life' and usage credited to Lilly and Stilwell (1965). It is defined live microorganisms which beneficially affect the host animals or humans by improving its intestinal microbial flora during the ingestion (FAO/WHO, 2002). Generally bifidobacteria and lactic acid bacteira are common bacteria microbes which have been used as probiotics and have been commonly consumed as fermented foods form such as yogurts and/or dietary

supplements. Probiotics known as feed supplements which also beneficially affect the host animal by improving its intestinal microbial balance, thus enhancing the health of host and usually inhibiting pathogens and toxic substance (Figure 3). For instance consuming probiotics help to reduce antibiotic associated with diarrhea (Black et al. 1991), short rotavirus diarrhea (Saavedra et al. 1994; Sugita & Togawa 1994, Guandalini et al. 2000), reduce recurrence of superficial bladder cancer (Aso et al. 1994), regulate immune modulation (Kaila et al. 1992, Nagao et al. 2000), improve oral vaccination (Link-Amster et al. 1994), reduce colonization by *Helicobacter pylori* (Felley et al. 2001), relief irritable bowel syndrome (Gupta et al. 2000, Brigidi et al. 2001; Niedzielin et al. 2001), reduce LDL-cholesterol (Bukowska et al. 1998), prevent allergy (Isolauri et al. 2001; Kalliomaki et al. 2001; Majamaa & Isolauri 1997), reduce symptoms of inflammatory bowel disease (Malchow 1997; Guslandi et al. 2000; Mattila-Sandholm et al. 1999), and reduce incidence of travellers diarrhea (Black et al. 1989).

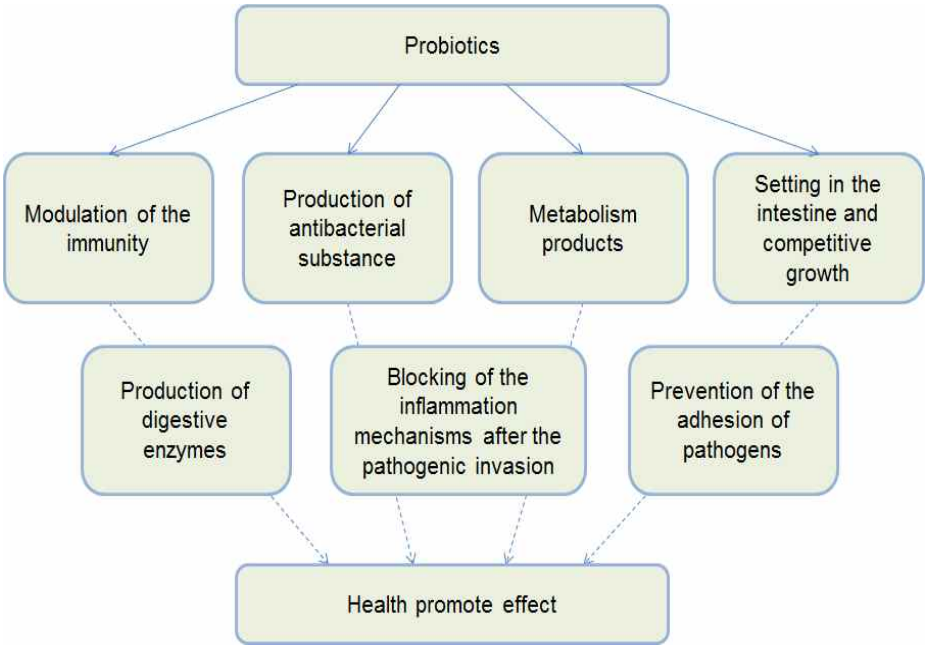


Figure 3. The mechanisms implied in the positive effects of probiotics on the animals' growth and health.

2) Intestinal regulatory effect of probiotics

The major purpose of probiotics is to change the composition of the normal intestinal microflora from a potentially harmful composition towards a microflora that would be beneficial for the host. This suggests that probiotics help to reduce harmful bacteria such as coli form bacteria, salmonella and clostridia and increase beneficial bacteria such as lactic acid bacteria, bifidobacteria. Previous studies have been carried out the protection effect of probiotics as follow. Cell culture medium of probiotics prevent necrotizing enterocolitis (NEC) by accelerating the maturation of intestinal innate immune response gene (Kriston Ganguli et al, 2013). Also, probiotics protect the

epithelial barrier and keep tight junction protein in clinical colitis induced-mouse (mennigen Rudolf et al, 2009). In murine model, administration of *Lactobacillus reuteri* down-regulated multiple enterocyte genes which function to stabilize enterocytes against movement. As a result of these change, enterocyte migration rate and crypt cell proliferation were increased. Moreover this probiotics increase microbial diversity and community evenness (Preidis et al, 2012). These research support that probiotics not only itself but also secretion factor play a important role for protect intestinal tract.

3. Properties of probiotics

1) Lactic acid bacteria

Lactic acid bacteria have many beneficial effects on host animals in terms of producing antimicrobial molecules with activity against gastric and intestinal pathogens and other microbial groups. Also LAB compete with pathogens for mucosal cell surface and mucin binding sites (Neeser, 2000). This could be protective effect against many pathogens which develope cancer, inflammation and allergy.

Receptor-specific binding, Glycolipid-binding haemagglutinating avtivity, charge and hydrophobic interaction are the mechanisms of attach to epithelial cell surface. Lactic acid bacteria commonly express cell surface hydrophobicity which facilitate contact angle and adhesion to xylene (Wadström et al., 1987; Strus et al., 2001). Lactic acid bacteria also express extracellular matrix

binding molecules such as fibronectin, collagens and vitronectin (Aleljung et al., 1994; Toba et al., 1995; Howard et al., 2000; Lorca et al., 2002). Many strains including *L. acidophilus*, *L gasseri*, *L. johnsonii*, and *L. crispatus* identified Surface layer (S layer) which covers the cell surface. S layer used as delivery vehicles for antigen delivery and has a protective function from host defence mechanism (Toba, 1995; Smit, 2001).

Oxidative damage is one of the reason for inducing cancer, cirrhosis, atherosclerosis and other chronic diseases. According to the recent studies, lactobacilli produce high antioxidant activity (Annuk et al., 2003), and *B. longum* and *L. acidophilus* inhibit linoleic acid peroxidation and eliminate free radicals (Lin and Chang, 2000).

Bacteriocin is peptide which has an anti-microbial activity. Some of lactic acid bacteria strain produce bacteriocin to enhance their survivability in complex intestinal ecosystem (Pinchuk, 2001; Jamuna, 2004). *Helicobacter pylori*, known as a gastric pathogen, was inhibited by secreted bacteriocin from *L. acidophilus*. In the same manner feeding *S. cerevisiae* spp *boulardii* exterminate escherichi coli O157:H7 in rumen fluid (Lorca et al., 2001; Bach et al., 2003).

Increasing production of mucin is one of defensive mechanism in the gut. This physicochemical barrier inhibits viral replication and provide receptors for microbes (Yolken et al., 1994). Most of intestinal mucin consist of MUC2 and MUC3 gene products whereas colonic mucin presents only MUC2. *L. plantarum* 299v effectively increases the expression of MUC2 and MUC3 mRNA in HT29 intestinal cells, and consequently adhesion of enterovirulent *E.coli* was inhibited (Mack et al., 1999). Similar

results were reported in other *Lactobacillus* strains which tested in different cell lines, Hep-2 and Caco-cells (Forstner and Forstner, 1994; Smith et al., 1995; Mack et al., 2003), suggesting that *Lactobacillus* has benefecial effects on the intestinal barrier composition.

(1) *Pediococcus acidilactici*

Pediococcus acidilactici is gram positive coccus and facultative anaerobe with lesser sensitivity to oxygen. It can grow in a wide range of pH, temperature and osmotic pressure, which gives better ability to colonize the gut. Pediococci inhibit enteric pathogens by lactic acid and bacteriocins known as pediocins.

Pediococcus acidilactici grows in MRS (deMann, Rogosa, Sharpe) media and optimum temperature is approximately 40℃. The optimum pH is 6.2 but decreased almost 3.6 during culture. This bacteria has broad range of beneficial effect so that treated many disorders. Such as immune modulation, digestive problem, inhibit pathogenic bacteria and alleviate intestinal microorganism which was disrupted by antibiotics.

(2) *Lactobacillus plantarum*

Lactobacillus plantarum is gram positive bacilli and also facultative anaerobic bacterium which produce lactic acid. *Lactobacillus plantarum* cultred in MRS media and optimum growth temperature between 30 and 40℃. They are commonly used in many fermented food production such as yogurt, cheese, pickles and so on. Lactobacilli are also important in silage

production. Lactic acid produced by Lactobacilli makes low pH and high level of heterologous protein inhibits growth of bacteria.

It has been shown that *Lactobacillus plantarum* can protect epithelial cells in *E.coli*-induced damage by preventing changes in host cell morphology, attaching lesion formation, stimulating immune response and enhancing the intestinal integrity.

3) *Bacillus subtilis*

Bacillus subtilis is gram positive and known as grass bacteria. Optimal temperature for growth is between 25 and 35℃. It has the ability to form endospore, allowing the organism to tolerate extreme environmental conditions (Figure 4). *Bacillus subtilis* has been considered as normal gut commensal bacteria and secrete various enzymes, such as protease, amylase, lipase, pullulanase, chitinase, xylanase and so on.

Bacillus are responsible for producing antibiotics such as polymyxin, difficidin, subtilin and mycobacillin.

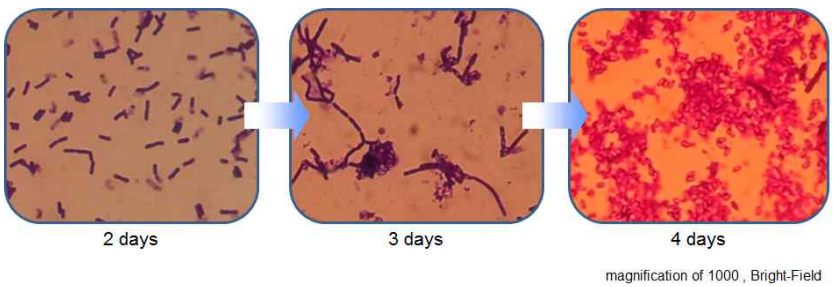


Figure 4. Endospore formation of *Bacillus subtilis*. Endospore can endure harsh environment and finally gives better survivability in any condition.

4) *Saccharomyces boulardii*

Saccharomyces boulardii is one of Yeast strain which has been shown to be non-pathogenic and non-systemic, and grows at the temperature of 30 ℃.

S. boulardii is used for preventing diarrhea, digestion problems, and lactose intolerance. *S. boulardii* is also shown to be prevented travelers′ diarrhea which associated with the use of antibiotics, irritable bowel syndrome, acute adult diarrhea, and Crohn′s disease.

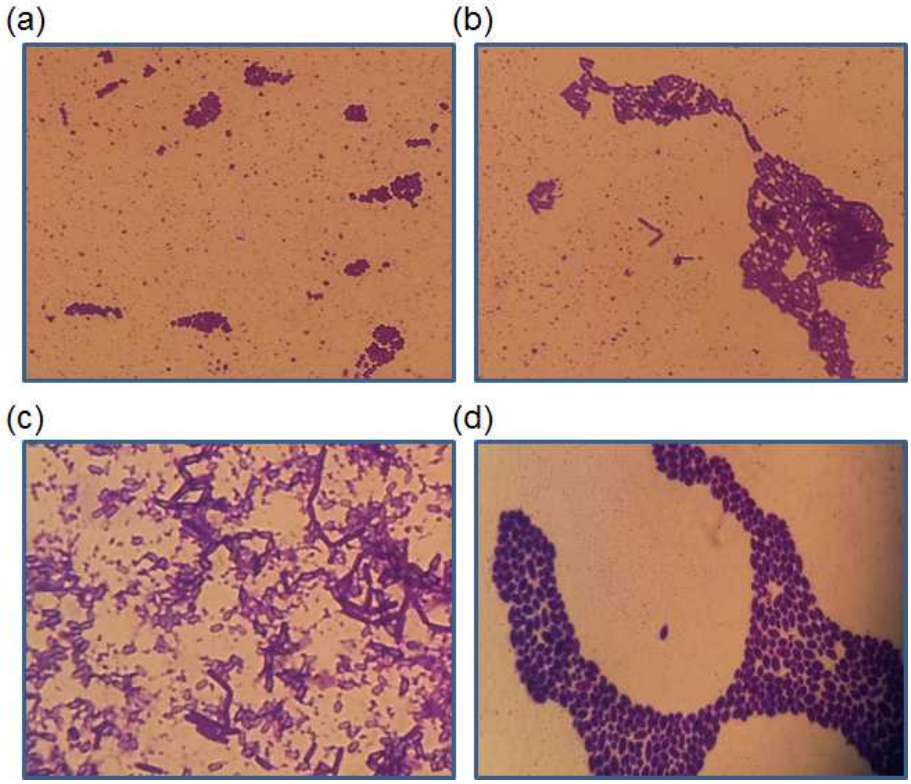


Figure 5. Morphology of probiotics strain. (a) *P. acidilactici*, (b) *L. plantarum*, (c) *B. subtilis*, (d) *S. boulardii*

III. Materials and Methods

1. Preparation of multi-species probiotics

1) Microbial strains and media

Multi-species probiotics contain lactic acid bacteria (LAB), a Bacillus and an yeast. *Lactobacillus plantarum* 177, GS1 and *Pediococcus acidilactia* 175, GS4 was tested in this study. All of LAB strains were cultured in MRS broth (De Man, Rogosa and Sharpe; BD/Difco, USA) at 37℃, Bacillus was cultured in BSM4 broth at 30℃ and Yeast was cultured in YPD broth (Yeast Extract Peptone Dextrose; BD/Difco, USA) at 30℃. (Table 1)

Expected effects of each strains are as follows; (i) LAB, for improved anti-microbial activity against *E.coli* K99, *E.coli* O157 and other harmful bacteria; (ii) Bacillus, as a source of digestive enzyme; (iii) Yeast, for protein and mineral source and immune boosting by β-glucan of cell wall.

