RESEARCH REPORT

PUBLIC HEALTH/WORKER SAFETY

Title: Use of *Lactococcus lactis* as a probiotic feed additive against porcine postweaning enteric colibacillosis- **NPB #14-075**

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Industry Summary

Background:

Bacterial resistance to antibiotics is considered to be one of the most important problems currently encountered in animal and human health. According to the World Health Organization, "Our grandparents lived during an age without antibiotics; so could many of our grandchildren", if the problem is not addressed immediately. The major generator of bacterial genes encoding antibiotic resistance is the overuse of antibiotics in both human medicine and animal agriculture. In food-producing animals, the major concern is the use of antibiotics as routine feed additives to prevent diseases and promote faster animal growth. The focus of our research program is to investigate natural non-antibiotic feed additives against porcine postweaning enteric colibacillosis to limit the development of bacterial resistance to antibiotics.

Enteric colibacillosis, caused by *Escherichia coli*, is the most common bacterial disease of neonatal and postweaning piglets. Neonatal colibacillosis is controlled to a certain degree by maternal colostral immunity. However, the control of postweaning colibacillosis is problematic in the modern swine industry. To ensure profitability, piglets are weaned at ~3 weeks of age (when their immune system is still weak). Consequently, postweaning diarrhea (PWD) caused by *E. coli* is a major health problem associated with high morbidity & mortality that is currently controlled in part by antibiotic feed additives.

Pathogenesis of postweaning diarrhea is only partially understood and multiple predisposing factors seem to be involved. However, the sudden termination of milk intake by relatively young piglets having naïve (weak) immune system has been considered to be one of the most important factors resulting in reduced exposure of piglets to immediate and long-term protection mediated by various defense components present in the sow's milk. This sudden termination of lactogenic protection results in decreased resistance to infectious diseases. Intestinal colonization by *E. coli* is the first necessary step in the pathogenesis of enteric colibacillosis. This colonization is mediated by fimbriae (bacterial hands) attaching to intestinal receptors (handles).

Our research group isolated various glycoproteins from porcine milk, which are recognized by the *E. coli* fimbria (bacterial hands) as its receptor analogue (false handle). Once *E. coli* binds to these receptor analogues (false handle), it is prevented from attaching to intestinal receptors (real handles), which is a necessary step for

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colonization and development of diarrhea. We demonstrated that these receptor analogues (false handle) are carbohydrate portions of milk proteins; identification and subsequent production of these carbohydrates (sugars) could potentially be used as non-antibiotic feed additives against colibacillosis. During our investigation of the identity of these complex carbohydrates, we discovered that certain strains of *Lactococcus lactis* consistently and reproducibly inhibited adherence of *E. coli* to cultured porcine enterocytes *in vitro.* Based on this discovery, we hypothesized for this project that *L. lactis* added to post-weaning diet as a probiotic has potential to prevent intestinal colonization by *E. coli*.

To test this hypothesis, we first had to determine whether it is safe to feed piglets high doses of live *L. lactis* bacteria. Accordingly, three groups of weaned piglets were fed non-medicated starter diet (control group), and starter died sprinkled with low dose (10^{10}cfu/kg) and high dose (10^{11}cfu/kg) of live probiotic *L. lactis* bacteria. No adverse effects (e.g. diarrhea, depression, weight loss) were noticed in any of the experimental groups and both low dose and high dose were colonized by *L. lactis* bacteria 1-2 days after initiation of probiotic diet.

The second major objective was to determine whether exposure of piglets to *L. lactis* may reduce colonization by enterotoxigenic *E. coli*. In order to perform this experiment, we had to make sure before each experimental trial that all the experimental piglets possess receptors for F4 positive *E. coli* (this was done by PCR).

First we performed a pilot study using 6 piglets (3/group) to determine if our infection/colonization model works. Piglets were weaned at day 21 and fed non-medicated starter diet (control) and non-medicated starter diet with probiotic bacteria (10^{10} cfu/kg) (probiotic group). After 3 days, both groups were challenge with F4 positive *E. coli* ($2x10^9$ cfu) (administered directly into the stomach). All piglets were colonized by *E. coli* bacteria and probiotic group seemed to clear the infection faster than control group (within 4 days). However, none of the animals developed diarrhea. Accordingly, for the main experiment we doubled the infectious dose of *E. coli*.

Main experiment was performed on 16 piglets (8/group) weaned at 21 days. Control group was fed a non-medicated starter diet and probiotic group the same diet sprinkled with live *L. lactis* bacteria (10^{10}/kg). At day three after initiation of probiotic diet, both groups were challenged with the double dose of F4 positive *E. coli*. Piglets were observed three times per day and fecal samples were collected daily until euthanasia at day 7 post-infection. Both groups were successfully infected/colonized by *E. coli* but none of the piglets developed diarrhea or had any gastrointestinal pathology. To determine if probiotic *L. lactis* interfered with the extent of colonization of intestines by *E. coli*, both bacteria were quantified in the feces of individual piglets and compared statistically between groups. Unfortunately, we did not see any differences between two experimental groups, which is contrary to our previous results obtained *in vitro* and to the results of the pilot study.

Conclusion and potential future study: Under experimental conditions implemented during the main experiment, we could not demonstrate any protective effects of probiotic *L. lactis* bacteria against colonization by *E. coli*. Based on this findings, it is not recommended to use of *L. lactis* as probiotic for prevention of porcine post-weaning diarrhea at this stage. At the same time, feeding live *L. lactis* bacteria did not seem to have any negative effects on weaned piglets and based on limited data generated by our pilot study, *L. lactis* seemed to interfered with colonization of E. coli in vivo. Accordingly, we are considering to test the effects of various doses of *L. lactis* and *E. coli* on colonization/infection in the near future.

Keywords
Postweaning colibacillosis; Probiotics; *Lactococcus lactis*; F4 fimbriae; *E. coli*.

Abstract

Enteric colibacillosis, caused by Escherichia coli, is the most common bacterial disease of neonatal and post-weaning piglets. Neonatal colibacillosis is controlled to a certain degree by maternal colostral immunity. However, the control of postweaning colibacillosis is problematic in the modern swine industry. To ensure

profitability, piglets are weaned at ~3 weeks of age (when their immune system is still weak). Consequently, postweaning diarrhea (PWD) caused by E. coli is a major health problem associated with high morbidity & mortality that is currently controlled in part by antibiotic feed additives. We previously demonstrated that *L. lactis* prevents attachment of F4 positive *E.coli* to enterocytes *in vitro*. Accordingly, objective of this project was to determine if *Lactococcus lactis* used as a probiotic feed additive can prevent E. coli infection (PWD) in weaned piglets.

