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Influence of Probiotics on Coccidia, *H.* Contortus and Markers of Infection in Goats

[1]Kwaku Barima Gyenai, [1]Mulumebet Worku, [3]Mehrdad Tajkarimi and [2]Salam Ibrahim

[1]Department of Animal Sciences, North Carolina Agricultural and Technical State University, B.C. Webb hall, 1601 East Market Street, Greensboro NC 27411, United States
[2]Department of Food Microbiology, North Carolina Agricultural and Technical State University, 171 Carver, 1601 East Market Street, Greensboro NC 27411, United States
[3]Joint School of Nanoscience and Nanoengineering, University of North Carolina at Greensboro NC 27401, United States

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Corresponding Author:
Mulumebet Worku
Department of Animal Sciences, North Carolina Agricultural and Technical State University, B.C. Webb hall, 1601 East Market Street, Greensboro NC 27411, United States
Tel: + 1336-285-4816
Email: worku@ncat.edu

Abstract: Immunostimulants can induce nonspecific resistance against parasites. The use of probiotics to control development of animal gastrointestinal parasites could help reduce the risks of infestation. In this study, we investigated the effects of probiotics administration on gastrointestinal parasites coccidia, *H.* contortus and markers of infection. A cocktail of probiotics mix including *Bifidobacterium longum, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus reuteri* and *Lactobacillus rhamnosus* (8.5 log CFU/mL) was drenched daily to three months-old male Spanish Boer kid-goats (n = 3) till four weeks of age. Control group of age matched kid-goats received sterile water daily for a 4-week period. Body weight, Fecal Egg Count, FAMACHA scores, Packed Cell Volume (PCV) and White Blood Cell Differential Count was determined weekly. Denaturing Gradient Gel Electrophoresis (DGGE) was used to monitor fecal bacteria using bacteria 16 sec rDNA primers. Pro-inflammatory cytokines, Prostaglandin2 (PGE2) and Immunoglobulin E levels in plasma were evaluated using commercial ELISAs. Results showed no significant difference in PCV, body weight, White Blood Cell Differential Count, FAMACHA score, PGE2 and IgE levels between probiotic drenched and control. However, eggs per gram were increased significantly ($p<0.05$) probiotics drenched 100, 90 and 120% at weeks 2, 3 and 4 for haemonchus and 70% for coccidia at week 3 respectively. An increase of 50 to 300% in pro-inflammatory cytokines was observed for probiotic drenched over control at weeks 2, 3 and 4. Pro inflammatory cytokine levels were significantly highest ($p<0.05$) for IFNr, G-CSF and IL-1α when pre-treatment levels were compared to week 4 for probiotic drenched. Results from PCR-DGGE analysis showed significantly increased fecal microbial DNA for probiotics drenched ($p<0.05$), with no difference in band pattern and staining intensity. Although increases in *H.* contortus count and Pro-inflammatory cytokine levels were observed, probiotics drenching had no effect against coccidia or *H.* contortus. This study supports the idea that use of probiotics in ruminants may be impacted by lack of microbial retention in the rumen. Further studies on establishment and retention are needed.

Keywords: Coccidia, Pro-Inflammatory Cytokines, Goat, *H.* contortus, Probiotics

Introduction

Probiotics are viable microorganisms that have positive effects on growth performance, nutrient synthesis, the microbial ecosystem, absorption, the reduction in the incidence of intestinal infection and restoration of gut microflora after bouts of diarrhea (Khalid *et al*., 2011). It has been reported that

Lactobacillus and *Propionibacterium* probiotic strains may stabilize ruminal pH and prevent subacute ruminal acidosis risk (Lettat *et al*., 2012). Application of probiotics has been suggested as a way to positively modulate ruminal flora and fermentation (Lettat *et al*., 2012). In a previous study, live ingestion of probiotics in sufficient amounts was shown to have a positive effect on the host via stimulation of the non-immune response and through the enhancement of immune protection (Böhmer *et al*., 2006). Probiotics use has also been linked to the enhancement of gut microflora, resulting in improved health status, gut microflora balance and increased resistance to pathogenic agents (Böhmer *et al*., 2006; de Moreno de LeBlanc *et al*., 2010).