Table 1. Media for growth of each strain. All quantities are g/L

Component	YPD	BSM4	MRS
$CaCO_3$	-	1	-
$MgSO_4 \cdot 7H_2O$	-	0.3	-
$FeSO_4 \cdot 7H_2O$	-	0.02	-
$ZnSO_4 \cdot 7H_2O$	-	0.02	-
Yeast Extract	10	2	5
Beef Extract	-	-	10
Peptone	20	2	-
Cottonseed flour	-	20	-
Glucose	-	15	-
Dextrose	20	-	20
Proteose Peptone No.3	-	-	10
Poly sorbate 80	-	-	1
$C_6H_{17}N_3O_7$	-	-	2
CH_3COONa	-	-	5
$MgSO_4$	-	-	0.1
MnO_4S	-	-	0.05
K_2HPO_4	-	-	2
pH	6.5	6.8~7.0	6.5

2) Lactic acid bacteria strain

(1) Anti-microbial activity test

Escherichia coli O157 (ATCC 43889), *Escherichia coli* K99 (KCTC 2617) are which already have. The interference of lactic acid bacteria with the growth of pathogenic strain was evaluated by pathogen-LAB co-culture assay. A tube containing 10ml of MRS broth was inoculated with 6.25 x 10^7 CFU/ml of *E.coli* O157 and 2 x 10^7 CFU/ml of either PA-WT or PA-GS4. Each

10ml cultures were used for viable cell counting by serial dilution method on MacConkey agar after 19hr incubation. 1.3 x 10^7 CFU/ml of *E.coli* K99 and 2.4 x 10^6 CFU/ml of either LP-WT or LP-GS1 were added to MRS broth for 6hr incubation.

Every co-culture assay was performed at 37℃, 250rpm shaking incubator. (Figure 6)

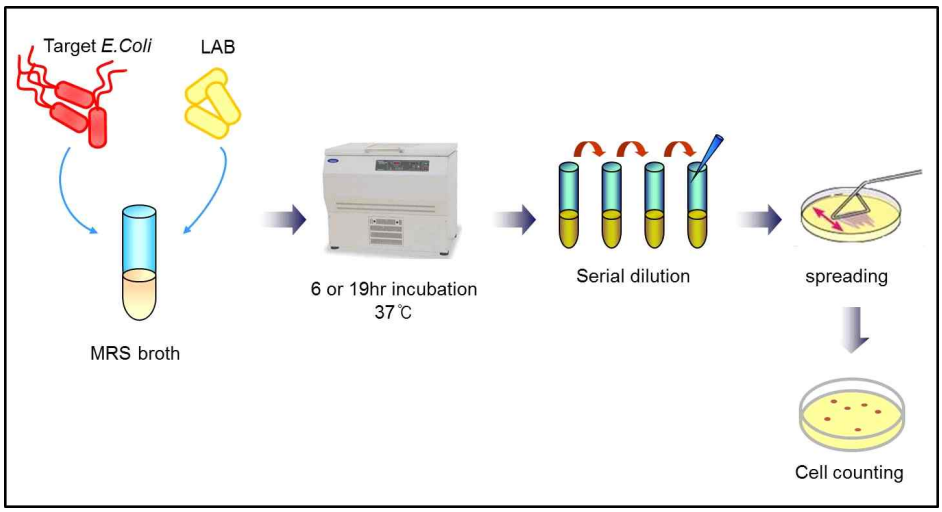


Figure 6. Analysis of the anti-pathogenic ability of each LAB strains.

(2) Physiological test

① pH curve

pH curve of wild and genome shuffled LAB were measured using pH meter. Before measurement, calibration was performed using calibration solution with pH 4.01, 7.00 and 10.01 according to manufacturer's instructions.

② Growth curve

Growth curve of wild and genome shuffled LAB were measured using UV spectrometer. Single colony of each LAB was inoculated into MRS broth media. The cells were cultivated until the end of each experiment. every 2hr, samples were analyzed by spectrometer with OD600 value.

(3) Acid tolerance test

WT and GS LAB strains were grown in MRS broth at 37℃ overnight. 3ml aliquot of each cultures were adjusted to pH 3.0 and 2.0 with HCl and incubated at 37℃ for 3hr. At indicated time points, viable cell was counted by serial dilution method with PBS (0, 30, 60, 120, 180 min).

(4) Bile resistance test

WT and GS LAB strains were grown in MRS broth at 37℃ overnight. Bile solution (oxgall) was added to total of 10ml culture media to achieve a final concentration of 0.3%. At indicated time points, viable cell was counted by serial dilution method with PBS (0, 1, 2, 3, 4 hr).

3) Sample preparation of multi-species probiotics

Single colony of *Pediococcus acidilactici* 175 (PA-175), *Pediococcus acidilactici* GS4 (PA-GS4), *Lactobacillus plantarum* 177 (LP-177) and *Lactobacillus plantarum* GS1 (LP-GS1) were

inoculated in a 50 ml corning tube which containing 45 ml MRS broth at 37℃ for 16hr with shaking at 150 rpm. After 16hr, 2 ml of culture media was inoculated 900 ml MRS broth in 1 L bottle and cultivate at 37℃ for 24hr with shaking at 150 rpm. Cells were harvested by centrifugation at 4℃ for 15 min at 5000 rpm.

Saccharomyces boulardii 796 (SB) was cultured in a 10ml tube which containing 6ml YPD broth at 30℃ for 24hr. And 5ml of culture media was inoculated 250 ml flask containing 50 ml YPD broth at 30℃ for 48hr with shaking at 200 rpm. After 48hr, 9ml of culture media was inoculated 400 ml YPD broth in 2 L flask and cultivate at 30℃ for 48hr with shaking at 200rpm. Cells were harvested by centrifugation at 4℃ for 15min at 5000 rpm.

Bacillus subtilis T4 (T4) was cultured in a 250 ml flask containing 40 ml BSM4 broth at 37℃ for 16hr with shaking at 200 rpm. After 16hr, 5 ml of culture media was inoculated 400 ml BSM4 broth in 2 L flask and cultivate at 30℃ for 96hr with shaking at 200 rpm to endospore formation. Endospore were harvested by centrifugation at 4℃ for 20 min at 7500 rpm.

Cell pellets of each strains were suspended in 200 ml PBS solution and stored at 4℃. Each strains are mixed 1 day before supply to the farm (Figure 7, 8). Two forms of multi-species probiotics were designed and tested in Holstein calves; WPM, PA-175 + LP-177 + T4 + SB; GPM, PA-GS4 + LP-GS1 + T4 + SB and all contain about 10^9 CFU/head in each probiotics (Table 2).

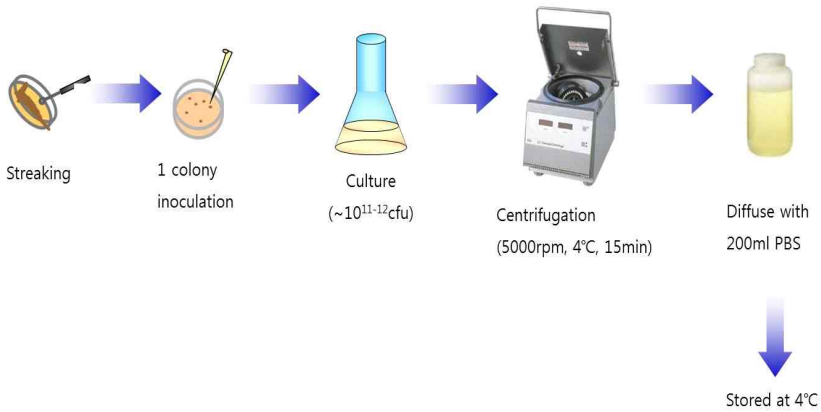


Figure 7. Single strain culture. Cells were diffused with 200 ml PBS. Diffused sample were stored at 4℃

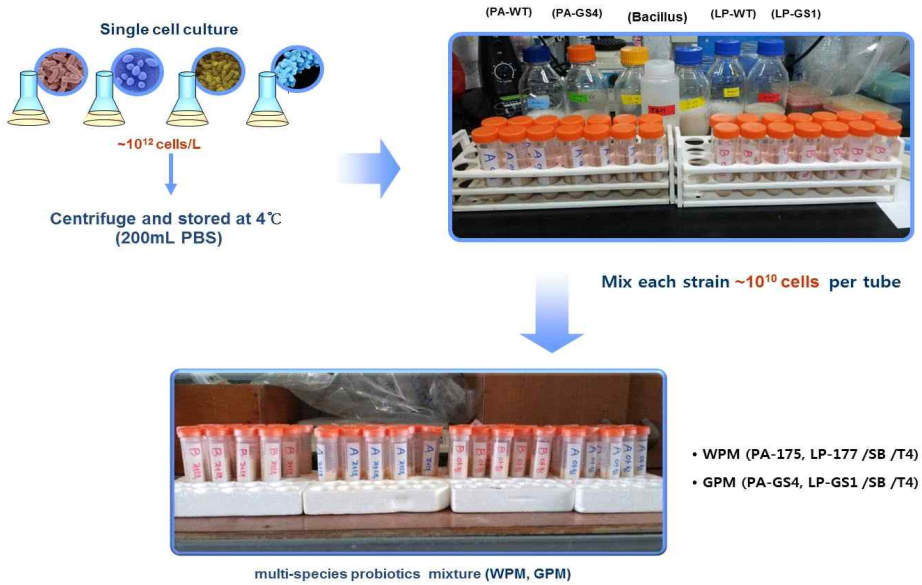


Figure 8. Production process of multi-species probiotics. Diffused samples were mixed 1 day before supply. Composition of each multi-species probiotics are as follows; PA-175 (WT), LP-177 (WT), T4 and SB (WPM); PA-GS4, LP-GS1, T4 and SB (GPM).

Table 2. Treatment and control groups

Group	Treatment	Note
Negative control (NC)	Basal diets	No treatment
Positive control (PC)	Basal diets + Antibiotics	Neomycin sulfate(0.01%)
WPM	Basal diets + Probiotics 1	PA175, LP177 (WT), T4, SB
GPM	Basal diets + Probiotics 2	PA-GS4, LP-GS1, T4, SB

4) Probiotics survivability in low temperature storage

Viable cells were counted during 8 weeks of 4℃ storage. 100 μl of each sample was suspended in 900 μl PBS. suspended sample was serially diluted and cell counted by plating on MRS agar (for PA-175, PA-GS4, LP-177, LP-GS1; BD/Difco, USA) and LB agar (for T4; BD/Difco, USA) and YPD agar (for SB; BD/Difco, USA). MRS and LB agar were incubated at 37℃ for 24hr (for measuring LAB and T4) or at 30℃ for 48hr (for measuring SB).

2. Holstein calves feeding test

A total of 40 holstein male calves aged 5-18 days were randomly assigned to four diets groups; NC(no treatment), PC(antibiotics treatment), WPM(*Pediococcus acidilactia* PA-175, *Lactobacillus plantarum* LP-177, *Saccharomyces boulardii* SB, and *Bacillus subtilis* T4), GPM(Genome shuffled *Pediococcus acidilactia* PA-GS4, Genome shuffled *Lactobacillus plantarum* LP-GS1, SB, and T4). Test and control group were fed milk replacer and calf starter with probiotics mixture (10^9 CFU each strain/d/head), with antibiotics (neomycin sulfate) or no treatment for 8 weeks (Table 3). Probiotics were supplemented twice a day with milk replacer (6:30 am, 5:30 pm). Calf starter and water were offered for ad libitum consumption from day 1 of the study. The schedule of *in vivo* experiment (Figure 9) and exprimental scheme (Figure 10) were as follow.

Table 3. Composition of basal diets

Ingredient	Milk replacer	Calf starter
Crude protein (%)	20	16.5
Crude fat (%)	10	2.5
Crude fiber (%)	3	10
Crude ash (%)	12.6	10
Ca (%)	0.6	0.6
P (%)	0.85	1.4
Vitamin (IU/kg)	25,000	
TDN (%)		70

Milk replacer from easybio and calf starter from seoulfeed, Korea.