To determine the ability of *Lactococcus lactis* to inhibit adherence of pathogenic *E. coli* and prevent diarrhea *in vivo*, we fist assessed the ability of ingested *Lactococcus lactis* to survive and colonize gastrointestinal tract of weaned piglets. The generated data demonstrate that ingested feed containing 10^{10} or 10^{11} CFU/kg of freeze-dried *Lactococcus lactis* is sufficient for effective intestinal colonization of weaned piglets. *Lactococcus lactis* was successfully isolated from fecal material and its identity was confirmed by bacterial culture, Western blot and PCR.

Experimental infection of recently weaned piglets demonstrated that gastrointestinal tract was successfully colonized by F4 positive *E.coli*. Bacteria were confirmed by bacterial culture, immunoprecipitation and PCR. The bacteria were quantified by Real Time PCR in piglets' fecal material. However, no clinical signs of diarrhea were reproduced during experimental infection.

Comparison of colonization by F4 positive *E.coli* of piglets exposed to *L. lactis* and control group indicated that both groups were susceptible to colonization by F4 positive *E. coli*. There was no statistically significant difference in amounts of *E. coli* in porcine feces between the groups of piglets during the whole period of the trial.

Introduction

Post-weaning diarrhea (PWD) caused by enterotoxigenic *Escherichia coli* (ETEC) is an important cause of morbidity and mortality in weaned piglets. Pathogenesis of PWD is considered to be complex and multifactorial. Accordingly, the current and future efforts for prevention and control of PWD should also be multifaceted including *inter alia* various management strategies (e.g. high biosafety and hygiene management practices), selective breeding for resistance to the disease, stimulation and enhancement of the naïve and weak immune system of early weaned piglets, and various antibiotic, prebiotic and probiotic feed additives.

Biosafety and hygiene management practices focus on the exclusion of pathogens from the production site and/or on the decreased exposure of hosts to pathogens. The rest of the prevention and control measures listed above are focused on the ability of the host to resist infection when encountering potential pathogens. Most of these approaches primarily focus on the early stages of pathogenesis of PWD, namely, prevention of the attachment and colonization of *E. coli* in gastrointestinal tract. For example, selective breeding for the resistance to colibacillosis focuses on the genetic subset of pigs that do not have receptors for F4 fimbria; induced immune prophylaxis (vaccinology) focuses on the induction of immunity (e.g. specific anti-F4-fimbrial antibodies) that prevents attachment of *E. coli* to the intestinal surface. Prebiotics and probiotics focus on the prevention of attachment of *E. coli* to the intestinal surface usually by competitive inhibition and/or by stimulation of innate resistance of the host to pathogens. Prophylactic use of antibiotics (e.g. antibiotic feed additives) will most likely be banned or strictly regulated and reduced in North America, as it happened recently in the European Union. Accordingly, alternative measures for control of PWD are urgently needed.

Our research group isolated a glycoprotein from porcine milk, called lactadherin, which interacts specifically with the most prevalent *E. coli* fimbria F4ac. We demonstrated that carbohydrate(s) located on lactadherin are recognized by the *E. coli* F4ac fimbria as its receptor analogue [1]. Once *E. coli* binds to these receptor analogues, it is prevented from attaching to intestinal receptors, which is a necessary step for colonization and development of diarrhea. Subsequently, we demonstrated that several other proteins from MFGM interact

similarly with F4ac fimbria and E. coli in vitro (Predrag). At the same time, an increasing number of solved structures of microbial exopolysaccharides suggest that it is possible that lactic acid bacteria may also synthesize carbohydrates which specifically bind F4 fimbria of E. coli. In search for bacterial strains expressing exopolysaccharides capable of inhibiting E. coli attachment to enterocytes, we discovered that a spcific strain of Lactococcus lactis, consistently and reproducibly inhibited F4-dependent adherence of E. coli to cultured porcine enterocytes in vitro (Figure 1). Based on this discovery, we hypothesized that *Lactococcus lactis* added to post-weaning diet as a probiotic would prevent intestinal colonization by F4 positive E. coli

Objectives:

The purpose of this study was first to determine if we can successfully and safely colonize intestinal tract of weaned piglets with our strain of L. lactis and determine if L. lactis will interfere with colonization of F4 positive E. coli in vivo.

1. Determination of the dose of *Lactococcus lactis* in feed for successful establishment of this probiotic in the gastrointestinal tract of weaned piglets.
2. Experimental infection with F4 positive *E. coli* of weaned piglets fed non-medicated starter diet either without *L. lactis* (control group) or with *L. lactis* (treatment group).
3. Statistical analysis of differences between three treatment groups to determine the potential protective effects of *Lactococcus lactis* against infection by F4 positive *E. coli*.

Materials and Methods

1. Experimental design

1.1. Determination of the dose of *Lactococcus lactis* in feed for successful establishment of this probiotic in the gastrointestinal tract of weaned piglets

1.1.1. Pilot study (not originally proposed) was included to rule out potential animal welfare issues related to exposure of piglets to low and high dose of *Lactococcus lactis* bacteria. We used two piglets and exposed them to low (10^{10} CFU/kg of feed) and high dose (10^{11} CFU/kg of feed) of bacteria over 7 days and did not observe any adverse side effects. Following this experiment we carried experiment outlined in 1.1.2. and confirmed absence of any side effects in piglets fed with various doses of *L. lactis*.

1.1.2. To determine the dose of *L. lactis* in feed for successful establishment of this probiotic in the gastrointestinal tract of weaned piglets we used 11 piglets weaned at day 21. They were divided into three groups (high dose n=3, low dose n=3, and control =4). Each group was fed *ad libidum* non-medicated pig starter diet (CO-OP® Whole Earth* Pig Starter) containing 0, 10^{10} or 10^{11} colony forming units (CFU) of freeze-dried *Lactococcus lactis* per kilogram of feed for 7 days. On days -1, 0, 1, 2, 3, 4, 5, 6, 7, 9, and 11, the fecal samples were collected and stored at -20C for subsequent quantification.

1.2. Experimental infection with F4 positive *E. coli* of weaned piglets fed non-medicated starter diet either without *L. lactis* (control group) or with *L. lactis* (treatment group).