In a related study, probiotics administration was reported to influence both innate and adaptive immunity by modifying the gut microbiota through direct contact with epithelial and immune cells (Lebeer *et al*., 2008). During this process, a protective effect was exerted via multiple immune and non-immune mechanisms and by direct antimicrobial activity against pathogens. This resulted in increased phagocytosis and modified cytokine levels via different cell populations (de Moreno de LeBlanc *et al*., 2010; Latvala *et al*., 2008; Vizoso Pinto *et al*., 2009).

Research interest on the use of probiotics in decreasing pathogen load and ameliorating gastrointestinal diseases in animal agriculture has been increasing. *In vitro* tests to identify the best potential probiotics for animals and *in vivo* studies have been conducted utilizing multi or mono strains of probiotic organisms as additives in ruminant feeding. In ruminants, most probiotic applications have addressed its use in cows and calves, with limited studies in sheep and goats. In young calves, incorporating live yeasts into grain reduced the duration of diarrhea (Galvão *et al*., 2005). Two different probiotic preparations containing six *Lactobacillus spp.* of bovine and human origin successfully reduced overall mortality, incidence of diarrhea and fecal coliform counts in calves (Timmerman *et al*., 2005). In a large-scale trial, steers fed standard steam-flaked corn-based finishing diet with L. acidophilus NP51 showed reduced *E. coli* O157 faecal shedding of 57% (Younts-Dahl *et al*., 2004). In a subsequent, though different, trial lasting two years, a 35% faecal shedding of *E. coli* O157:H7 in beef cattle was observed following daily administration of *L. acidophilus* strain NP51 (Peterson *et al*., 2007). Despite the advances made in the use of probiotics in animal agriculture, their use in the treatment and/or prevention of diseases of economic importance (coccidia and/or *H.* contortus) in goat production remains limited. Goats are markedly susceptible to infection with gastrointestinal nematodes. However, the frequency of anthelmintic resistance is higher than in sheep, with which they share the same nematode parasites (Waller, 1997). Integrated control of strongylosis in goats is required due to anthelmintic resistance. Parasitic infection, which occurs in young and stressed animals, is of economic importance in goat production (Kusiluka *et al*., 1998). The clinical state of infection is one of the main factors responsible for kid mortality in pre- and post-weaning periods (Donkin and Boyazoglu, 2004). Using commercial probiotics, Whitley *et al.* (2009) evaluated production performance in meat goats and reported no significant gains. However, limited studies have looked at the role probiotics play in disease progression in goats. This study was initiated to evaluate the influence of probiotics administration on gastrointestinal parasites coccidia, *H.* contortus and markers of infection.

Materials and Methods

Animals and Treatment

Six, three-month-old male Spanish Boer kid-goats from the North Carolina Agricultural and Technical State University Farm were used in the study. The protocol was approved by the university animal care and use committee. The kid-goats were all raised in the same pen in a barn on a sand rock floor covered with wood shavings. The animals were allowed daily access to pastures, fed hay and basal diets from Southern States SSC-31-911800 goat feed (17% crude protein) and given water ad-libitum until 28 days-of-age. The non-probiotic control group (n = 3, initial average BW: 43) and probiotic drenched kind-goats (n = 3, initial average BW: 45).

Probiotic Mix Preparation

A cocktail of probiotics, *Bifidobacterium longum, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus reuteri* and *Lactobacillus rhamnosus*, each at 8.5 Log CFU/mL was used in this study. Samples were cultured in MRS broth anaerobically at 37°C for approximately 14 h to a titer of 10^9 Log CFU/mL. Bacteria were then harvested by centrifugation (4000g, 10 min, 4°C), washed twice in 0.1% Peptone Water (wt/vol) and adjusted to final concentrations of 8.5 Log CFU/mL (vol) sterile skimmed milk (skimmed milk powders were dissolved in distilled water). Quantitative measurement using MRS agar plates was done to confirm the total number of bacteria per ml. The animals in the treatment group were drenched daily with 10 ML of probiotics where as control animals received 10ML of sterile water daily for 4 weeks.