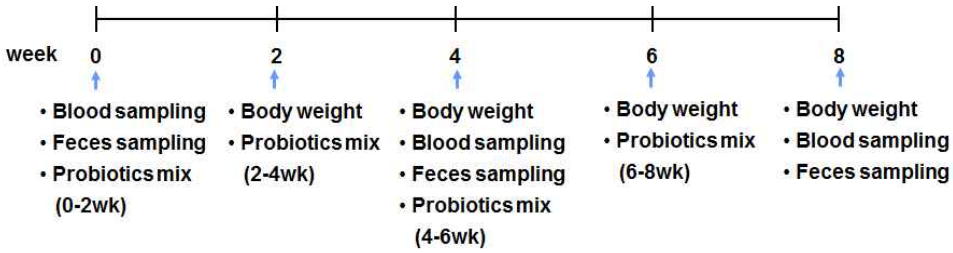


Figure 9. The schedule of *in vivo* experiment. New probiotics mixture was supplied every two weeks.

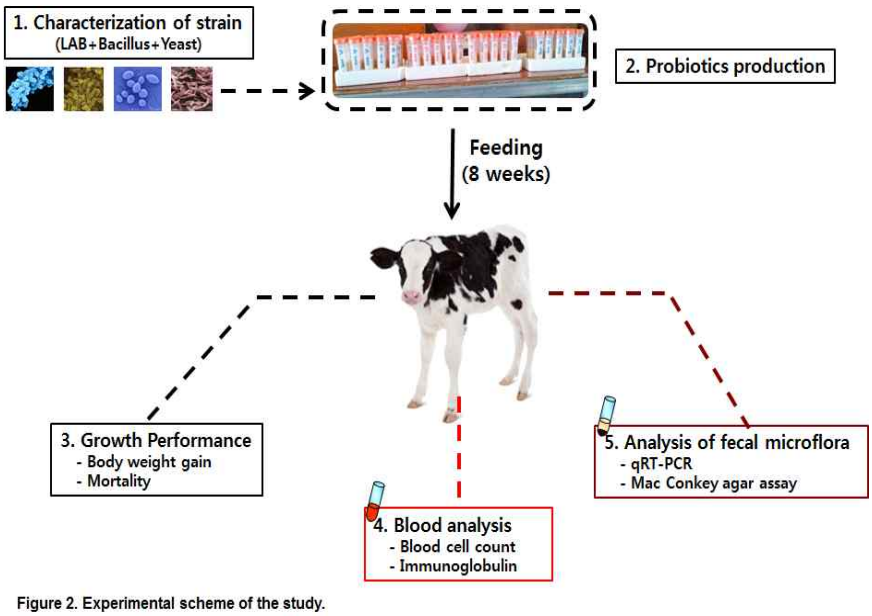


Figure 10. Experimental scheme of the study.

3. Growth performance

Growth performace was evaluated such as body weight, average daily gain and mortality. Body weight was measured 4 times (2, 4, 6, 8 weeks of experiment) and mortality was measured every day.

4. Fecal microflora analysis

1) Quantitative real-time polymerase chain reaction (qRT-PCR)

Fecal samples are collected every 4 weeks from each holstein calf. Samples were stored at -80℃ until DNA extraction. fecal DNA extraction was performed as described by Yu and Morrison (2004).

Oligonucleotide sequences were checked by using the probe match function of the Ribosomal Database Project software package (Larsen, 1993) and tested for uniqueness by BLAST (Basic Local Alignment Search Tool). Primers designed to target different genus or species and purchased from the Bioneer (Daejeon, Republic of Korea). The designed primer used for the real-time PCR are described in Table 4.

PCR was performed with MyiQ single color Real-Time PCR Detection System (Bio-Rad, USA). Each reaction mixture (25 μl) contained 0.5 μg of template DNA, 12.5 μl TOPreal qPCR 2X PreMIX (SYBR Green) (Enzynomics, Daejeon, Republic of Korea), 0.5 μl of each specific primers at a concentration of 10 pmol/μl

and distilled water. The following amplification program was used : 95℃ for 10 min, 40 cycles consisting of 95℃ for 30 sec, annealing temperature (Table 4) for 20 sec, 72℃ for 30 sec and then one cycle of 95℃ for 1 min. Total bacterial primer set was used for normalization of the data. Delta-delta Ct method was used for comparing fecal microflora composition among the groups. (Schmittgen, 2008).

Table 4. Real-time PCR primers used to measure fecal microflora composition

Target bacterium	Primer sequence (5'→3')	AT[1] (℃)	PCR product size (bp)	Reference
Escherichia coli	F[2] : GTTAATACCTTTGCTCATTGA R[3] : ACCAGGGTATCTAATCCTGTT	46	340	Malinen *et* al., 2003
Clostridium perfringens	F : ATGCAAGTCGAGCGAGTG R : TATGCGGTATTAATCTCTCCTTT	49	120	Rinttilä *et* al., 2004
Lactobacillus spp.	F : AGCAGTAGGGAATCTTCCA R : CACCGCTACACATGGAG	46	341	Kim *et* al., 2011
Pediococcus acidilactici	F : CGAACTTCCGTTAATTGATCAG R : ACCTTGCGGTCGTACTCC	50	872	Mora *et* al., 1997
Bacillus subtilis	F : AAGTCGAGCGGACAGATGG R : CCAGTTTCCAATGACCCTCCCC	55	595	Wattiau *et* al., 2001
Total microbes	F : TCCTACGGGAGGCAGCAGT R : GGACTACCAGGGTATCTAATCCTGTT	56	467	Nadkarni *et* al., 2002

[1]AT : annealing temperature ; [2]F : forward ; [3]R : reverse

2) MCK agar assay

Fecal samples are collected from 3 calves selected randomly per each group. MCK agar assay was performed the day of collecting fecal samples for live coliform bacteria counting.

0.3 g of fecal sample was suspended in 2.7 ml PBS. Suspended sample was serially diluted and plating on MCK agar at 37℃ for 24hr. After 24hr red colonies were counted.

5. Blood collection and analysis

Blood samples were collected every 4 weeks. Blood was obtained by puncture of the jugular vein using evacuated tubes (Vacutainer Systems; Preanalytical Solutions, USA) containing either no anti-coagulant for serum separation or K2 EDTA. Tubes were placed on ice immediately and serum samples were centrifuged 1,600 x g for 20 min at 4℃. Collected plasma was stored at -80℃ until serum immunoglobulin analysis.

Total serum IgG and IgA were measured by ELISA using immuno plate (SPL, Gyeonggi, Republic of Korea) following the manufacturer's instruction. Add 100 μl of diluted coating antibody (Sheep anti-bovine IgG affinity purified; Bethyl laboratory, USA) with carbonate-bicarbonate buffer (Sigma, USA) and incubate at a room temperature for 1hr. After washing 5 times, add 200 μl of blocking solution to each well and incubate 30 min. After washing 5 times, add 100 μl of standard (Reference serum; Bethyl laboratory) or sample and incubate 1hr. After washing 5 times, add 100 μl of diluted HRP detection antibody (Sheep anti bovine IgG; Bethyl laboratory) and incubate 1hr. After washing 5 times, develop with 100 μl TMB solution for 15 min and stop reaction by adding 100 μl of 0.18M H_2So_4. Measure absorbance on a plate reader at 450 nm by Infinite M200 PRO (TECAN, Switzerland).

Red blood cell (RBC), white blood cell (WBC), hematocrit,

platelet and hemoglobin were measured from blood containing K2 EDTA.

6. Statistical analysis

All statistical analysis were performed using SAS (SAS Inst. Inc.). Results were expressed as the mean and Standard error of the mean (SEM). Analysis of variance (ANOVA) test and student *t*-test were used as significance tests.

IV. Results and Discussion

1. Characterization of probiotics

1) Anti-microbial activity test

To evaluate anti-microbial activity, pathogen-LAB co-culture assay was performed. 1.3 x 10^7/ml of *E.coli* K99 and 2.4 x 10^6/ml of LP-177 or LP-GS1 were cultured together in MRS broth for 6hr. *E.coli* K99 was increased by 7, 3.3 and 1.9 fold higher than control in K99, LPW and LPG group, respectively, after 6hr (Figure 11).

Also, 6.25 x 10^7/ml of *E.coli* O157 and 2 x 10^7/ml of PA-175 or PA-GS4 were cultured together in MRS broth for 19hr. *E.coli* O157 was increased in O157 group but it was decreased in PAW and PAG group, respectively (Figure 12).

Overall both GS LAB show strong inhibition activity to target pathogenic *E.coli* than wild type LAB.

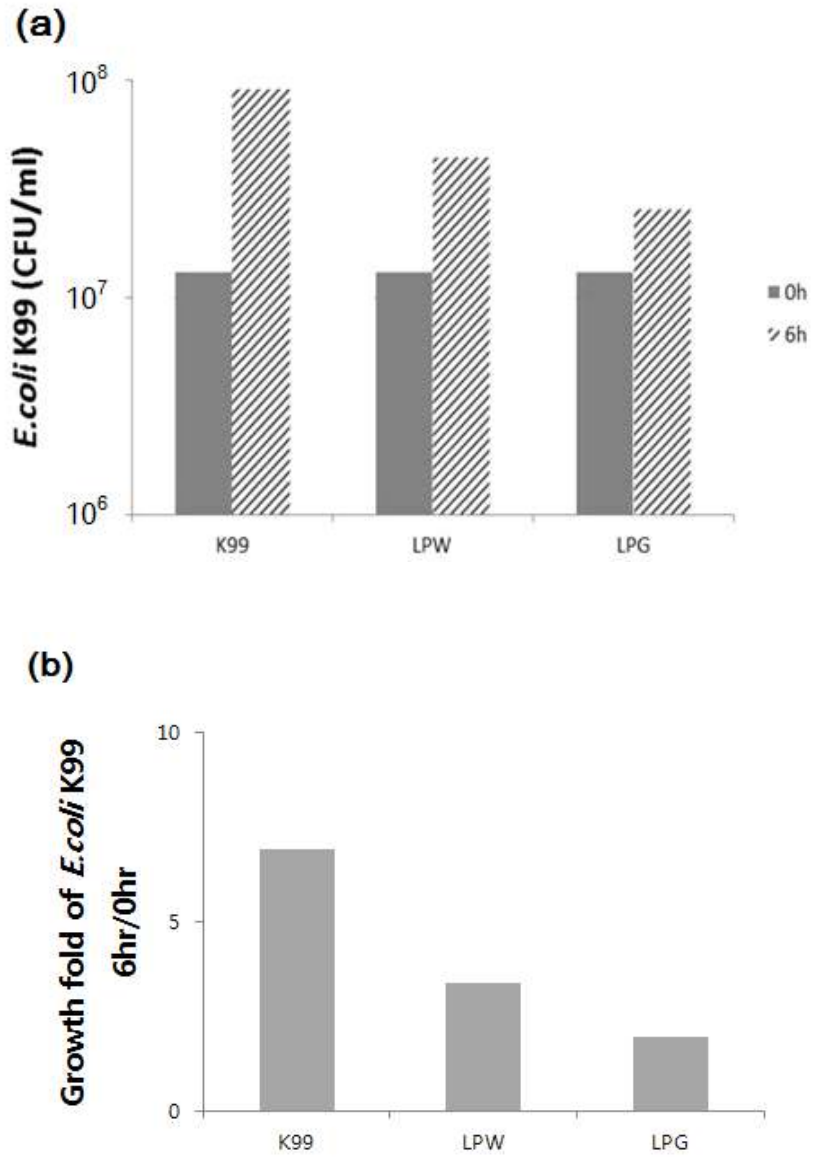


Figure 11. Viable cell counts of *Lactobacillus plantarum*177(WT), *Lactobacillus plantarum*177-Genome shuffling mutants with *E.coli* K99 co-culture medium at 6hr. (a) viable cell counts of *E.coli* K99(cfu/ml). (b) Growth fold of *E.coli* K99