1.2.1. Pilot study (not originally proposed) was included to determine if experimental infection protocols with F4 positive *E. coli* will result in colonization and infection of weaned piglets. Out of twenty suckling piglets that were tested for the presence of F4 receptors by PCR, as describe below, 6 were identified as positive, which were weaned at day 21 and divided into 2 groups of 3 animals each. They were housed in the same room but each group was kept in their separate wire mesh pen. Each pen was equipped with separate feeder and drinking water source. Animals were not allowed to mingle, each group had its dedicated cleaning supplies, and entering a pen had to be preceded by

sanitization of footwear. Piglets were provided *ad libitum* water and non-medicated starter diet (CO-OP® Whole Earth Pig Starter, Federated Co-operative Limited). Starting two days after weaning, feces samples were collected from each individual piglet and frozen at -20C for further analysis. At day 3 post-weaning, one group continued to receive the non-medicated starter diet (control group) while the second group received the non-medicated starter diet supplemented with 10^{10} CFU of *L. lactis* per kilogram of feed (probiotic treatment group). After three days (day 6 post-weaning) all piglets were infected with F4 positive *E. coli* as described below.

1.2.2. For the main trial, 60 suckling piglets were tested for the presence of the gene encoding the receptor for an F4 fimbrial adhesin of *E. coli*, and 16 piglets tested positive. They were weaned at day 21, individual weight was recorded for all piglets that were randomly divided into two experimental groups (n=8 per group) which were housed in two separate rooms with free access to drinking water and non-medicated starter diet. At day 3 post-weaning, one group continued to receive the non-medicated starter diet (control group) while the second group received the non-medicated starter diet supplemented with 10^{10} CFU of *L. lactis* per kilogram of feed (probiotic treatment group). After three days (day 6 post-weaning) all piglets were infected with F4 positive *E. coli* as described below.

2. Animals

Animal care was carried out in accordance to the guidelines of the Canadian Council on Animal Care (CCAC) and animal protocol was approved by the University Committee on Animal Care and Supply. Piglets were obtained from the Prairie Swine Center, transferred to the WCVM Animal Care Unit and monitored 3 times a day for quantity and quality of feces, behaviour, dehydration, and weight loss for the entire duration of the trial.

3. Screening animals for the presence of F4 receptors.

To ensure that experimental animals used in this study were susceptible to infection with F4 positive *E. coli* a pool of suckling piglets was tested for the presence of the gene encoding one of receptors for the F4 fimbrial adhesion as describe previously [2]. The test relies on a single nucleotide polymorphism in intron 7 of *Sus scrofa* mucin 4 (*MUC4*) gene which was associated with either susceptibility or resistance to adherence of F4 positive *E. coli*. Briefly, we isolated genomic DNA of piglets and amplified a fragment of the *MUC4* gene using polymerase chain reaction (PCR) with synthetic oligonucleotides, XPNf and XPNR (Table 1). PCR fragments were then digested in the presence of restriction endonuclease *XbaI*. The reaction products were analyzed by polyacrylamide gel electrophoresis Approximately 30% of tested piglets were considered to possess the receptor for F4 fimbria based on presence of 151 and 216 base pairs (bp) fragments generated by *XbaI* digestion of amplified 367 bp products (Figure 1).

4. Preparation of probiotics (*Lactococcus lactis)*

Lactococcus lactis bacteria from a frozen (-70^{0}C) stock were streaked on Trypticase soy agar and incubated overnight at 37^{0}C until individual colonies were formed. Single *L. lactis* colony was inoculated into 1 liter of Trypticase soy broth (TSB) and incubated at 37^{0}C with gentle shaking for aeration. After reaching stationary phase of growth, the culture was harvested by centrifugation, washed with phosphate buffered saline (PBS) and dehydrated at -80^{0}C (lyophilized).
Viable bacteria were quantified in lyophilized powder as follows. Ten milligram of lyophilized powder was weighed and diluted in PBS. Serial dilutions of this suspension were prepared and plated on Trypticase soy agar. After overnight incubation, developed colonies were counted and the titer of viable bacteria was determined. Typically, it amounted to 10^{11} colony forming units (cfu) per gram of lyophilized powder. Lyophilized bacteria were stored at 4^{0}C until use.

5. Preparation of probiotics supplemented starter diet

Lyophilized *L. lactis* (100 mg) was weighed and re-suspended in small volume of PBS (5 ml). The suspension was evenly sprayed on a thin layer of starter diet (1 kg) in a large flat tupperware container with subsequent vigorous shaking. This allowed distribution of re-suspended bacteria evenly throughout the granulated feed. The spraying and mixing continued until all moisture of the bacterial suspension was absorbed by the granulated feed. This feed mixed with bacteria *L. lactis* was added to the pre-emptied feeder of the probiotic treatment group of piglets.
Viable bacteria in the feed were quantified as follows. Twenty gram of feed was mixed with 200 ml of sterile PBS. The mixture was shaken to extract bacteria from the solid feed granules into liquid phase. Serial dilutions of the aqueous extract were plated on Alcan agar plates and the titer of live *L. lactis* was determined. Alcan agar is a medium allowing growing selectively *Lactococcus* strains. The results of the titration indicated that probiotics-supplemented feed contained approximately 10^7 cfu of live *L. lactis* per gram.

6. Experimental infection with F4 positive *E. coli*

On day 3 post exposure of probiotic treated group of piglets to *L. lactis* diet, animals in both groups were challenged with approximately 10^{10} live F4 positive enterotoxigenic *E. coli*. Using oral intragastric tube, each animal was first administered 10 ml of sterile PBS (to ensure that the tube was placed in the stomach and to decrease the gastric acidity) and subsequently 10 ml of F4 positive *E. coli* bacterial suspension (containing ~ $2.2x10^9$ cfu per ml) was inoculated into stomach of each piglet in the pilot study (described under 1.2.1) and dose was doubled in the main experiment described under 1.2.2).

7. Sample collection

Fecal samples were collected each morning from individual piglets from day 2 after weaning until euthanasia (day 7 post *E. coli* challenge). The animals were euthanized by I/V injection of euthanyl forte (1 ml per 5 kg of BW). Gross pathology of gastrointestinal tract was assessed, and jejunal, ileal and colonic content samples were collected and stored at -20^0C until needed. Fecal samples were analyzed for the presence of *L. lactis* and haemolytic F4 positive *E. coli*. Both species were quantified using either microbiological or molecular methods as described below.

8. Raising anti-*Lactococcus* antibodies.

Polyclonal serum against *L. lactis* bacterial cells was raised in a New Zealand male adult rabbit. Fresh bacterial culture was washed in PBS, diluted to $1.25x10^8$ in PBS and exposed to ultraviolet light at 300,000 J for 45 min using Stratalinker UV Crosslinker (La Jolla, California, USA). Bacterial suspension was emulsified with equal volume of Titermax Gold Adjuvant (Cedarlane Laboratories, Hornby, Ontario). The mixture (0.2 ml) was injected subcutaneously into New Zealand rabbit at 4 different sites. Boost immunization was repeated with 2 weeks intervals. On day 35 post initial immunization, serum was collected and anti-*Lactococcus* titers were determined by dot-blot, Western blot, and glass slide precipitation reaction.