Fecal Egg Count and Blood Collection

Initial fecal egg counts were below 500 epg, prior to drenching. Following drenching, body weights and blood samples were collected once a week for a 4 week period. Fresh, individual fecal samples were collected by rectal

swab and analyzed on the day of collection for eggs per gram of feces from 0, once a week, up to 4 weeks-of-age. The fecal samples were analyzed using a modified McMaster technique (Paracount-EPG^{TM}, 1984). Slides were read using an Olympus B 201 microscope (Optical Elements Corporation) at 10x magnification. The egg count was focused to determine the presence of *H.* contortus and coccidia. The number of eggs counted on the McMaster slide was multiplied by 50 to obtain the parasite eggs per gram of feces per animal. Blood samples were collected from the jugular vein of both treatment groups in test tubes containing 0.1 mL of acid citrate dextrose to prevent blood coagulation and plasma was collected weekly through week 4 FAMACHA® Scores, Packed cell volume and *Differential Leukocyte count* the color of the ocular mucous membranes was scored weekly using FAMACHA® cards (SSCRP.org). On a scoring scale where: 1 = optimal, 2 = acceptable, 3 = borderline, 4 = dangerous and 5 = fatal, the numbers were used to evaluate membrane color (Kaplan *et al*., 2004).

Packed Cell Volume (PCV) as a measure of anaemia was evaluated using an aliquot of blood with anticoagulant from each goat. Blood was collected in micro-capillary tubes and then centrifuged for 10 min at 14,000 rpm in an IEC MB Micro Hematocrit centrifuge (Damon/IEC Division). After centrifugation, samples were analyzed for percentage of packed cell volume using a micro-capillary reader (Damon/IEC Division).

White Blood Cell Differential Counts (WBCDC) were performed on blood smears stained using the Sure Stain Wright CS-432 stain as described by the manufacturer (Fisher Scientific). White blood cell differential counts were performed using an Olympus B 201 microscope (optical elements corporation) using 100× magnification.

Fecal DNA Isolation and PCR-DGGE Analysis

Isolation of fecal DNA was conducted weekly on individual animals drenched with probiotics and control from 1 to 4 weeks using the QIAamp DNA stool Assay kit (Qiagen, Gaithersburg, MD). Extracted DNA was evaluated using the bacterial 16s rDNA gene for PCR. Approximately 200 bp of the bacterial 16S rDNA gene were amplified using the following primers: Forward 5'GCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGCCTACGGGAGGC AGCAG-3', Reverse-5'-ATTACCGCG GCTGCTGG-3' (McEwan *et al*., 2005). The reverse primer had a 45 nucleotide GC-clamp linked to it. Previous research has supported the GC-clamp's effectiveness in obtaining a better profile on the denaturing gel (Muyzer *et al*., 1998). The following PCR conditions were employed: 95°C denaturation for 120 sec, followed by 35 cycles at 95°C for 60 sec, 60°C for 45 sec and 72°C for 90 sec and a single step of 72°C for 30 min. Amplicons from PCR were analyzed on a 2.0% agarose that was stained using ethidium bromide.

Denaturing Gradient Gel Electrophoresis (DGGE) analyses were performed on PCR products using the DCodeTM Universal Mutation Detection system (15 cm system; Bio-Rad). PCR products were loaded onto a 6% (wt/vol) acrylamide gel containing a denaturant gradient of 30 to 60% (100% denaturant consisted of 7 M urea and 40% [vol/vol] formamide) parallel to the direction of electrophoresis by using the D-Code system (Bio-Rad, Hercules, Calif.). Gels were electrophoresed at 60°C at a constant voltage of 85 V for 6 h prior to being stained with ethidium bromide (Promega Corp., Madison, Wis.). DNA bands in the DGGE gel were visualized by standard ethidium bromide staining and photographed prior to data analysis.

Analysis of DGGE Profile

Using the BioNumerics software version 6.6 (Applied Maths, Kortrijk, Belgium), images obtained from the DGGE gel were evaluated (Martínez *et al*., 2009). Bands from DGGE fingerprints were transformed to peak profile and the staining intensities of individual bands were evaluated as a proportion of the peak surface area relative to the surface area of the entire molecular fingerprint of the sample. The influence of probiotics drenching on kid-goats was evaluated on normalized fragment intensities of all bands in DGGE fingerprints. Diversity of microbiota was calculated by Shannon's and Simpson's indices as discussed by (Tran *et al*., 2012).