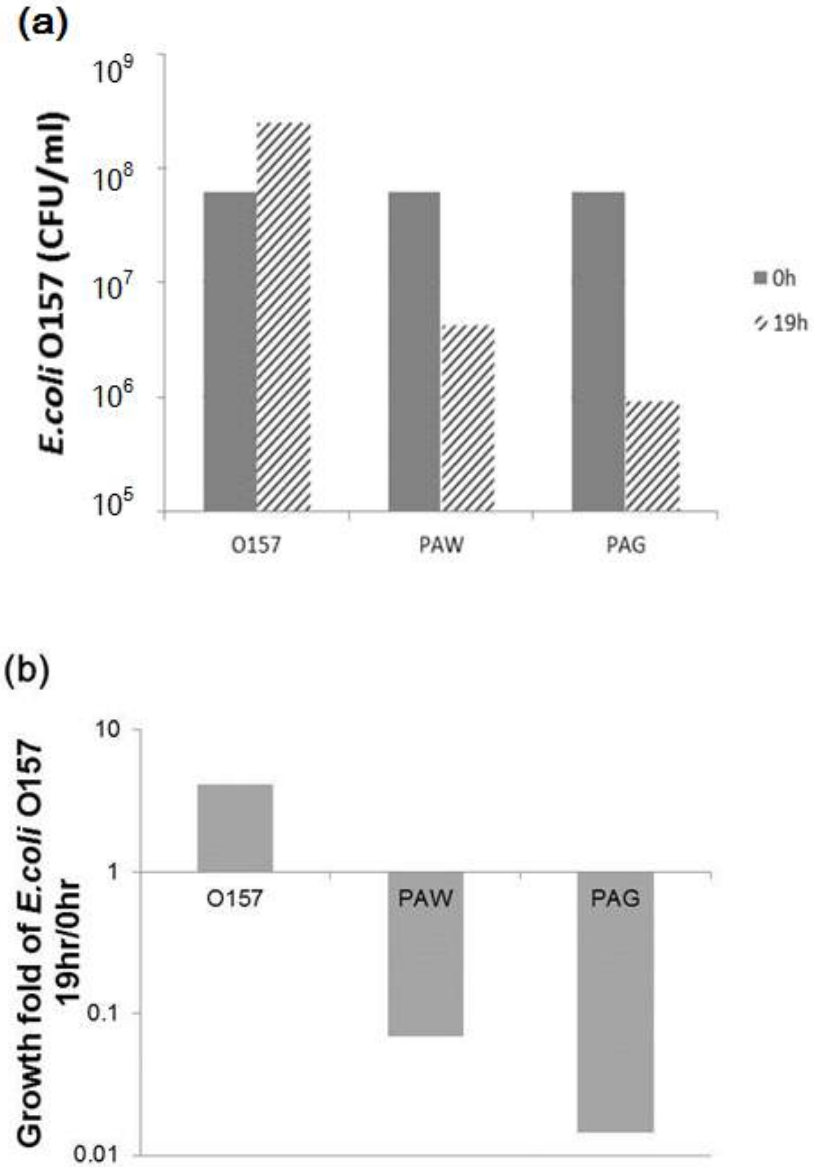


Figure 12. Viable cell counts of *Pediococcus acidilactici*175(WT), *Pediococcus acidilactia*175-Genome shuffling mutants with *E.coli* O157 co-culture medium at 19hr. (a) viable cell counts of *E.coli* O157(cfu/ml). (b) Growth fold of *E.coli* O157

2) Physiological test

To confirm whether GS mutant could retain physiology of lactic acid bacteria, both growth and pH curve were evaluated.

Despite the minor differences in magnitude of the activation, there are no major differences in growth curve between wild type and genome shuffled lactic acid bacteria. Thus, GS mutant has retain normal physiology as wild type lactic acid bacteria.

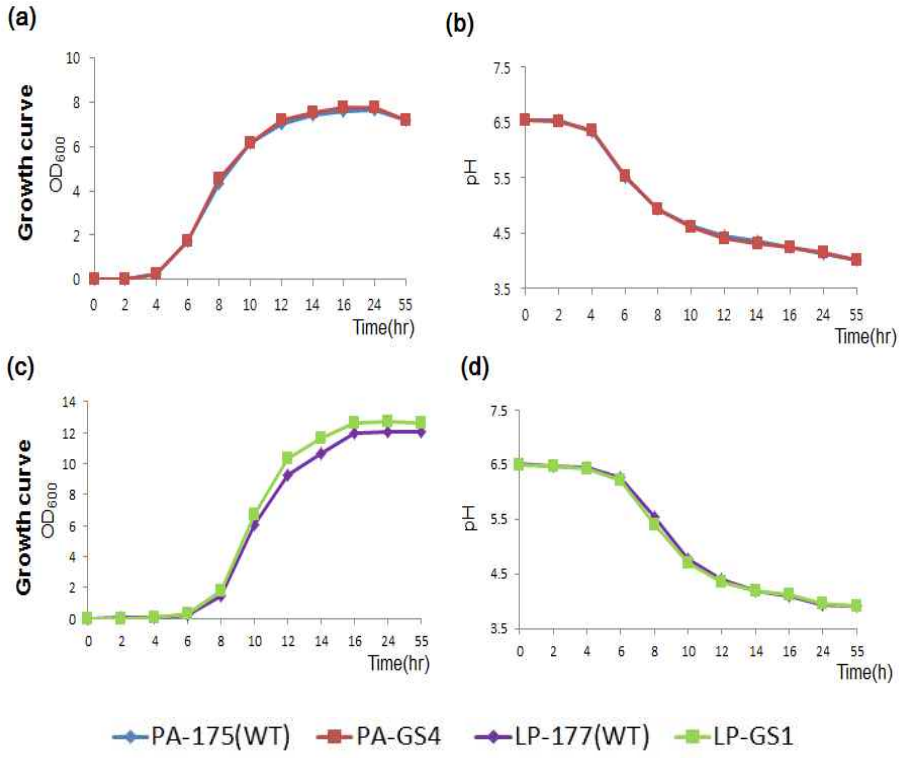


Figure 13. Physiological characterization of of Lactic acid bacteria. (a) Growth curve of WT and GS *P.acidilactici*. (b) pH curve of WT and GS *P.acidilactici*. (c) Growth curve of WT and GS *L.plantarum*. (d) pH curve of WT and GS *L.plantarum*.

3) Acid tolerance test

LP-GS1 and PA-GS4 showed better acid tolerance than LP177 and PA175 at pH 2.0 and 3.0 condition (Figure 14).

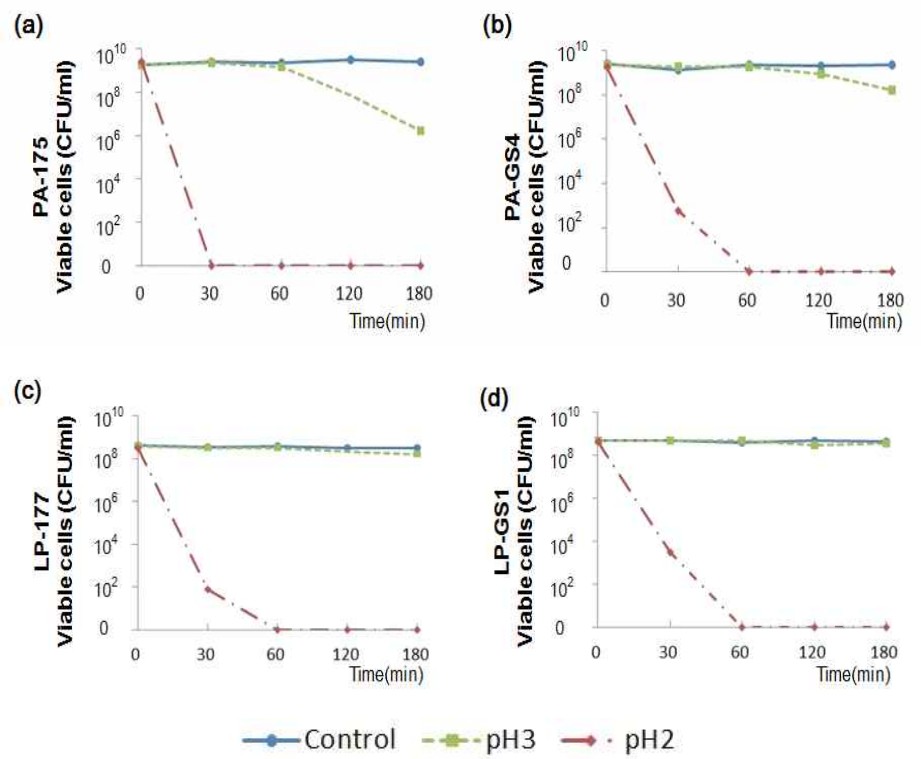


Figure 14. Acid tolerance of LAB strains. (a) Viable cell counts of PA175. (b) Viable cell counts of PA-GS4. (c) Viable cell counts of LP177. (d) Viable cell counts of LP-GS1.

4) Bile resistance test

PA175 and PA-GS4 had no difference in bile resistance. However, LP-GS1 improved bile resistance more than 10 times

compared to LP177 at 4hr incubation (Figure 15).

When lactic acid bacteria were orally administered, low pH of stomach and bile acid of duodenum are major obstacle for reach to the intestine. Thus, enhanced acid tolerance and bile resistance gave better survivability at stomach and duodenum which consequently more LAB reach to the intestine.

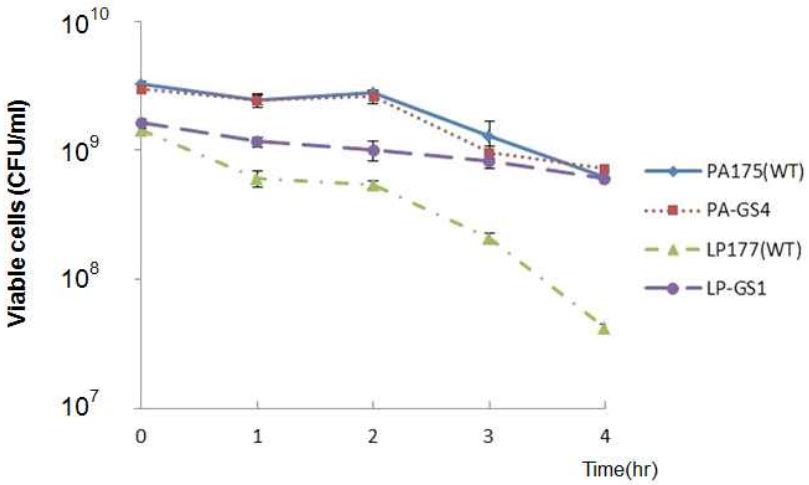
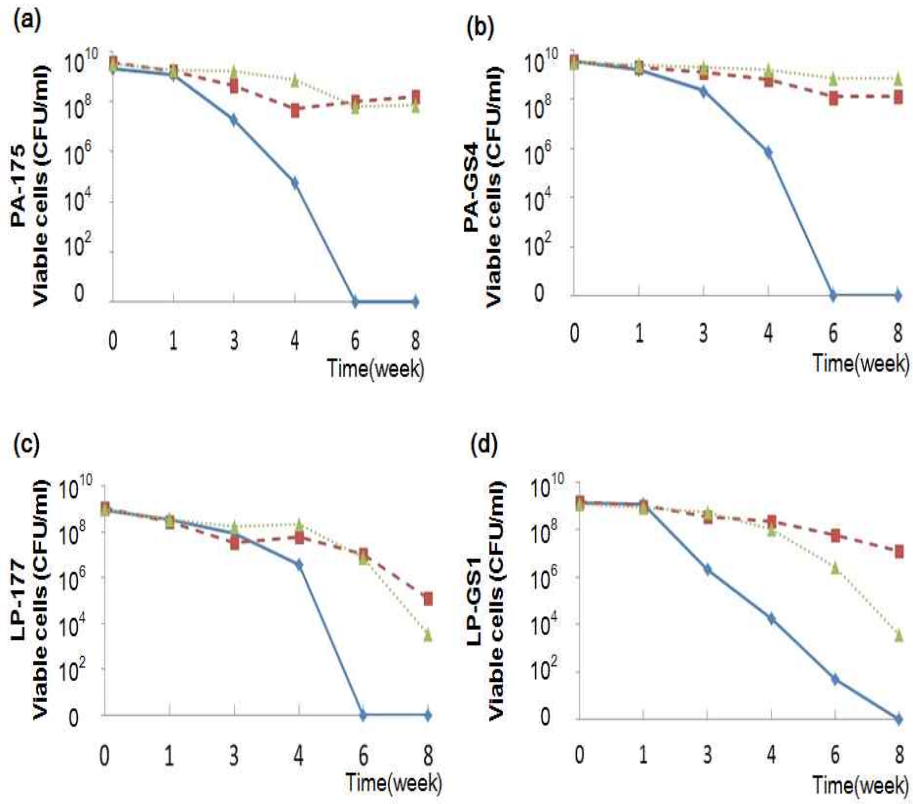


Figure 15. Bile resistance of LAB strains (0.3% oxgall). PA175, PA-GS4 showed similar bile resistance. However LP-GS1 showed increasing bile resistance than LP177.

5) Survivability in low temperature

During the experimental period, probiotics mixture (GPM and WPM) was stored at 4℃ for 2 weeks until new probiotics mixture supplied. Therefore survivability of each strain was evaluated to confirm whether constant amount of probiotics could be supplied or not.

There are no differences between PA175 and PA-GS4 in viable cells. LP177 and LP-GS1 also show similar tendency of survivability at 4℃ of storage. Also, I found that PBS and New media (MRS broth) show better survivability than spent media. This results indicate that low pH is critical to LAB survivability. PBS group shows almost same cell number during 2 weeks of storage period. Finally both SB and T4(endospore) show similar cell number during 8 weeks.