9. Quantification and immunological identification of *L. lactis* and *E. coli* in porcine feces

Feces was re-suspended in sterile PBS (100 mg/ml). Bacteria were extracted into aqueous phase by vortexing the suspension till it reached a homogenous state. Serial dilutions of the extract were plated on Columbia blood agar plates (Figure 2) on Alcan agar medium (Figure 3) for *E. coli* and *L. lactis*, respectively, incubated at 37^0C for 24-48 hours, number of individual colonies was counted and CFU/g of feces determined (Figure 2).
Identity of bacterial isolates were confirmed by slide agglutination test using rabbit anti-F4 polyclonal antibodies (Dr. J.M. Fairbrother, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Quebec) or anti-*Lactococcus* polyclonal rabbit serum obtained as described above.

10. Detection of bacteria in feces by Polymerase Chain Reaction (PCR).

Total DNA was isolated from approximately 200 mg of porcine frozen feces using QIAamp Fast DNA Stool Mini Kit (QIAGEN) as directed by manufacturer. Two hundred nanograms of each DNA sample were subjected

to PCR using Taq PCR Kit (New England BioLabs). Oligonucleotides used for PCR-based detection of bacteria are listed in Table 1 (Figure 4).

11. Quantification of *E. coli* in porcine fecal samples

Total DNA was isolated from approximately 200 mg of porcine frozen feces using QIAamp Fast DNA Stool Mini Kit (QIAGEN) as directed by manufacturer. Fifty nanogram of each DNA sample were subjected to Real Time PCR using CybrGreen Q-PCR Kit (New England BioLabs) in Stratagene MX5000 real-time PCR machine. Oligonucleotides used for PCR-based detection and quantification of bacteria are listed in Table 1. Oligonucleotide pairs LLF/LLR and F4Qf/F4QR were used for quantification of *L. lactis* and F4 positive *E. coli*, respectively. Total DNA was isolated from 0.2 ml of bacterial suspension of known titers and served as standards for quantification of bacteria in fecal samples. Detection limit of quantitative PCR was determined as 10^4 CFU for *L. lactis* and 10^2 CFU for F4 positive *E. coli*.

Table 1. Synthetic oligonucleotides used in this study.

Oligonucleotide Name	5' to 3' Nucleotide Sequence	Tm, melting temperature	Fragment Size, base pair	Purpose of Application
LLF	TGAAGAATTGATGGAACTCG	60.3	126	Detection of *L. lactis*
LLR	CATTGTGGTTCACCGTTC	60.0		
F4Qf	CACTGGCAATTGCTGCATCT	66.3	87	Detection and Quantification of F4 *E. coli*
F4QR	ACCACCGATATCGACCGAAC	65.9		
XPNf	GTGCCTTGGGTGAGAGGTTA	64.0	367	Detection of F4 receptors
XPNR	CACTCTGCCGTTCTTCTTTCC	65.0		

Results

1. Establishment of probiotic *Lactococcus lactis* in the gastrointestinal tract of weaned piglets

The first objective of this study was to expose weaned piglets to a low (10^{10} CFU/kg) and high (10^{11} CFU/kg) dose of *Lactococcus lactis* in feed in order to determine if *L. lactis* could be established in the gastrointestinal tract of weaned piglets without any side effects (as described in experimental design under 1.1.2). One to two days after initiation of feeding probiotic diet, high titers of *L. lactis* were detected in feces from both groups and no clinical side effects were observed in any of experimental animals. Accordingly, the lower dose was chosen for the challenge study describe below.

2. Experimental infection with F4 positive *E. coli* of weaned piglets fed non-medicated starter diet either without *L. lactis* (control group) or with *L. lactis* (treatment group).

2.1 Pilot study was first performed to test the infection protocol/model.

Twenty suckling piglets were tested for presence of F4 receptors by PCR (as described above) and 6 of them tested positive based on presence of 151 and 216 bp fragments generated by *XbaI* digestion of amplified 367 bp products of mucin 4 (*MUC4*) gene (Fig. 1). These 6 piglets were weaned at 21 days and 3 were fed starter diet with *L. lactis* probiotic (10^{10} CFU/kg) (probiotic group) and the rest three were fed only starter diet (control group). After three days both groups were challenged with intra-gastric administration of $2.2x10^{10}$ CFU of F4 positive *E. coli* suspended in 10 ml of PBS. None of the experimental animals developed diarrhea, but all of them were colonized by both *L. lactis* and F4 positive *E. coli*. Probiotic treated group had higher number of *L. lactis* bacteria in feces right from the beginning of the experiment, while control group initially had lower numbers during the first few days, but subsequently the number of *L. lactis* increased in feces and became

comparable to probiotic group (Figure 5). Since both groups were kept in the same room, it was considered that control group was exposed over time to *L. lactis* in spite of our attempts to implement various measures to prevent cross contamination (separate pens, disinfection footwear baths etc). At the same time this pilot study generated potentially promising results regarding *E. coli* colonization. Namely, all piglets were colonized by *E. coli* after infection, but probiotic group seemed to be more effective in clearing the colonization (2 out of 3 piglets did not have *E. coli* in feces after day 4 post-infection) (Figure 6).

Based on observations in this pilot study the following actions were taken for the main experiment: *i)* Experimental groups were kept in the separate rooms to prevent cross-contamination with probiotic bacteria between groups, and *ii)* Since none of infected animals developed diarrhea in the pilot study, the infection dose was doubled in the main study describe below.

2.2 Main study: Experimental infection with F4 positive *E. coli* of weaned piglets fed non-medicated starter diet either without *L. lactis* (control group) or with *L. lactis* (treatment group).

Sixty suckling piglets were tested for the presence of the gene encoding the receptor for an F4 fimbrial adhesin of *E. coli*, and 16 piglets were determined to be positive. They were weaned at day 21, individual weight was recorded for all piglets that were randomly divided into two experimental groups (n=8 per group) which were housed in two separate rooms with free access to drinking water and non-medicated starter diet. At day 3 post-weaning, one group continued to receive the non-medicated starter diet (control group) while the second group received the non-medicated starter diet supplemented with 10^{10} CFU of *L. lactis* per kilogram of feed (probiotic treatment group). After three days (day 6 post-weaning) all piglets were infected with F4 positive *E. coli*.

One piglet in control group developed septic arthritis in left hind hock on the day 1 post challenge and had to be euthanized. Bacteria isolated from the joint swab at necropsy did not form haemolytic colonies on Colombia blood agar and none of the bacteria isolated from joint swab were tested positive for F4 antigen by glass slide immunoprecipitation. Therefore, this infection was considered to be incidental and the animal was excluded from the study.