Pro-Inflammatory Cytokine Analysis

Blood was collected into red top 10ml plain vacutainer tubes (Becton Dickinson and Company.) The serum was separated and stored at -20°C until ready for use. Duplicate serum samples were assayed for pro-inflammatory cytokines: Interleukin (IL)-1a, IL-8, interferon (IFN)-γ, Tumour Necrosis Factor (TNF-α), Granulocyte-Colony Stimulating Factor (G-CSF) and G-CMSF, Interferon Gamma-Induced Protein 10 (IP-10) and Rantes using an optimized commercial human Inflammation enzyme-linked immunosorbent assay (Signosis, Sunnyvale, CA). The concentration of ProstaglandinE2 (PGE2) was measured in both probiotics drenched and control samples using a commercially available enzyme immunoassay kit in triplicate according to the manufacturer's suggested protocol (Cayman Chemical, An Arbour, MI). Immunoglobulin E (IgE) levels were measured in plasma using IgE assay kits based on the manufacturers suggested protocol (NovaTeinBio, Cambridge, MA).

Statistical Analysis

A two-way analysis (treatment x week) of variance test using SAS statistical analysis software (SAS Inst.

Inc., Cary NC) was used to analyze BW, FEC, PCV, WBCDC and PGE, IgE and cytokine values. Statistical significance was determined using $p<0.05$. Dunnett's method was used with the GLM procedure to compare differences between the control and treatment to determine the response to probiotic drenching.

Results

All animals were in good health at the start of the study and no diseases occurred in the probiotics drenched and control group. Body weight measurements did not differ between the two treatment groups from zero to 4 weeks-of-age (Table 1). Probiotics drenching did not result in any changes in PCV levels and FAMACHA scores from zero to 4 weeks-of-age between the two treatment groups (data not shown). Similarly, differential white blood cell count levels did not differ between probiotic drenched and control from zero to week 4 (Fig. 1).

The influence of probiotics, if any, on fecal egg count is presented in Table 2. Results of the fecal egg count (*H.* contortus and coccidia) analysis for probiotics showed low to no significant difference between the two treatment groups at week 1 for *H.* contortus. However, from 2 to 4 weeks-of-age, a much different trend of 100, 10 and 15% significant increase in *H.* contortus levels was observed for kid-goats drenched with probiotics ($p<0.05$) (Table 2). In contrast, *H.* contortus values in control animals were low during the first two weeks and increased slightly at week 3 and 4 (Table 2). Probiotics drenched kid-goats had higher epg; however, the epg did not impact PCV or FAMACHA levels.

Coccidia oocyte counts showed a much different trend for kid-goats drenched with probiotics vs. control. A 30% increase was observed for control over probiotics drenched (Table 2). At week 1, control animals showed a 280% fold increase ($p<0.04$) compared to that of probiotics drenched (Table 2). The trend of increased coccidia oocyte continued; however, values did not differ between the two different treatment groups by week 2. An inverse relation of 70% increased coccidia oocyte in probiotics drenched over control was observed at week 3 ($p<0.05$). Coccidia levels continued to increase for probiotic drenched up until week 4 when a sharp decline was observed with no significant difference between the two different treatment groups (Table 2).

Figure 2 shows cumulative averages obtained from 8-different Pro-Inflammatory Cytokine (PIC). As shown, from pre-treatment (day 0) to one week after probiotic drenching, there was no difference in PIC levels between the two different treatment groups. Cumulative PIC levels of probiotics drenched animals increased significantly ($p<0.05$) 100 to 400% over control from week 2 through 4 (Fig. 2). Figure 3 shows PIC levels of probiotic drenched pre-treatment and week 4, when PIC levels were significantly highest for IFNr, G-CSF and IL-1α as compared to pre-treatment levels. However, GM-CSF levels increased the highest pre- and after probiotic drenching without any significant change (Fig. 3). For controls, levels were high pre-treatment, declined sharply after week 2 and then remained unchanged through the duration of the study (data not shown).

Probiotics drenching had no significant effect on PGE2 concentration when compared to control at week 1. At weeks 2, 3 and 4, an 8% increase was observed for probiotics drenched over control (Fig. 4). PGE2 levels declined by as much as 10 to 20% for both treatment groups in week 4 (Fig. 4). Analysis of IgE levels showed a slight but non-significant increase at week 1 and 2 for control (Fig. 5). However, an inverse relation was observed at week 3 and 4 where the IgE levels of animals drenched with probiotics increased 15% compared to control.