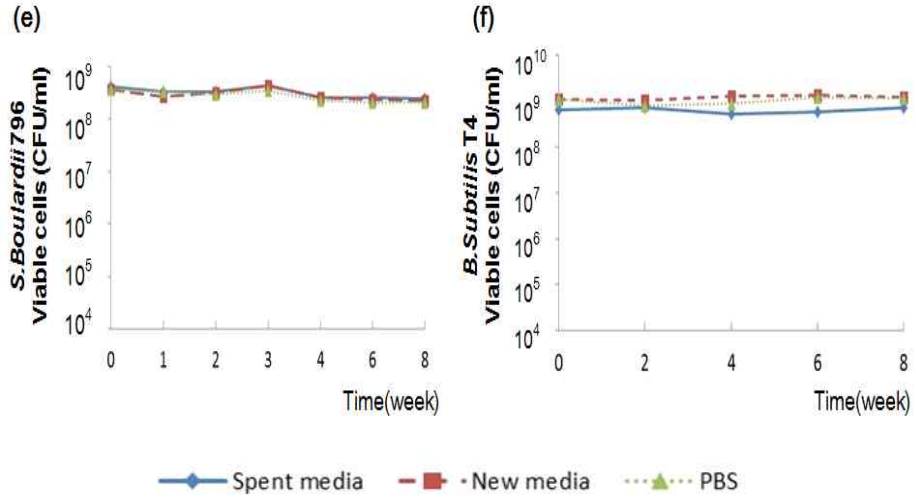


Figure 16. Survival rate during cold storage. Viable cell counts of (a) PA175, (b) PA-GS4, (c) LP177, (d) LP-GS1, (e) *S.boulardi*, and (f) *B.subtilis* T4 (endospore);

2. Probiotics preparation

Probiotics were cultured and prepared in PBS solution. Each strain produced at least more than 10^{11}CFU/ml. One day before supply to farm, probiotics were mixed in 50ml corning tube which contains 10^{10} cell per strain. Viable cells were present as described (Table 5). These results confirmed that both WPM and GPM contain sufficient microbes as probiotics. Together, cell morphology was measured by gram staining to confirm contamination during cell culture (Figure 17).

Table 5. Viable cell counting in probiotics products (CFU/head)

Probiotics		1st production	2nd production	3rd production	4th production
WPM	PA-175	2.64×10^9	3.01×10^9	5.76×10^9	7.8×10^9
	LP-177	1.84×10^9	1.47×10^9	5.46×10^9	5.95×10^9
	SB	2.16×10^9	1.53×10^9	2.28×10^9	2.88×10^9
	T4	1.6×10^9	1.05×10^9	1.85×10^9	1.8×10^9
GPM	PA-GS4	2.48×109	3.33×10^9	6.09×10^9	6.65×10^9
	LP-GS1	1.28×10^9	1.7×10^9	4.97×10^9	6.47×10^9
	SB	2.16×10^9	1.53×10^9	2.28×10^9	2.88×10^9
	T4	1.6×10^9	1.05×10^9	1.85×10^9	1.8×10^9

PA : *Pediococcusacidilactici*; LP : *Lactobacillus plantarum*;
SB : *Saccharomyces boulardii* 796; T4 : *Bacillus subtilis* T4;

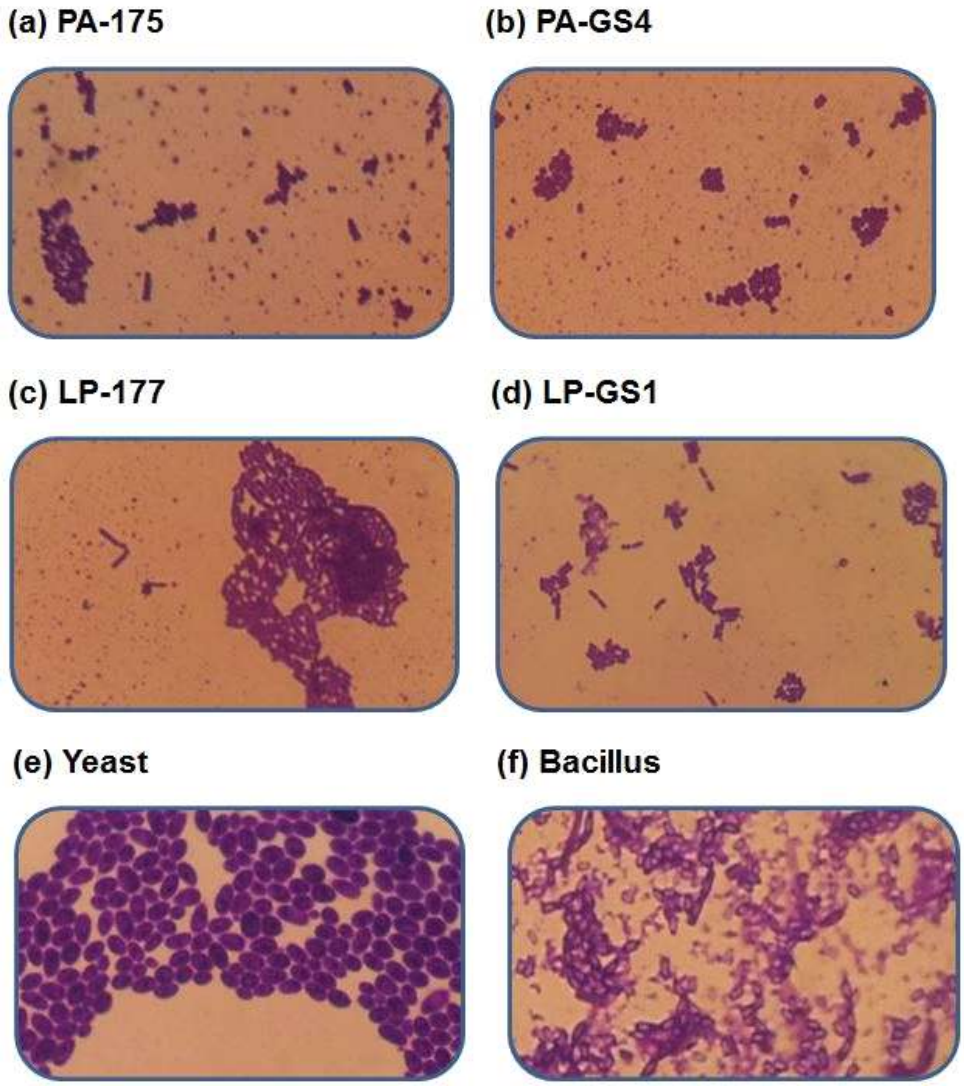


Figure 17. Photomicrograph of each strain. There is no contamination during cell culture. (a) *Pediococcus acidilactici* 175, (b) *Pediococcus acidilactici*-GS4, (c) *Lactobacillus plantarum* 177, (d) *Lactobacillus plantarum*-GS1, (e) *Saccharomyces blourdii* 796, (f) *Bacillus subtilis* T4

3. Growth performance in Holstein calves

To confirm the effect of multi-species probiotics on the growth performance, body weight and mortality were measured for 8 weeks.

Body weight gain of each group had no significant difference. However GPM group showed same average daily gain (ADG) as PC group (Table 6). Furthermore PC and GPM group showed 90% survival rate whereas NC and WPM showed 50% mortality (Figure 18). All mortality was occurred before 4 week when immune system and intestinal environment were not stabilized. This results suggest that both PC and GPM help to maintain healthy state and decrease mortality in pre-weaning period.

Table 6. Comparison of average daily gain among the groups.

	NC	PC	WPM	GPM	SEM	p-value
Calves (No.)	5	9	5	9		
ADG(kg)	0.81	0.73	0.69	0.73	0.046	0.3514

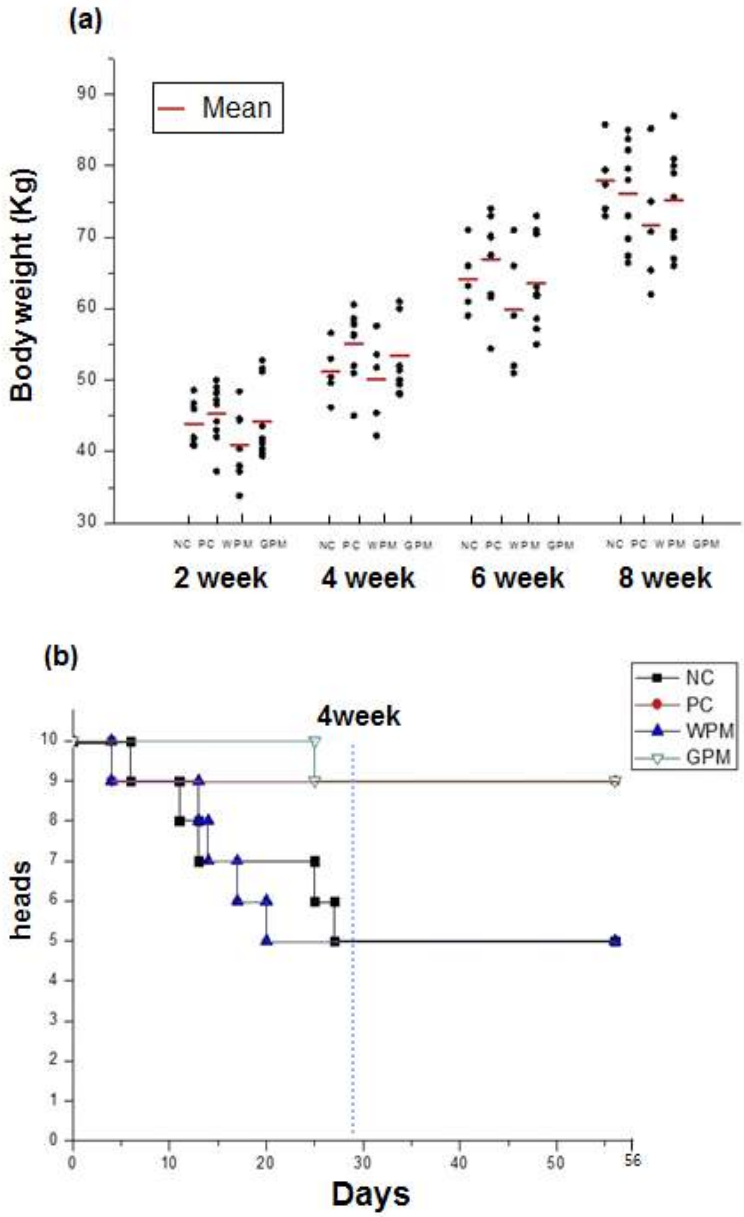


Figure 18. Growth performance in commercial environment. (a) body weight gain of each calf (dot plot), (b) comparison of mortality among the groups.

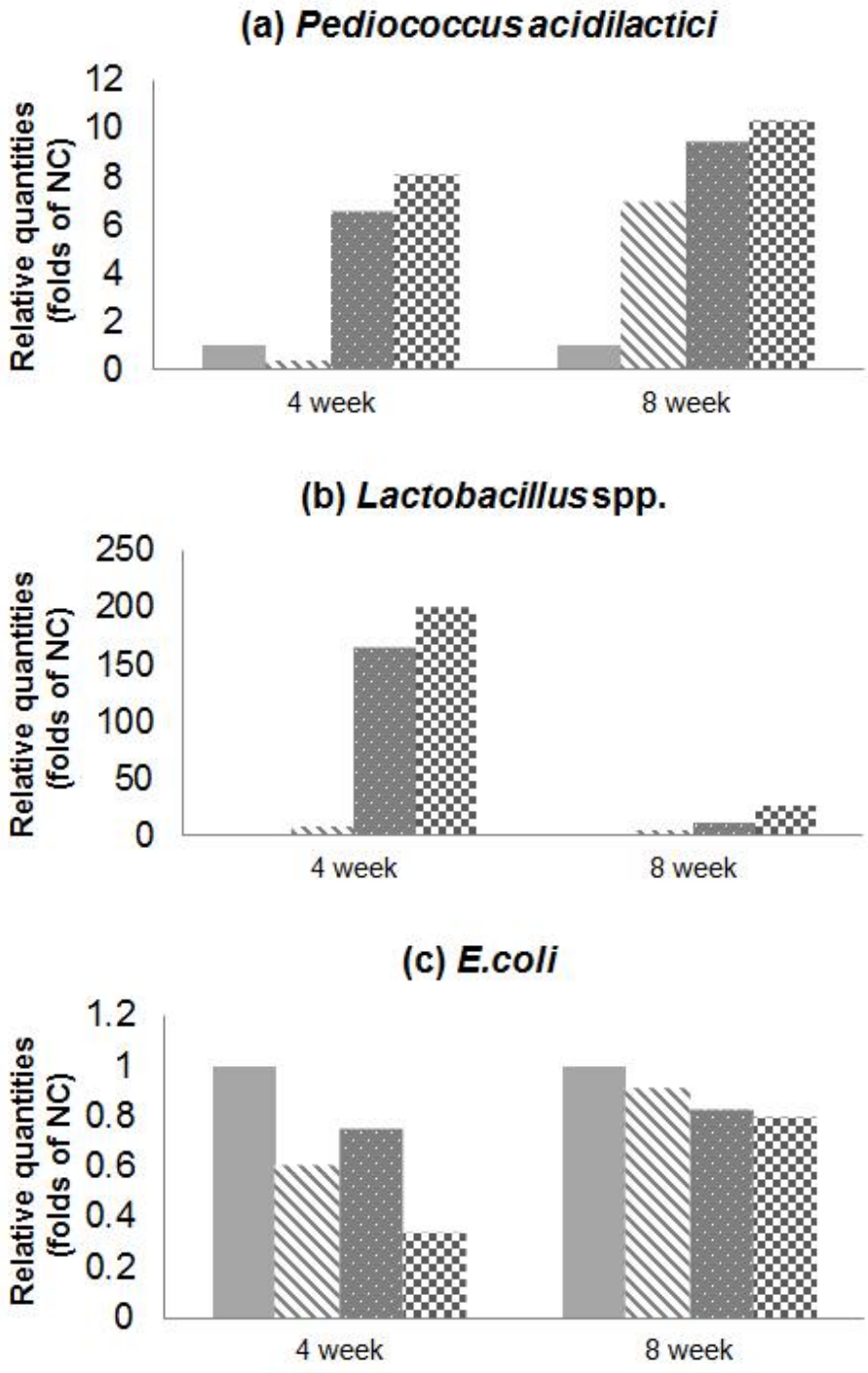
4. Intestinal microflora composition

1) Quantitative real-time polymerase chain reaction

Fecal microflora was evaluated by qRT-PCR which could check both living and death bacteria. Relative quantities to NC were also calculated (Figure 19).