In spite of separating experimental groups in two different rooms and careful implementation of biosecurity measures (control room was always accessed first, PPE was completely changed before entering experimental rooms, etc), *L. lactis* was detected in both groups, but the titer differed between experimental groups. Namely, in probiotic treated group, *L. lactis* titers were high right from the onset of experiment (1-2 days after initiation of feeding diet containing probiotic bacteria 10^{10} CFU/kg) while *L. lactis* titers in control group increased slowly over time and became similar to probiotic group by the end of experiment (day 7 post *E. coli* infection) (Figure 7).

In spite of doubling the infection dose of intra-gastrically inoculated *E. coli* with an attempt to decrease gastric acidity by prior inoculation of PBS, none of experimental animals developed diarrhea and none of them had any evidence of gastrointestinal pathology. However, based on fecal bacteriological (culture), immunological (immunoprecipitation) and PCR analysis, all of them were colonized with F4 positive *E. coli* except one animal in probiotic group. Bacterial titers were determined along the length of the trial for each individual piglet by quantitative PCR and average of *E. coli* titers were plotted for control and probiotic groups (Figure 8).

Bacterial populations of both *L. lactis* and *E. coli* in porcine feces were fluctuating substantially both between individual piglets and within each piglet at different time points of sampling. Data was log transformed for each individual piglet in experimental groups. No correlation between the load of *L. lactis* and *E. coli* in fecal matter was found (coefficient of correlation -0.047).

Weight gain by piglets in probiotics group was on average lower than in control piglets during the period of trial (Fig. 9). However, the difference was not statistically significant (t-test, P=0.146). This indicates that accelerated colonization of recently weaned piglets with Lactococcus lactis does not necessary lead to worsening of piglets' performance.

Discussion/Summary:

To determine if *L. lactis* interferes with F4 positive *E. coli* colonization and infection we fed weaned piglets with starter diet supplemented with or without probiotic bacteria and analyzed fecal contents of individual piglets of both groups. No correlation between the load of *L. lactis* and *E. coli* in fecal matter was found (coefficient of correlation -0.047). Accordingly, it was concluded that under the experimental conditions implemented, there was no protective effects of probiotic L. lactis bacteria against colonization by *E. coli*. This is contrary to our previous results obtained *in vitro* and to the apparent results of the pilot study (even though the sample size was low).

Discrepancy between *in vitro* and *in vivo* results are not unusual. *In vitro,* we used assay that quantifies attachment of *E. coli* to a culture of enterocytes with or without presence of *L. lactis*. *In vivo,* intestinal environment is complex and dynamic with numerous additional factors influencing potential preventive effects of *L. lactis* against *E. coli* colonization. However, we are intrigued by the results of our pilot study. They are not definitive, but it seems that *L. lactis* did interfere with colonization of E. coli when we used lower infection dose of *E. coli*. Accordingly, we are wondering if there is a saturation effect exhibited in interaction between *L. lactis* and *E. coli*. Unfortunately, we cannot determine this based on current data. Accordingly, we are considering to investigate further effects of various doses of *L. lactis* on intestinal colonization by *E. coli*.

There were no negative side effects of *L. lactis* weaned piglets. The analysis of the dynamics of *L. lactis* population in piglets' feces is visualized in Figure 7. It indicates that oral exposure to large doses of viable probiotics bacteria allowed establishing a stable population of L. lactis in probiotics group faster that in control piglets. It may be viewed as "accelerated" colonization, as opposed to slower rate of colonization in control group. This advantage may be explored, providing that our additional studies indicate potential protective effects of *L. lactis* against *E. coli*.

Pathogenesis of PWD is complex and multifactorial. Therefore, experimental reproduction of disease is challenging and unpredictable [3] (and personal communication with Drs. Ngeleka and Fairbrother). Accordingly, it is not surprising that we were not able to induce diarrhea in infected piglets; at the same time, we did successfully achieve intestinal colonization with low and high infection doses in our pilot and main trials, respectively. The high *E. coli* infectious dose in the main trial may have saturated potential beneficial effects of low dose *L. lactis* probiotics in experimental piglets. Accordingly, it is our intention to investigate further effects of various doses of *L. lactis* on intestinal colonization by *E. coli*.

Figures

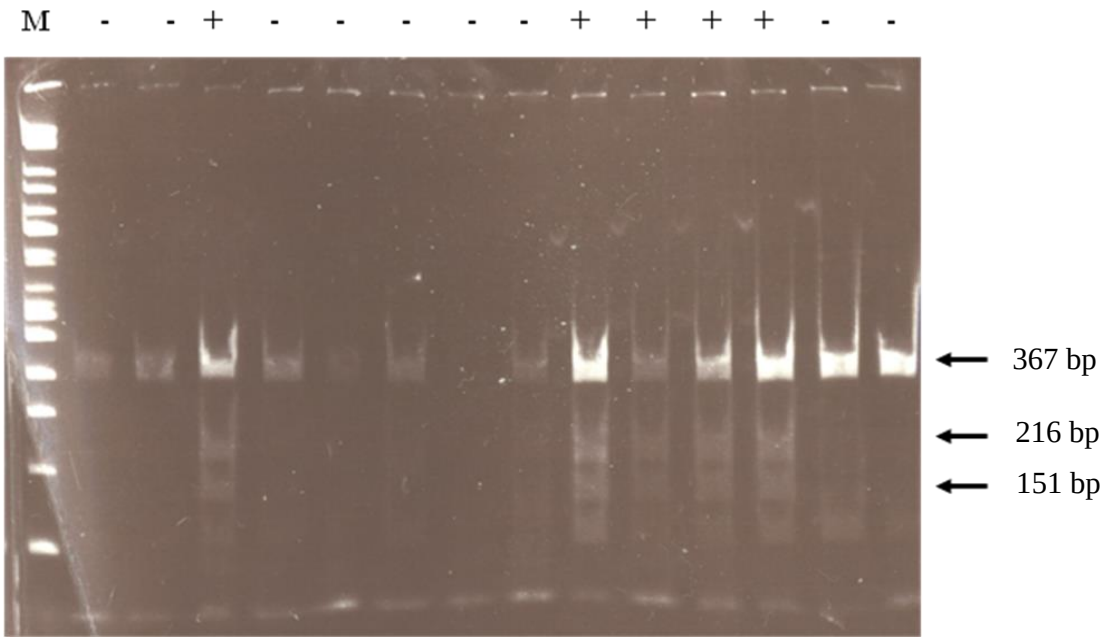

Figure 1. A representative example of analysis of the piglet genome for the presence of F4 receptor.
M, molecular weght standards; + = piglets positive for receptor based on presence of 151 and 216 bp fragments generated by XbaI digestion of amplified 367 bp products of mucin 4 (*MUC4*) gene; - = piglets negative for resceptor; arrows indicate positions of PCR product of 367 bp and products of digestion (151 and 216 bp) in the presence of restriction endonuclease *XbaI*.