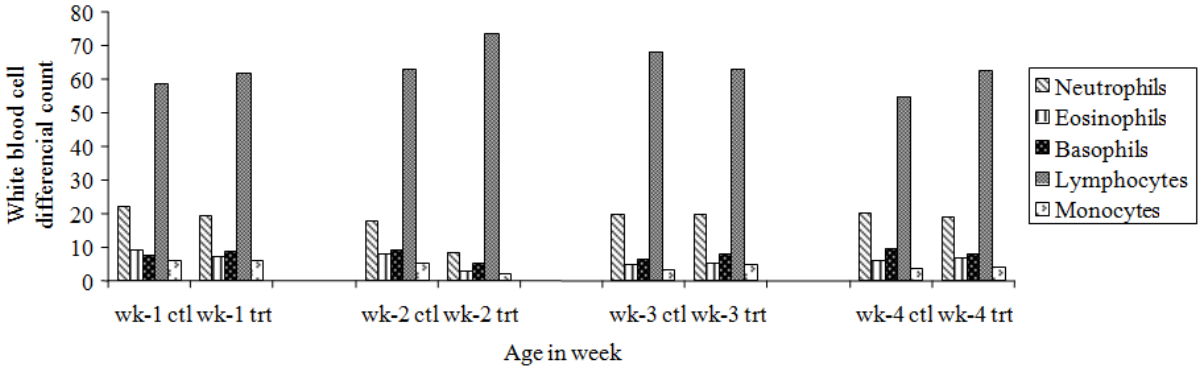

Fig. 1. The results are expressed as means of n = 3. Results presented in millions mL-1 where trt and ctl are kid-goats drenched with and without probiotics respectively

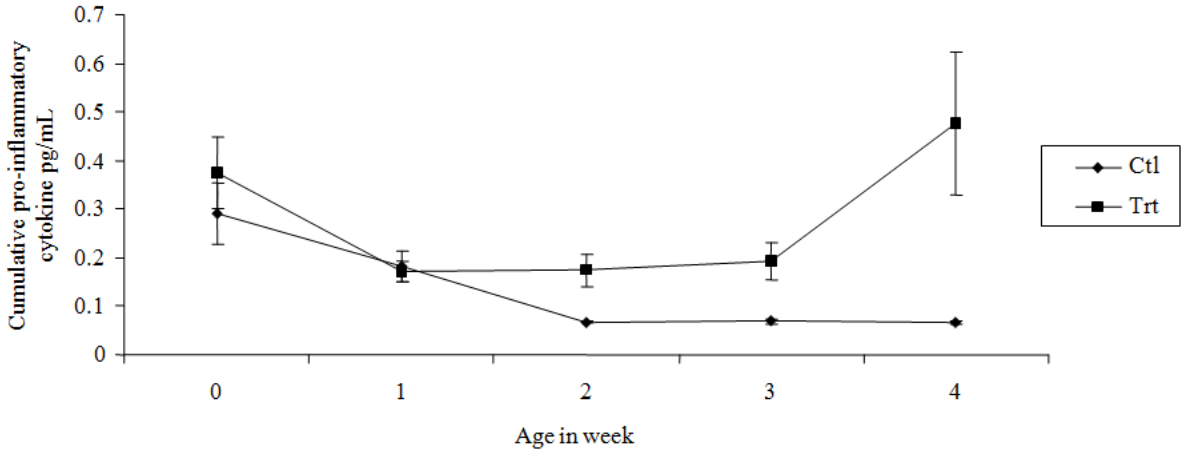

Fig. 2. Pro-inflammation cytokine levels of kid goat drenched with (Trt) and without (Ctl) probiotics from day 0 to 4 weeks-of-age. Results presented as mean ± standard deviation ($p<0.05$)