E.coli and *Clostridium perfrigens* are decreased in PC, WPM and GPM group compared to NC group. *E.coli* is well known pathogenic bacteria which cause diarrhea, acute mastitis in dairy cows and food poisoning in humans (BP Bell *et* al., 1994; Josefa M. Rangel *et* al., 2005). Also *C.perfringens* is causing food poisoning and producing large amounts of enterotoxin. The result of *E.coli* show similar tendency to MCK agar assay. Especially GPM is more effective than WPM in suppression of harmful bacteria because GS LAB not only improved anti-microbial activity to *E.coli* but also enhanced both acid and bile resistance. Increasing evenness among the groups may result from rumen development at 8 week.

On the other hand, *Pediococcus acidilactici*, *Lactobacillus* spp and *Bacillus subtilis* which considerd as beneficial bacteria to host are increased in PC, WPM and GPM group compared to NC group. Alike *E.coli* qRT-PCR result, *Lactobacillus* spp improving evenness among the groups at 8 week which result from rumen development (Figure 19 b,c).


(a) Pediococcus acidilactici
Relative quantities (folds of NC)
12
10
8
6
4
2
0
4 week
8 week
(b) Lactobacillus spp.
Relative quantities (folds of NC)
250
200
150
100
50
0
4 week
8 week
(c) E.coli
Relative quantities (folds of NC)
1.2
1
0.8
0.6
0.4
0.2
0
4 week
8 week

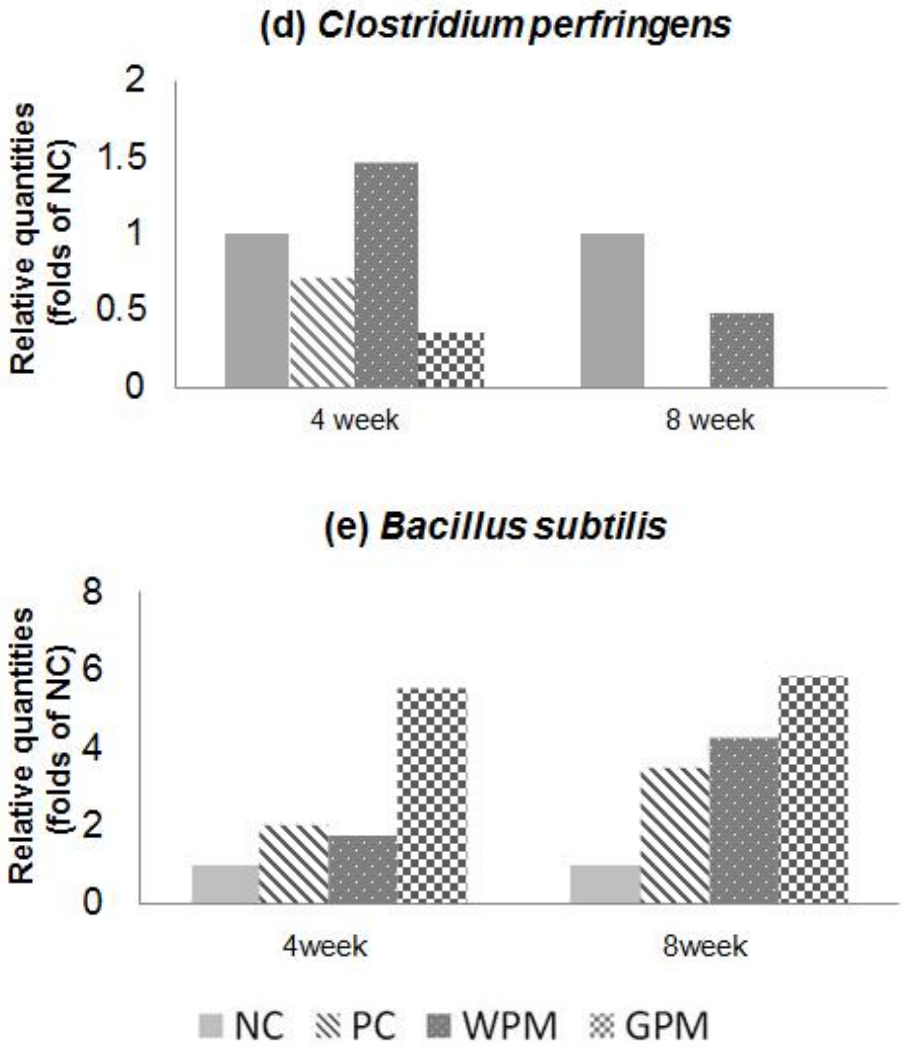


Figure 19. Analysis of fecal microflora composition by using qRT-PCR. Quantities of microflora were represented as folds of NC. (a) *Pediococcus acidilactici*. (b) *Lactobacillus* spp. (c) *E.coli* (d) *Clostridium perfringens*. (e) *Bacillus subtilis*.

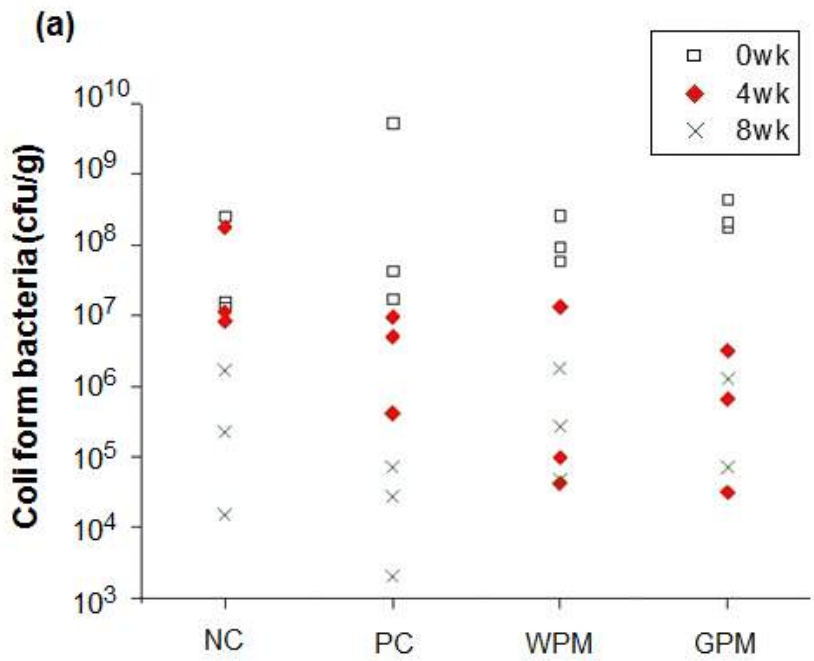
NC : no treatment, PC : neomycin treatment,

WPM : PA175+SB+T4, GPM : PA-GS4+LP-GS1+SB+T4

2) MCK agar assay

MacConkey agar is selective media for coli form bacteria known as a potential pathogen such as *E.coli* and *Salmonella*.

Pathogenicity is mainly represented as living cell. qRT-PCR method is based on DNA suggesting that both live- and killed-bacteria can be detected. Therefore viable coliform bacteria was evaluated by MCK agar assay. From 0 to 4 week, the most severe mortality period, NC did not alter coli form bacteria number. However coli form bacteria were reduced in PC, WPM and GPM at the same period. Especially only GPM has significance. This result indicate that GPM effectively inhibits pathogenic bacteria, thereby lowering mortality in early stage of development.



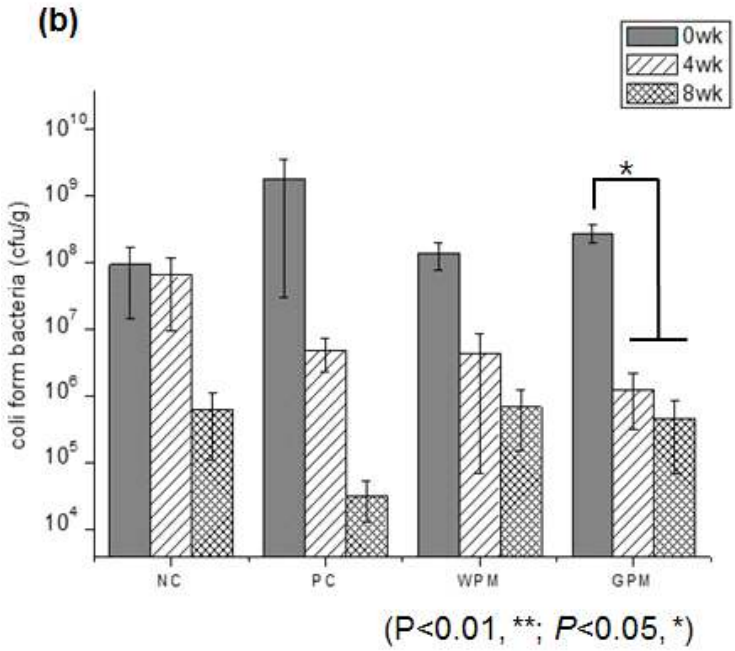


Figure 20. Viable fecal coliform bacteria counting. (a) viable coliform bacteria (dot plot), (b) bar graph.

5. Blood analysis

1) Serum Immunoglobulin

Total serum IgG and IgA level were measured by ELISA. IgG increases steadily during experimental period due to acquired immunity (figure 21a). Serum IgA level has no significant difference among the groups.

Generally calf which feed colostrum has high IgG level and gets more resistance to disease in pre-weaning period. In this study, over 6 mg/ml of IgG calves may feed colostrum (Figure 22). Because IgG level of colostrum fed calf is decreased as time

goes by (Klaus, 1969). Interestingly, the low mortality of GPM group is not associated with colostrum effect since 3 calves of PC group and 4 calves of GPM group were fed colostrum which is almost same ratio (Figure 22b, d). In this respect low mortality of GPM group has a colostrum effect (Figure 18b).