Figure 2. Representative Columbia blood agar plate containing bacteria streaked from two fecal samples. The sample on the left half of the plate has bacterial colonies without heamolytic zone, while hemolytic zones are present around colonies on the right half of the plate which confirms presence of F4 positive *E. coli*.

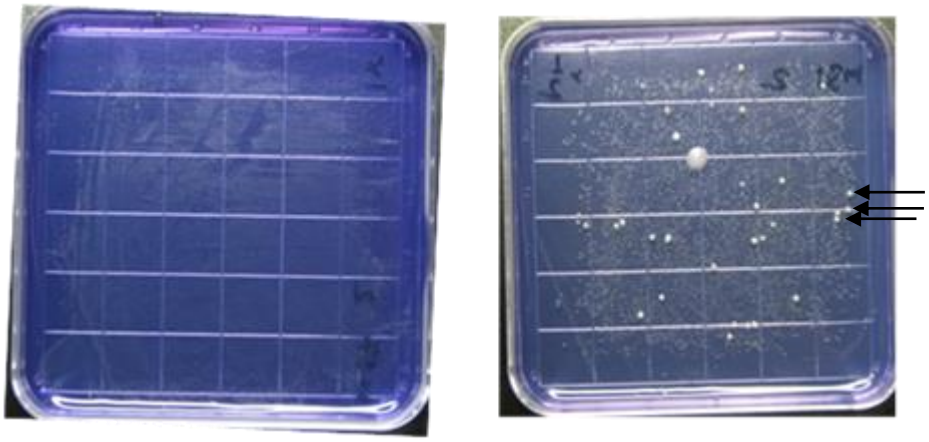

Figure 3. Representative plates of the aqueous extracts of porcine feces plated on Alcan medium. Both plates represent 100 microliters of 1:10 dilutions of feces extracts on Alcan medium. The left plate did not detect *L. lactis* in feces (considered negative, titer 0). The right plate contains *L. lactis* (arrows) colonies at approximately $3x10^3$ cfu/g of feces.

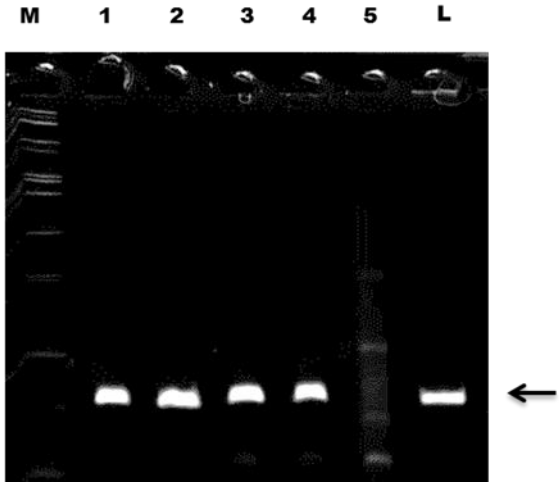

Figure 4. An example of the PCR analysis of bacteria cultured from fecal material of piglets exposed to *Lactococcus lactis* as a feed additive. An arrow indicates the PCR fragment specific for *Lactococcus*; molecular weight standards (Lane M); *Lactococcus lactis* colonies isolated from feces of experimental animals (Lane 1, 2, 3, 4); *E. coli*, which served as a negative control (Lane 5); *Lactococcus lactis* strain used to prepare feed additive (Lane L)

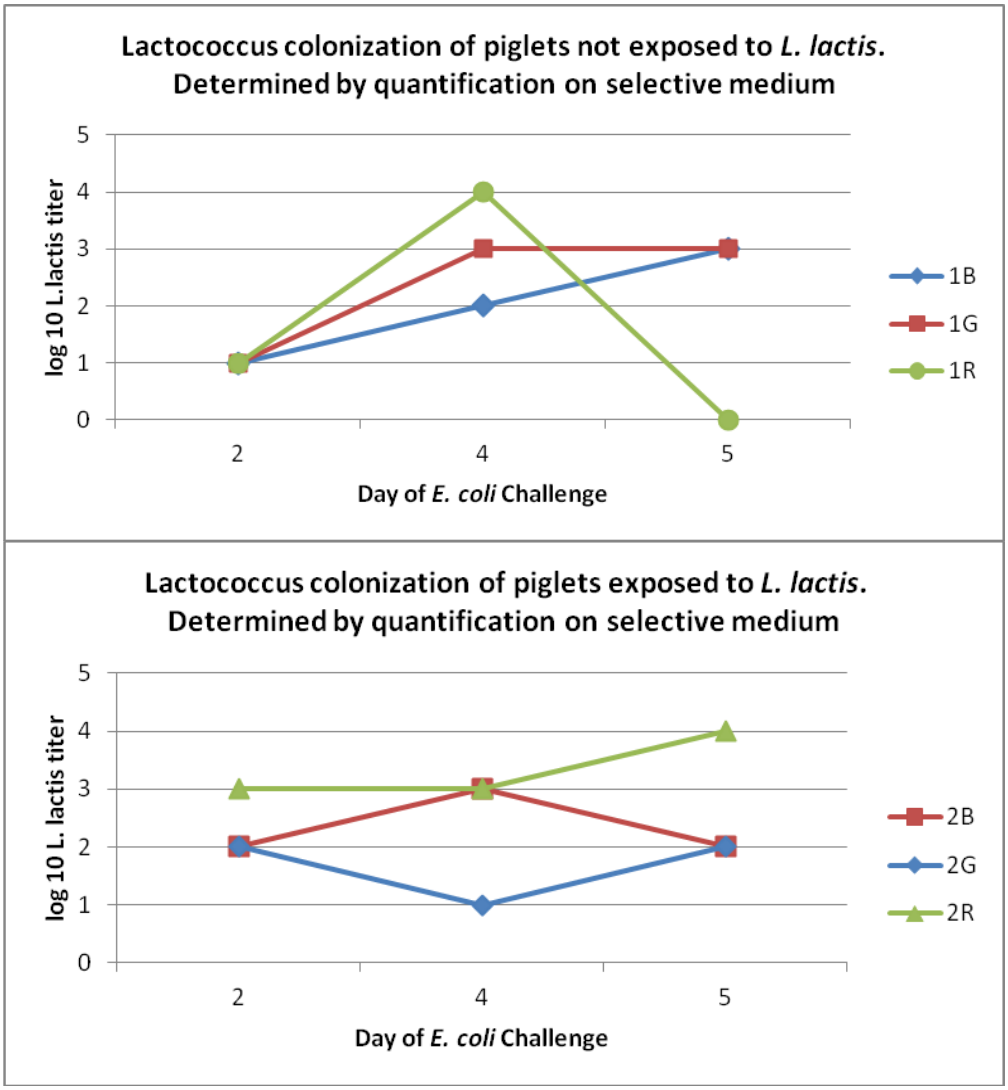

Figure 5. Quantification of *L. lactis* in feces of piglets during pilot trial. Samples quantified on days 2, 4 and 5 post exposure to *L. lactis*. The upper panel illustrates quantities of *Lactococcus* shed by each of 3 piglets (1B, 1G, 1R) not exposed to probiotics supplemented feed. The lower panel shows quantities of *Lactococcus* shed by each of 3 piglets (2B, 2G, 2R) exposed to 10^{10} cfu of *L. lactis* per kilogram of feed. *Lactococcus* was quantified by culturing of serial dilutions of feces aqueous extract on Alcan selective medium.