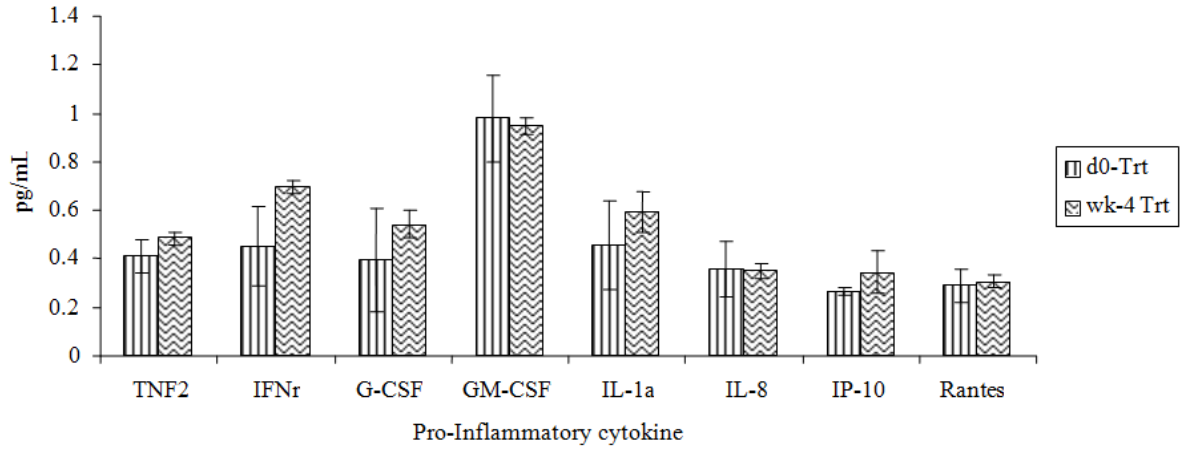

Fig. 3. Pro-inflammation cytokine levels of probiotics drenched (Trt) kid-goats at day zero versus week 4.Results presented as mean ± standard deviation ($p<0.05$)

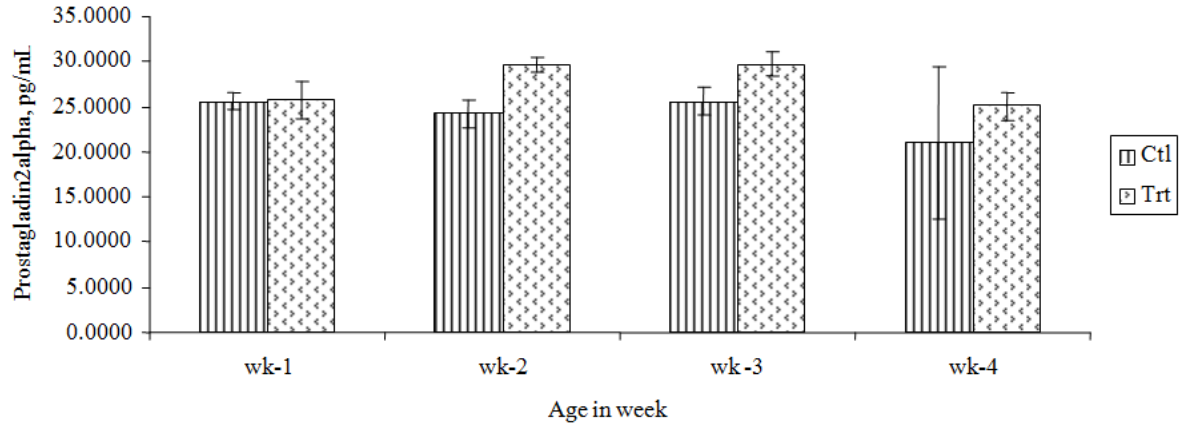

Fig. 4. Prostaglandin (PG)E levels of kid goat drenched with (Trt) and without (Ctl) probiotics from 1 to 4 weeks-of-age. Results presented as mean ± standard deviation ($p<0.05$)