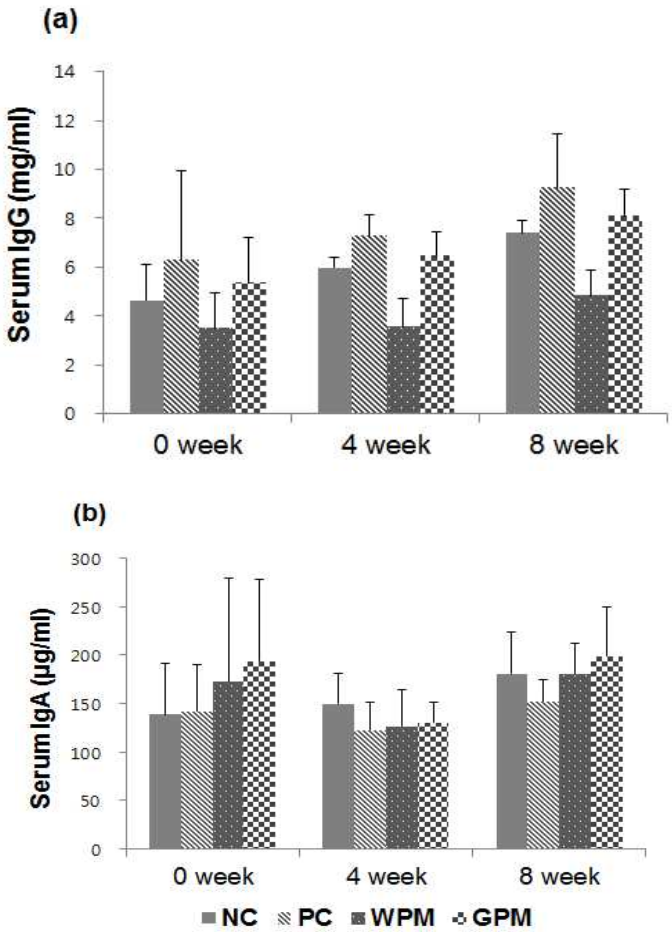
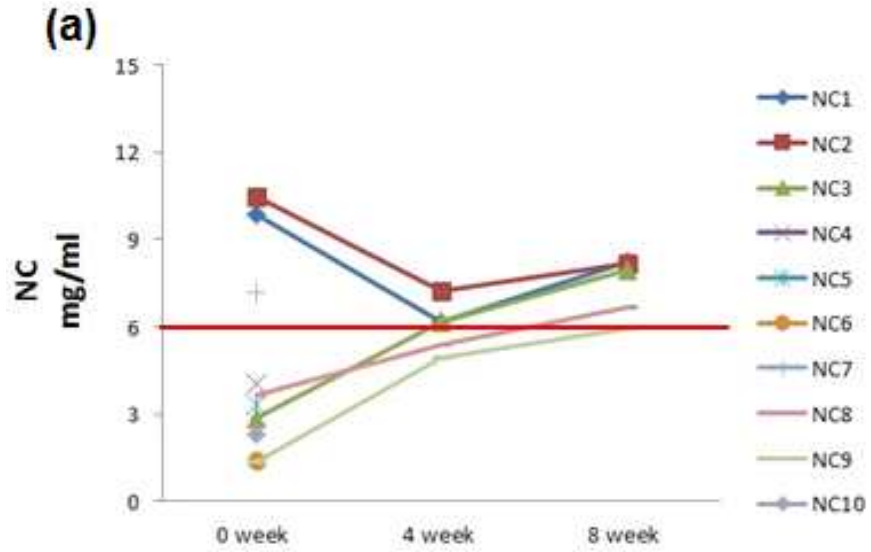
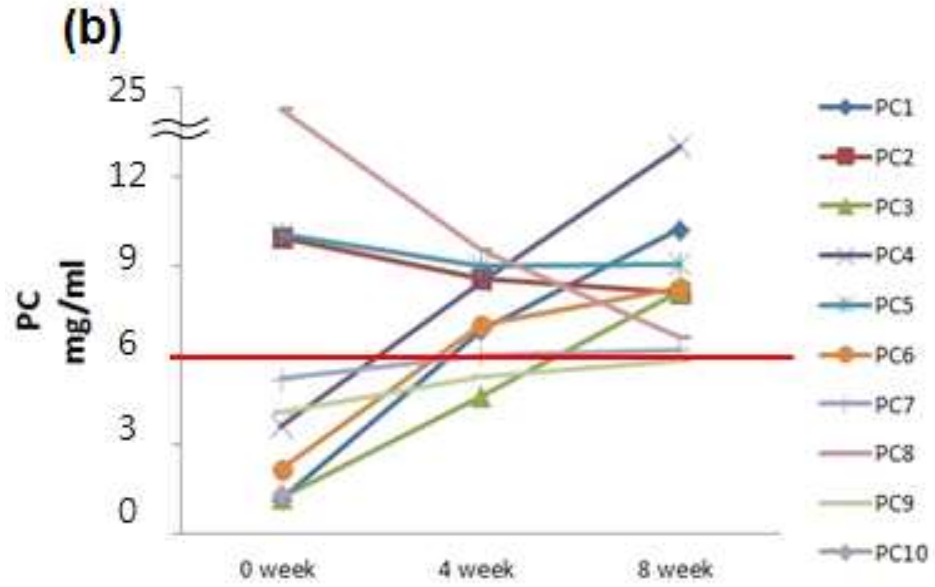


Figure 21. Effect of different probiotics on serum immunoglobulin. (a) Serum IgG (mg/ml), (b) Serum IgA (μg/ml)


(a)
NC
mg/ml
15
12
9
6
3
0
0 week
4 week
8 week
NC1
NC2
NC3
NC4
NC5
NC6
NC7
NC8
NC9
NC10


(b)
PC
mg/ml
25
12
9
6
3
0
0 week
4 week
8 week
PC1
PC2
PC3
PC4
PC5
PC6
PC7
PC8
PC9
PC10

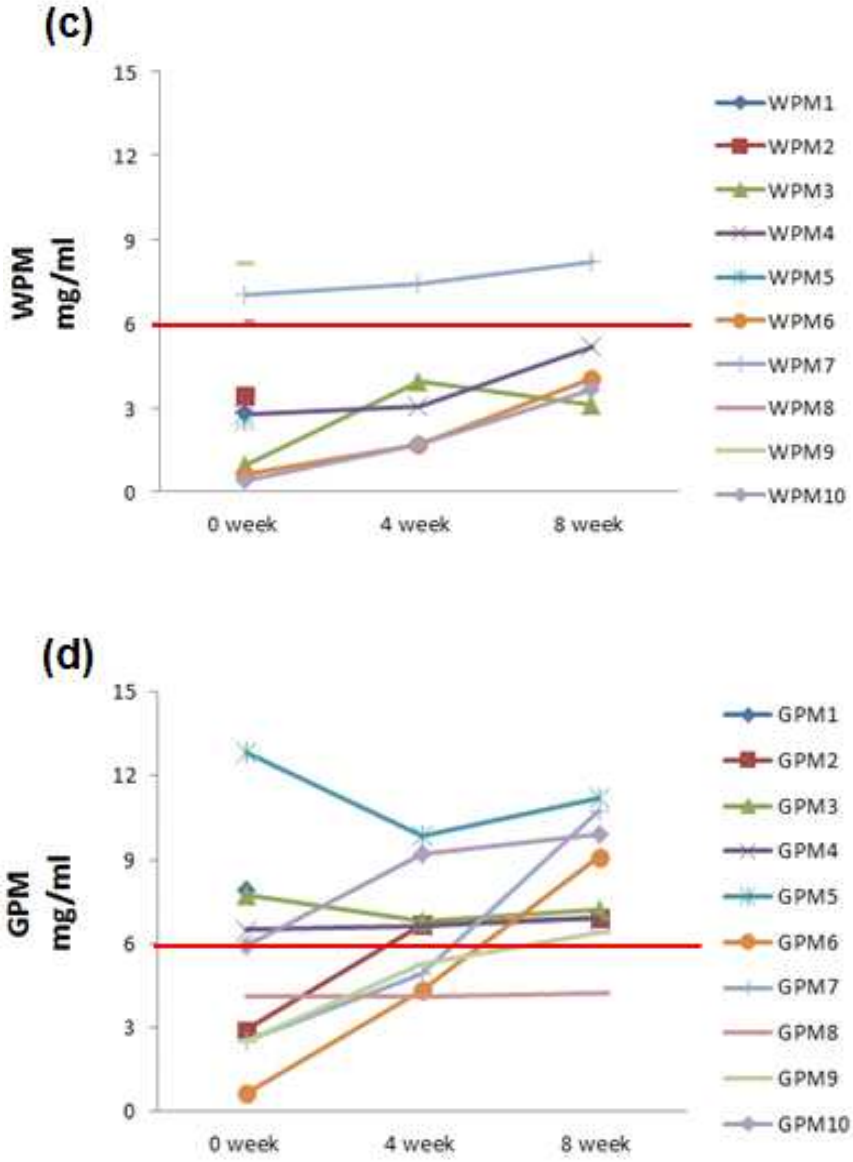
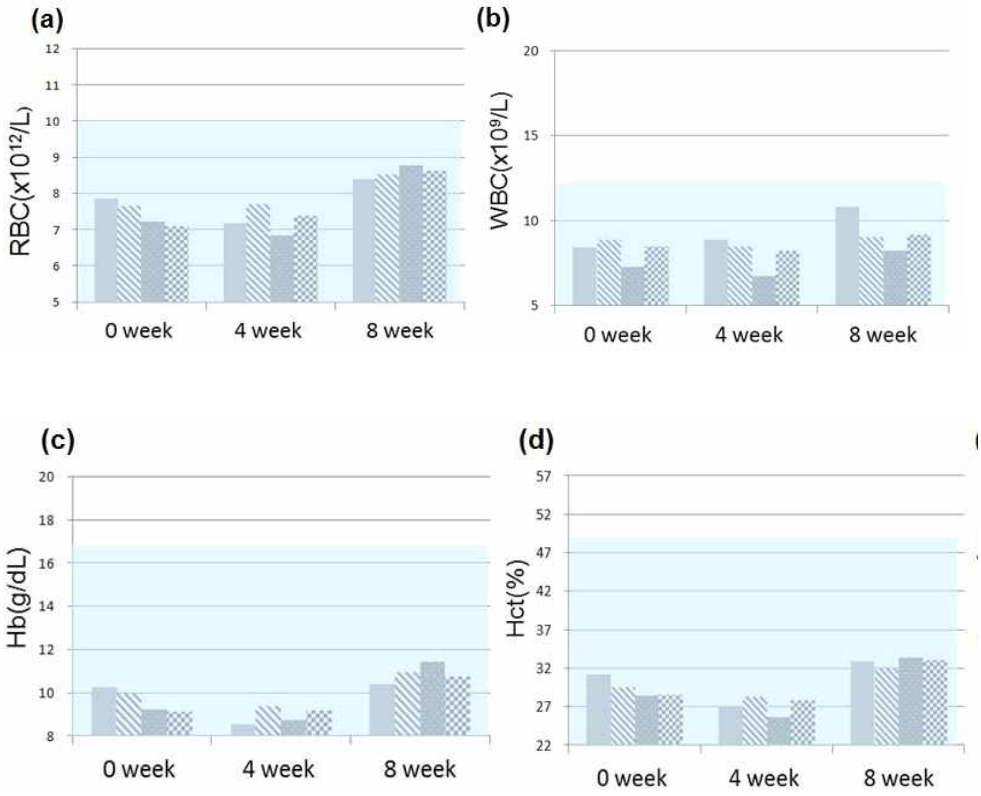


Figure 22. Total serum IgG level of each calf during experiment period. (a) Negative control, (b) Positive control, (c) Wild type LAB added probiotics mixture, (d) Genome shuffled LAB added probiotics mixture

2) Blood cell counting

Blood hematological profile including red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), hematocrit (Hct) and platelet (Plt) were no significant difference among the groups (Table 7), showing that all group remains normal range (Figure 23). This result indicates that multi-species probiotics are safe to holstein calf.



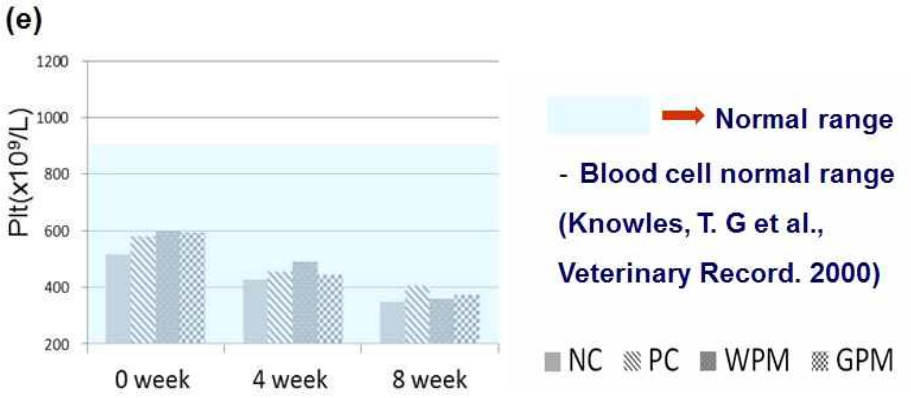


Figure 23. Effect of different probiotics on blood parameter.
(a) Red blood cell. (b) white blood cell. (c) hemoglobin.
(d) hematocrit. (e) platelet

Table 7. Effect of different probiotics on blood parameter.

Experiment	Trt	Experiment , week			SEM	P-value		
		0	4	8		Trt	Time	Trt x T
RBC, M/ul	NC	8.41	7.54	11.55	0.75	0.13	0.0315	0.65
	PC	8.88	8.48	9.00				
	WPM	7.27	6.72	8.20				
	GPM	8.47	8.19	9.13				
WBC, K/ul	NC	7.86	6.15	8.45	0.42	0.66	0.0002	0.36
	PC	7.66	7.71	8.51				
	WPM	7.22	6.84	8.77				
	GPM	7.08	7.36	8.62				
Hb, g/dL	NC	10.23	7.62	10.55	0.55	0.67	0.0001	0.35
	PC	9.95	9.37	10.93				
	WPM	9.19	8.74	11.42				
	GPM	9.10	9.16	10.71				
Hct, %	NC	31.23	38.25	33.15	2.60	0.23	0.2608	0.61
	PC	29.52	28.28	31.88				
	WPM	28.38	25.66	33.36				
	GPM	28.59	27.78	33.02				
Plt, K/ul	NC	514.60	357.20	346.75	38.86	0.19	<.0001	0.96
	PC	577.90	456.22	403.78				
	WPM	596.30	490.40	360.80				
	GPM	594.50	446.00	374.89				

6. Conclusion

The aim of this study is to confirm the effect of multi-species probiotics on neonatal holstein calves. Two lactic acid bacteria strains were developed by genome shuffling method as previously described (Choi, 2011; Seo, 2012). These strains show same growth and pH curve as wild type LAB, suggesting that genome shuffled *pediococcus acidilactici* and *lactobacillus subtilis* maintain original characteristic of lactic acid bacteria. Also, acid tolerance and bile resistance are tested in this study. LP-GS1 and PA-GS4 are improved acid tolerance and especially LP-GS1 increased bile resistance. Enhancing these resistance lead to more GS-LAB reach to the intestine and effectively inhibit pathogenic bacteria than wild type LAB.