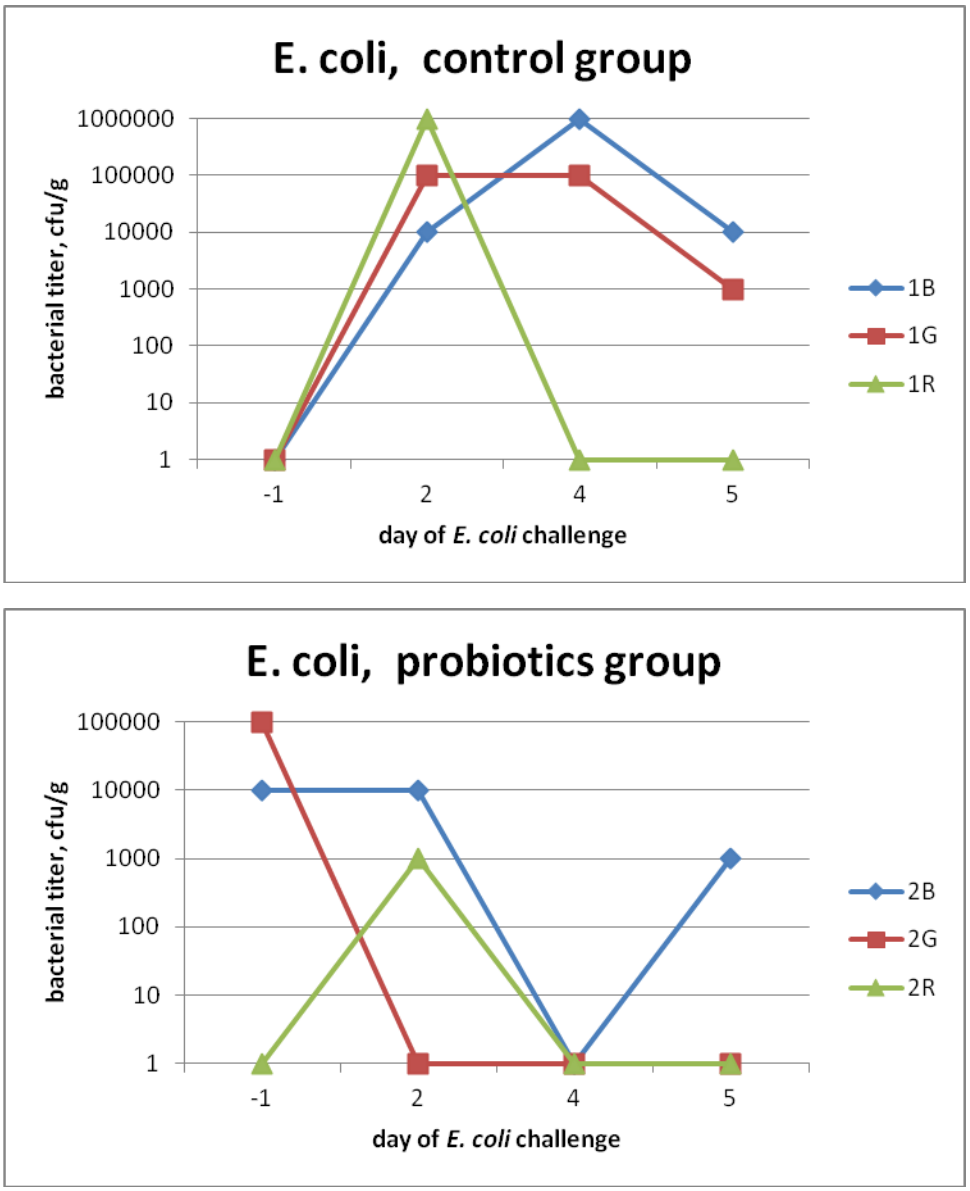

Figure 6. Quantification of *E. coli* in feces of piglets during pilot trial. Samples quantified on days -1, 2, 4 and 5 post-infection with *E. coli*. Upper panel illustrates quantities of *E. coli* shed by each of 3 piglets (1B, 1G, 1R) not exposed to probiotics supplemented feed (control group). Lower panel shows quantities of *E. coli* shed by each of 3 piglets (2B, 2G, 2R) exposed to 10^{10} cfu of *L. lactis* per kilogram of feed (probiotics group). . *E.coli* was quantified by Real-Time PCR using oligonucleotide primers specific for the gene coding F4 fimbriae.

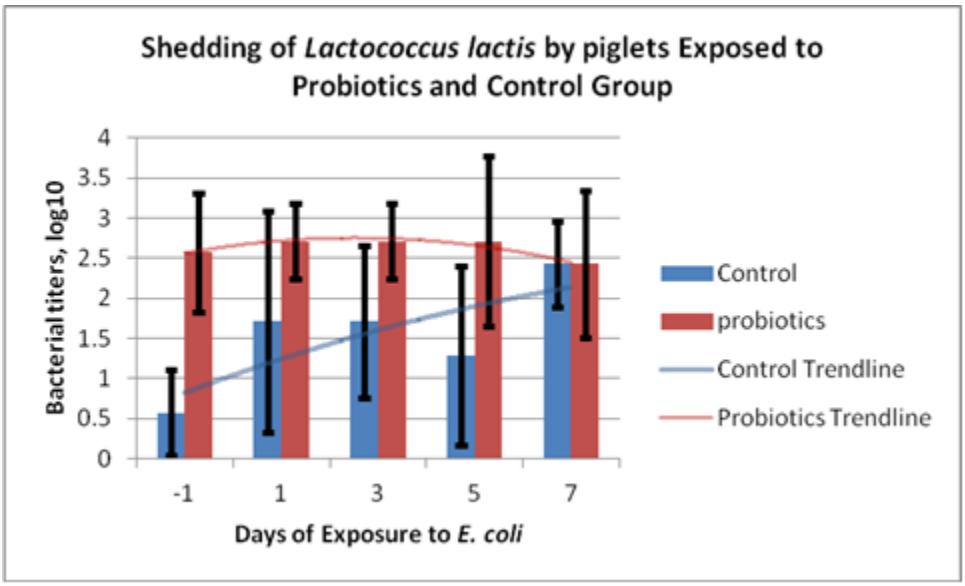

Figure 7. Average titers of *L. lactis* in feces of experimental animals along the length of the trial. Error bars, standard deviation. Trend lines suggest gradual establishment of *Lactococcus* population in gastrointestinal tract of control group, as opposed to accelerated establishment of the *Lactococcus* population in probiotics group.