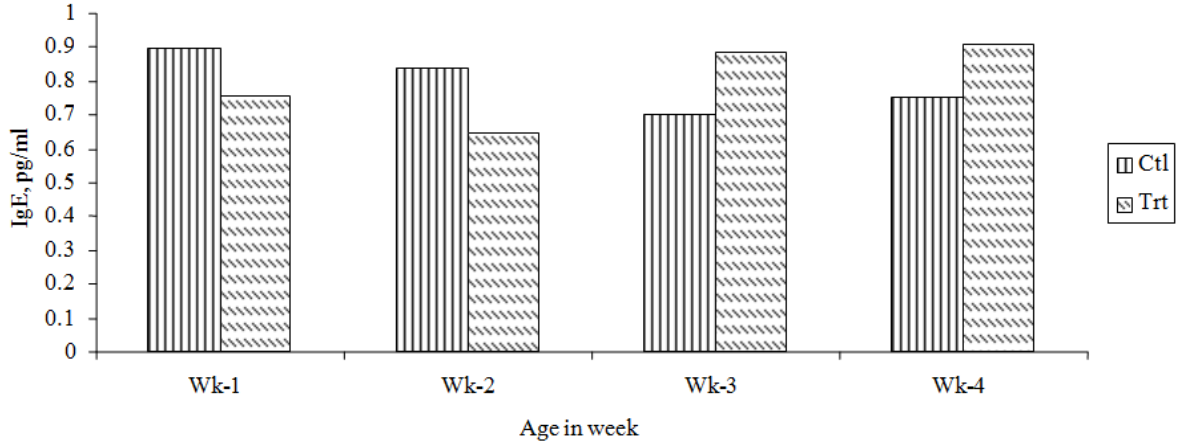

Fig. 5. Immunoglobulin E (IgE) levels of kid goat drenched with (Trt) and without (Ctl) probiotics from 1 to 4 weeks-of-age. Results presented as mean ($p<0.05$)

Table 1. Body weight of kid-goats drenched with (Trt) and without (Ctl) probiotics

	Body weight	
Goat ID	Ctl	Trt
Day-0	42.66±3.78^{a3}	45.00±2.64^{a3}
wk-1	60.66±10.06^{a2}	62.00±9.64^{a12}
wk-2	65.66±11.02^{a2}	65.33±8.08^{a12}
wk-3	66.33±10.06^{a2}	68.00±11.26^{a1}
wk-4	83.00±12.05^{a1}	76.00±11.53^{a1}

Results are shown as means ± standard deviation.
^{a,b}Within a row, means without a common superscript differ ($p<0.05$).
^{1,2,3}Values within a column without a similar numeric superscript differ ($p<0.05$)

Table 2. Fecal egg count of kid-goats drenched with (Trt) and without (Ctl) probiotics

	Fecal egg count			
	H. contortus		Coccidia	
Week	Ctl	Trt	Ctl	Trt
Day-0	0±00^{a5}	0±00^{a4}	233±189^{ab3}	150±50^{b5}
wk-1	33±28^{a3}	33±28^{a3}	1550±848^{a2}	525±176^{b4}
wk-2	16±28^{b4}	33±57^{a3}	2316±202^{a1}	2300±377^{a2}
wk-3	66±57^{b1}	116±76^{a12}	1550±444^{b2}	2800±912^{a1}
wk-4	50±0^{b2}	133±189^{a1}	1700±424^{a2}	1666±642^{a3}

Results are shown as means ± standard deviation.
^{a,b}Within a row, means without a common superscript differ ($p<0.05$).
^{1,2,3,4}Values within a column without a similar numeric superscript differ ($p<0.05$).

Table 3. Effects of probiotics drenching on diversity of fecal microbiota

	Shannon's index$^{¥}$ (mean ±SEM)		Simpson's index§ (mean ±SEM)	
Week	Ctl	Trt	Ctl	Trt
wk-1	2.78±0.42^{b1}	3.82±0.31^{a1}	0.898±0.005	0.907±0.005
wk-2	2.36±0.45^{b1}	3.63±0.34^{a1}	0.887±0.014	0.906±0.012
wk-3	2.61±0.45^{b1}	3.85±0.34^{a1}	0.889±0.014	0.910±0.011
wk-4	2.60±0.45^{b1}	3.81±0.34^{a1}	0.895±0.013	0.913±0.009

Each value represents means ± standard deviation of 3 observations of kid-goat drenched with (Trt) or without (Ctl) probiotics.
^{a,b}Within a row, means without a common superscript differ ($p<0.05$).
^{1}Values within a column without a similar numeric superscript differ ($p<0.05$).
$^{¥}$The larger the Shannon's index, the more diverse the microbial population.
§The smaller the Simpson's index, the more diverse the microbial population.

The effect of probiotics drenching on kid-goats' total microbial composition on the diversity indices (Shannon's and Simpson's) using bacterial 16S rDNA gene primers for PCR-DGGE are presented in Table 3. Results showed 20 to 30% increase differences in microbial population based on Shannon's diversity index at week 1, 2, 3 and 4, for probiotic drenched ($p<0.05$). The variation observed in the fecal microbiota population can be attributed to residuals from the probiotic drenching. Simpson's index, by contrast, showed no difference in the two treatment groups. Analysis of DGGE revealed no changes in the composition of the fecal microbiota bands for kid-goats drenched with probiotics and control (Supplementary Fig. S1). Analysis of the ratio of staining intensity of all bands observed for each week as a proportion of total fingerprint intensity using bacterial 16S rDNA gene primers for PCR-DGGE are presented in Table 4. Similar to the band pattern observed, results showed no differences between kid-goats drenched with probiotics and control from 1 to 4 weeks-of-age.