It is noteworthy that PC (antibiotics treat) and GPM (Genome shuffled LAB treat) show 90% survival rate while NC (no treat) and WPM (Wild type LAB treat) have only 50% mortality. Although body weight gain has no significant difference among the groups, GPM show same ADG with PC. In this connection, Mac conkey agar assay was performed to detect coli form bacteria which is potential pathogen. Within 0-4 week, the most severe mortality period, PC, WPM and GPM were shown to reduce the coli form bacteria, whereas NC has no change. In this regard, GPM seems to prevent calf diarrhea and reduce mortality by inhibition of potentially pathogenic bacteria.

Gut microflora was also changed in GPM group. The result of qRT-PCR shows similar tendency with MCK agar assay in *E.coli*. Furthermore GPM is comparable to PC in terms of lowering pathogenic bacteria and enhancing beneficial bacteria in

feces.

Based on *in vitro* and *in vivo* examination on GPM with improved antimicrobial activity to specific pathogens, UV mutation and genome shuffling approaches can be applied to develop novel type of probiotics for a wide variety of animal diseases.

It is expected that this multi-species probiotics can be used as valuable alternative to antibiotics in the environment-friendly livestock product and antibiotics free farming.

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VI. Summary in Korean

송아지 설사병은 송아지 사양에 있어 초기 폐사율에 가장 큰 영향을 미치는 질병으로 알려져 있다. 이는 아직 면역체계가 발달되지 않은 생후 30일 이내의 송아지가 외부로부터 바이러스나 세균의 침입을 받았을 때 발병하게 되는데, 특히 병원성 대장균인 *E.coli* K99가 주 원인균으로 밝혀져 있다.

기존 사양에 있어서 송아지 설사병을 예방하기 위해 항생제를 사용하였으나 축산물 내 잔류 문제와 내성균의 발생으로 인해 사료내 항생제 사용이 전면 금지되었고 그 대체제로서 생균제가 대두되었다. 선행 연구에서 UV mutation과 genome shuffling 기법을 이용하여 병원성 대장균에 대해 항균활성이 증가된 유산균 두 종을 선정하였고, 본 연구에서는 이를 포함하는 복합 미생물 생균제를 개발하여 송아지에서 그 효과를 검정하였다.

실험은 평균 10일령 홀스타인 수송아지 40두를 대상으로 8주간 진행되었다. 시험구는 미처리구, 항생제 처리구, Wild-type 유산균 혼합구, GS 유산균 혼합구로 구성되었고 모든 생균제 처리구는 *S.boulardii*와 *B.subtilis* T4가 포함되었다.

사양시험 결과 미처리구와 Wild-type 유산균 혼합구는 50%가 폐사하였음에 반해 GS 유산균 혼합구에서는 항생제구와 동일한 90%의 생존율을 보여주었다. 증체량에 있어서 유의차는 없었으나 GS 유산균 혼합구는 항생제구와 동일한 증체율을 보여줌으로써 항생제 대체제로서의 가능성을 보여주었다.

또한 혈액분석 결과 일반 혈액성상은 모든 처리구에서 정상을 나타내며 복합 미생물 생균제의 안전성에 문제가 없음을 확인하였다.

초유를 급여 받은 송아지는 초기 IgG 수치가 높게 유지되며 외부로부터 침입하는 병원균에 대해 초유를 급여받지 못한 송아지보다 강한 저항성을 가지게 된다. 혈액 내 IgG 조사 결과 모든 처리구에서 비슷한 숫자의 송아지가 초유를 급여 받은 것으로 나타났고 따

라서 GS 유산균 혼합구의 성장성적은 초유에 의한 효과가 아님을 알 수 있다.

잠재적 병원균인 coliform 생균수는 미처리구 대비 모든 처리구에서 낮게 나타났다. 특히 가장 폐사율이 심한 0-4주의 기간 동안 미처리구는 변화가 없는 반면, 생균제 급여구와 항생제 처리구는 모두 감소하는 효과를 보여주었다. 그리고 8주차에는 반추위가 발달되고 장내 균총이 안정됨에 따라 모든 처리구에서 낮은 수준의 coliform 생균수를 보여주었다.

잠재적인 병원균인 *E.coli*와 *Clostridium perfringens*은 미처리구에 비해 생균제 처리구에서 낮게 나타났고 유익균으로 알려진 *Pediococcus acidilactici*, *Lactobacillus* spp, *Bacillus subtilis*는 생균제 처리구에서 미처리구보다 높게 나타났다. 이러한 결과는 복합 미생물 생균제가 장내 미생물 균총을 개선하는 효과가 있다는 것을 시사한다.

따라서 본 복합 미생물 생균제는 송아지에서 초기 장내 미생물 균총이 형성되지 않았을 때 효과적으로 병원성 미생물을 방어하여 폐사율을 감소시키고 건강한 사양을 가능하게 함으로서 항생제 대체제로서의 가능성을 보여주었고 이를 통해 무항생제, 친환경 축산물을 생산할 수 있을 것으로 기대된다.

주요어 : 복합 미생물 생균제, 송아지 설사병, 항생제, 장내 미생물, 유산균

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먼저 새로운 학문의 장을 열어주시고 소중한 배움의 기회를 주신 지도교수이신 최윤재 교수님께 큰 감사의 말씀을 드립니다. 교수님께서 보여주신 학문에 대한 열정과 엄격한 가르침, 그리고 제자들에 대한 애정은 제 삶에 큰 귀감이 되었습니다. 사회에 나가서도 항상 교수님의 가르침과 삶의 자세를 본받아 부끄럽지 않은 제자가 되도록 노력하겠습니다. 아낌없는 관심과 격려에 진심으로 감사드립니다. 항상 많은 조언과 도움을 주신 조종수 교수님께도 감사의 말씀을 드립니다. 또한 바쁜 와중에도 학위 심사를 맡아주신 백명기 교수님께 감사드립니다. 제가 석사과정에 입학하는데 조언을 주시고 학위과정동안에도 가장 많은 도움을 주신 선배님, 강상기 교수님께도 깊이 감사드립니다. 뺄줌할땐 셔플댄스를 밟으라던 중저음의 목소리가 그리울 것 같습니다. 실험설계와 결과분석을 할 때 많은 도움을 주신 복진덕 박사님께도 감사드립니다. 논문수정에 많은 도움을 주신 문현석 박사님 그리고 항상 도움이 되는 말씀을 해주신 이윤석 박사님께 감사의 말씀을 드립니다.

2년 동안 동고동락하며 큰 힘이 되어준 실험실 식구들에게도 고마움을 전합니다. 실험실의 맏형인 대천이형, 제가 신입생일 때부터 졸업할 때까지 실험실 생활과 연구하는데 큰 힘이 되어주셔서 고맙습니다. 형의 위로가 많은 도움이 됐어요. 언제나 기분 좋은 미소를 보여주며 친절하게 가르쳐주신 장도형에게도 감사드립니다. 형 덕분에 데이터 정리하는데 많은 도움이 되었어요. 시크하면서 친절한 혜선이, 너의 직설적인 멘트 덕분에 항상 즐거웠어. 앞으로도 실험실

의 많은 어록을 만들어 주길 바라. 만물박사 창윤이형, 논문부터 취업까지 형의 손을 안거친게 없네요. 요태까지 그래왔고 아패로도 계속 형의 도움이 몹시 기대가 됩니다. 신뢰와 열정의 밤박사 태은이, 배울 점도 많고 실험하는데 언제나 도와줘서 고마워. 옆자리에서 너의 넘치는 백치미를 보느라 지루할 틈이 없었던 것 같아. 동갑친구 준영이, 처음 입학했을 땐 엄청 강한 인상이었는데 실험실 생활을 같이하다보니 정말 정이 많은 것 같아. 내 몫까지 아자스방(이라고 쓰고 밤박사의 장난감이라고 읽는다)을 부탁해. 내추럴본 귀요미 수나, 언제나 넘치는 리액션과 표정은 보는 사람을 즐겁게 해. 실장을 맡아 고생이 많지만 웃음 잃지 말고 즐겁게 지내길 바라. 나와 같이 모텔에서 하룻밤을 보낸 건구, 학위 진행하는데 정말 도움 많이 받았어. 운전도 잘하고 귀엽고 듬직하고 누군진 몰라도 여자친구는 좋겠네. 인텔리한 원석이, 동창의 동창이라는 특이한 인연 계속 이어갔으면 좋겠어. 목표한바 꼭 성취하길 바랄게.

사랑스러운 동기들! LQ 인선이, 처음 입학하고 정말 많이 의지되고 힘이 되었어. 매사에 열정적인 모습이 정말 존경스러웠고 앞으로도 매력 터지는 손짓과 눈빛 기대할게. 긍정의 아이콘 정인이, 같이 취업준비하면서 정말 도움을 많이 받았네. 네가 구워온 쿠키는 잊지 못할 거야. 회사에서도 너의 긍정에너지를 팍팍 전파해줘. 듬직한 동생 동석이, 나보다 궂은일도 많이 하고 정말 고생 많았어. 동기로서 많이 도와주지 못해 미안하네. 넉살좋은 웃음과 털털한 성격으로 무슨 일을 하든 성공할거라 생각해.

실험실의 살림꾼 호빈이, 나보다 실험목장에 더 많이 가서 1저자를 넘겨줘야할 것 같아. 힘들겠지만 남은 학위 무사히 마치길 기도할게. 웃음이 매력적인 윤정이, 너의 선명한 웃음소리는 듣는 사람도 기분 좋게 만드는 마력이 있어. 학위주제 파이팅 하고 호빈이보다 먼저 졸업하는 걸 위안으로 삼자(호빈아 미안).

그리고 학위주제 준비하는 도운이, 힘들더라도 선배들 조언 흘려듣지 말고 항상 겸손한 자세로 많이 배우길 바란다. 이번에 들어온 신

입생 기준, 휘수, 나영, 소연이, 몇 번 얘기도 못해보고 가는 게 아쉽네. 훌륭한 교수님과 박사님, 선배들에게 많이 배우고 무슨 일이든 나중에 분명 도움이 되니까 어렵고 힘들더라도 잘 이겨내길 바라.

대학시절부터 인생의 멘토이자 아낌없는 격려를 준 영준이게도 이 자리를 빌려 고마움을 표시하고 싶습니다. 자치위원시절을 비롯하여 대학원에 들어와서도 너에게 항상 도움만 받는구나. 비록 말은 안했지만 언제나 감사하게 생각하고 있고 도움을 구할 때마다 흔쾌히 받아줘서 정말 고맙다. 곧 있을 결혼 진심으로 축하하고 행복하게 살길 바랄게. 나와 코드가 잘 맞는 정훈이, 이번에 취업 정말 축하하고 언제까지나 서로에게 힘이 되는 친구가 될게. 힘들 때 옆에서 도와줘서 고마웠고 앞으로도 잘 부탁한다.
뜻하지 않은 큰 인연 정글북 패밀리에게도 고마움을 전합니다. 지영이, 빛나, 주휘, 재곤이, 지한이 모두 소중하고 언제나 힘이 되어줘서 고마워. 고등학생 때부터 항상 가깝게 지낸 선우, 힘든 시기에 같이 있어주지 못해 미안하고 언제나 먼저 연락해 줘서 고맙다. 어서 완쾌하길 바랄게.

마지막으로 언제나 저를 믿고 지원해준 가족들에게 고마움을 전합니다. 저의 선택을 믿고 어긋나지 않도록 뒷바라지 해주신 아버지, 어머니 앞으로 더욱 효도하는 아들이 되겠습니다. 크나큰 은혜에 감사드리며 항상 건강하세요. 사랑합니다. 또한 언제나 주기만 하는 누나, 그동안 표현을 못했지만 고맙고 미안해. 매형이랑 계속해서 행복하길 바라고 앞으로 내가 더 잘할게. 그리고 못난 아들을 대신해서 부모님께 아들노릇까지 톡톡히 하고 계신 매형에게도 깊이 감사드립니다.

이 외에도 미처 이름을 부르지 못한 소중하고 고마운 분들 모두에게 감사의 인사를 드립니다. 여러분의 도움이 있었기에 지금의 제가

있을 수 있었습니다. 항상 감사하는 마음으로 다른 사람에게 도움이 되는 윤성현이 되겠습니다.

2014년 1월 늦은 시각에, 관악에서.