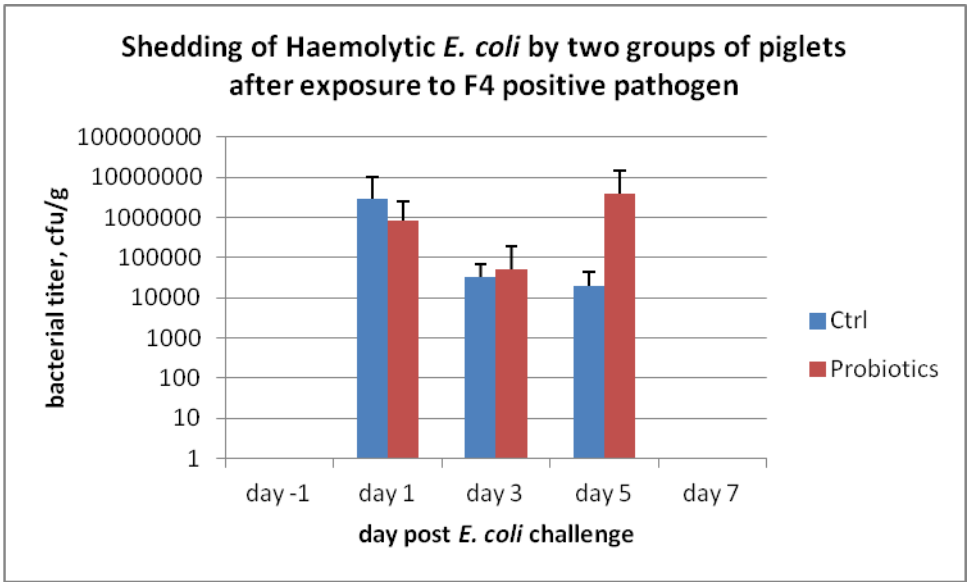

Figure 8. Average of *E. coli* titers in fecal samples of piglets from control and probiotic treatment groups. Error bars, standard deviation. Probiotic L. lactis did not have statistically significant effects on prevention of intestinal colonization by F4 positive *E. coli*.

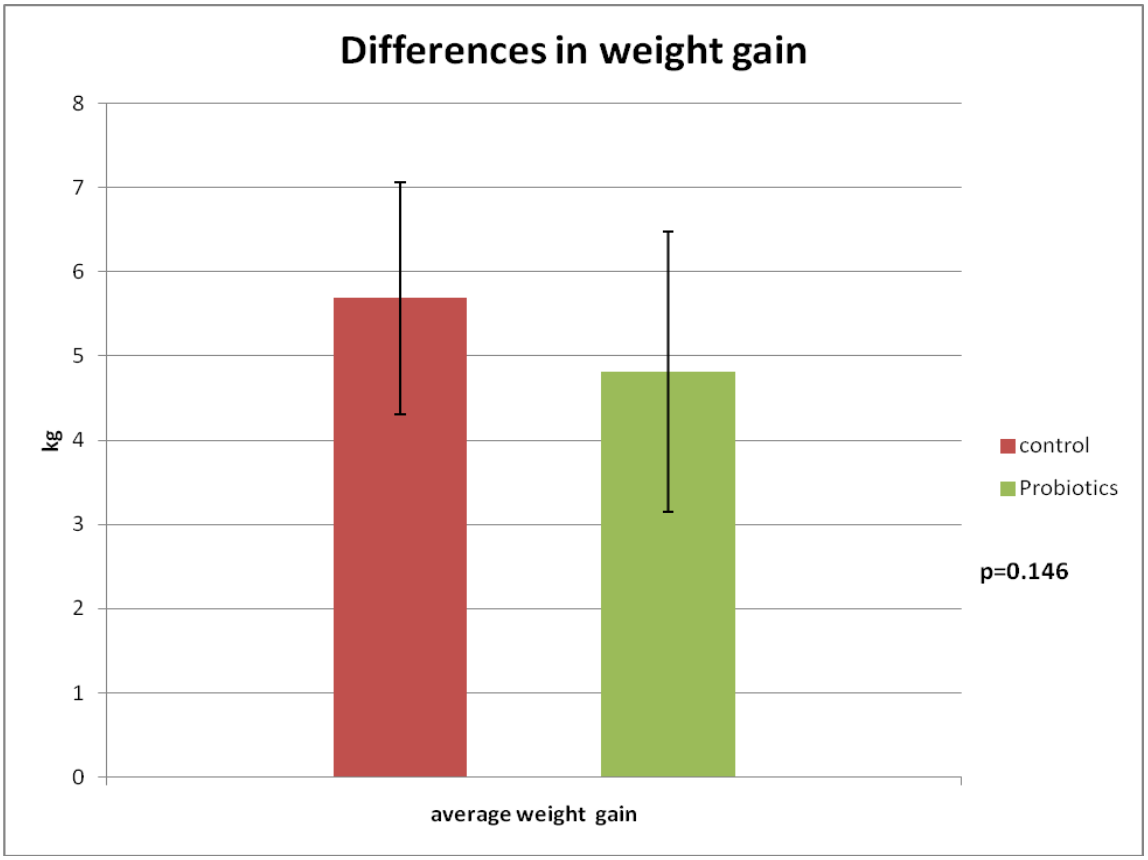

Figure 9. Average weight gain by piglets from control and probiotics groups over 14 days. Error bars, standard deviation. Piglets fed probiotic diet had lower average weight gain, but the difference was not statistically significant.

References

1. Shahriar, F., et al., *Identification by mass spectroscopy of F4ac-fimbrial-binding proteins in porcine milk and characterization of lactadherin as an inhibitor of F4ac-positive Escherichia coli attachment to intestinal villi in vitro.* Dev Comp Immunol. , 2006. **30**(8): p. 723-734.
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3. Fairbrother, J.M., É. Nadeau, and C.L. Gyles, *Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies.* Animal Health Research Reviews, 2005. **6**(01): p. 17-39.