Discussion

Probiotic administration in animal agriculture represents an excellent way to lower the risk of illnesses on farms. It can also result to good health, improved animal productive performance, through higher live-weight gain, increased feed efficiency, digestion and increments in milk and egg production (Lema *et al*., 2001). In dairy cattle, live yeast supplementation has been reported to improve performance by increasing dry matter intake and milk production (Jouany, 2006; Sniffen *et al*., 2004; Stella *et al*., 2007). However, our current findings showed probiotic supplementation to have no influence on body weight pre-weaning. In fact, increased fecal egg count (*H.* contortus and coccidia) levels were observed in probiotic drenched at the 3rd and 4th week of the study. This would indicate that the animals used in the study acquired sporulated oocysts from the environment and were sub-clinically infected. However, the presence of oocysts in feces did not necessarily imply a clinical state of infection. In addition, the increased fecal egg count may be indicative of decreased ability to fight. Diarrhea is one of the most typical symptoms of clinical infection. Diarrhea was however, not observed in either treatment or control group. Though increased *H.* contortus and coccidia levels were observed, there was no difference in FAMACHA scores, PCV, WBCDC, PGE2 and IgE between probiotics drenched and control.

Measurements of PIC levels showed a different trend of an initial decrease at week 1 marked by increased levels at week 4. The initial high levels of PIC prior to treatment may serve as an indicator of inflammation in the treatment group. However, the levels decreased in week 1 to reach control levels. Pro-inflammatory cytokine levels increased from 2 to 3 weeks with a significantly sharp increase observed by the end of the study for probiotics drenched over control. Changes in expression levels of pro-inflammatory and anti-inflammatory cytokines in both healthy and infected mice have been discussed (Lebeer *et al*., 2008). Probiotics have also been shown to modulate cytokine release of TNF-α, IFN-$_{\gamma}$, IL-10 and IL-12 (Arvola *et al*., 1999). *H.* contortus which is indicative of the high PIC levels observed in the probiotic drenched. In addition, cytokines play a central role in maintaining a delicate balance between necessary and excessive defense mechanisms in animal health. Our finding regarding the differences in PIC levels between the two treatment groups was consistent with changes in *H.* contortus and coccidia levels. Based on these findings, it can be inferred that a slight change in *H.* contortus and coccidia causes an increase in PIC levels.

An analysis of fecal microbiota from kid-goats drenched with probiotics and control using PCR-DGGE to evaluate the bacteria 16 sec rDNA gene showed no differences in band pattern. Shannon's index, on the other hand, showed increased microbial activity for kid-goats drenched with probiotics. The increased fecal microbiota could be a result of the probiotic drenching resulting to the of probiotics inability to bind to the intestinal wall to confer protection and/or survival in the gastrointestinal track (Gaggia *et al*., 2010).

Administration of probiotics had no influence on *H.* contortus and coccidia; rather, increased PIC levels were observed. The lack of probiotic activity on *H.* contortus and coccidia could be due to genera, species, or strains. One approach in probiotic application might involve the use of mixtures of strains belonging to different genera or species (Timmerman *et al*., 2004). Dose, timing and duration of the administration of probiotics may also have affected efficacy. In a Krehbiel *et al.* (2003) review of direct-fed microbials in ruminant diets, probiotics were reported to have no beneficial influence in healthy calves. In cattle, limited probiotic influence was observed due to the ability of ruminants to degrade most probiotics (Callaway *et al*., 2008). More recently, Whitley *et al.* (2009) reported that supplementing healthy, growing meat goats' diet with probiotics conferred no consistent benefits. Further study is warranted in two key areas: (1) on the influence of probiotics on coccidia, *H.* Contortus and pro-inflammatory cytokines in goats and (2) Supplemental treatment for parasite infection in addition to probiotic supplementation.

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Author's Contributions

Kwaku Barima Gyenai: Design and implementation of research, conducted research work, data analysis of research data and manuscript write up for publication.

Mulumebet Worku: Design of research, data interpretation and manuscript write up.

Mehrdad Tajkarimi: Assisted with the study plan, provided and formulated probiotics for research and manuscript write up.

Salam Ibrahim: Assisted with the study plan and provided probiotics for research.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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