Effect of Age and Probiotics on Immune Response in Lambs

Honors Thesis
Presented to the College of Agriculture and Life Sciences, Animal Science
of Cornell University
in Partial Fulfillment of the Requirements for the
Research Honors Program

by
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May 2018

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Abstract

Lambs rely on maternal colostrum for immune protection since they are born immunodeficient. Research indicates that immunodeficiency at birth is required for the development of immune tolerance of commensal microbiota. Furthermore, the role between commensal microorganisms and host interaction supports that commensals play a role in immune training, but work regarding the role of commensal microbiota in immune development in ruminants is minimal. Even with support from maternal immunoglobulin, lambs exhibit high mortality rates, so implementing early and effective vaccination schedules for disease prevention is important. Unfortunately, this has not been well-studied.

In this experiment, we investigated whether treatment with probiotics could enhance immune response in lambs. Lambs received probiotics weekly beginning at two days old, and then were vaccinated with keyhole limpet hemocyanin (KLH) at either 2, 16, or 30 days after birth, followed by a booster 2 weeks later. Blood samples were collected immediately prior to vaccination on sample day (SmplD 0), at booster two weeks later (SmplD 14), and two weeks after the booster vaccination (SmplD 28). Serum was isolated and anti-KLH specific IgG and IgM production was assayed by ELISA.

Statistical analyses indicated significant differences for vaccination age and sample time. Levels of anti-KLH specific IgG increased with age, supporting that older animals can generate stronger IgG specific immune responses. Additionally, significant differences in sample time showed increased levels of IgM production after the primary vaccination and further increased IgM levels after the booster vaccination. Although not statistically significant, general trends indicated that probiotics might enhance immune response in 30-day old lambs.

Acknowledgements

I thank Dr. Thonney for helping me formulate a thesis topic, assisting me in experimental design and statistical analysis, and helping edit the manuscript. I also thank him for guiding me through academics at Cornell as my academic advisor, especially his prompt, helpful replies to my emails.

I thank Dr. Gavalchin for her help during the development of the ELISA assays, answering my immunology questions, and helping with the manuscript.

I really appreciate both Dr. Thonney's and Dr. Gavalchin's willingness to mentor me through this thesis project and allowing me to experience all phases of the research process as an undergraduate.

Lastly, I thank my friends and family who kindly listened to me endlessly talking about the thesis project even when they had no idea what I was talking about. I thank my family especially for their support throughout my undergraduate career.

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List of Abbreviations Used

KLH:Keyhole Lymphocyte Hemocyanin

Ab: Antibody

Ig: immunoglobulin

Literature Review

Reducing mortality rates of lambs increases profitability of both large- and small-scale sheep farming operations. Mortality rates of lambs born alive range from 9 to 32% (Dalton et al., 1980; Daniels et al., 2000; Dawson & Carson, 2002; Hight & Jury, 1970; Hulet et al., 1987; Johnston et al., 1999; Matos et al., 2000; Oltenacu & Boylan, 1981; Scales et al., 1986) and is higher for twins and triplets. Reducing mortality, especially by vaccinating young, susceptible animals as early and effectively as possible is important to the farmer's ability to run a monetarily successful business that incorporates appropriate animal welfare practices.

Passive immunity derived from maternal colostrum plays a critical role in early immune protection in immunocompetent animals (De La Rosa et al., 1997; Hunter et al., 1977; Nowak & Poindron, 2006; Sawyer et al., 1977; Tizard, 1987). However, maternal antibodies may also interfere with vaccinations and the development of an independent, protective neonatal immune response (De La Rosa et al., 1997; Mutwir et al., 2000). While independent immunocompetence increases as an animal's age increases, there is also a marked decline in passive immunity derived from the mother, allowing for a window of susceptibility to disease that is generally targeted as the proper time to vaccinate (Chase et al., 2008).

Recent research has indicated that the importance of immune development in neonates centers around the necessity of immune system development to distinguish commensal microbiota from pathogenic bacteria (Belkaid and Hand, 2014). Commensal microbiota has proven critical for immune function in studies with germ-free mice, particularly in training the immune system to distinguish commensal microbiota from pathogenic bacteria. Initiation and maintenance of proper immune responses to pathogens is highly dependent on communication between the mucosal immune system and commensal bacteria (Belkaid and Hand, 2014). In the mucosal immune system, commensal organisms also benefit the host by competing with

pathogens for adhesion sites and decreasing the amount of nutrients available to pathogenic organisms (Abd El-Tawab et al., 2016). Early microbiota seeding in neonates begins with exposure to maternal commensal bacteria in the vaginal canal and continues to build and diversify in repertoire through all interactions with the environment and other animals, as well as food and water resources.

Probiotics, which refer to commensal microbacterial supplement/additive, have long been associated with increased health and feed efficiency in ruminants. Probiotics largely consist of lactic acid bacterial microorganisms. Multiple studies have shown the protective roles that these microorganisms play in regulating intestinal transit, gut barrier reinforcement, colonization resistance, vitamin synthesis, downregulation of Th2 immune responses, and development of both innate and adaptive immune function (Zimmermann & Curtis, 2017; Wu et al., 2016).

Although researchers have noted the potential of probiotics for increasing vaccination success, the route of administration of vaccine and probiotics, as well as probiotic strain type and dosage, play a critical role in increasing efficacy of vaccines as documented in humans (Zimmermann & Curtis, 2017). Thus, early probiotic administration has the potential to increase early immune response in neonatal lambs, but research with probiotics in small ruminants has mainly focused on probiotic's effect on acceleration of growth rather than immune development.

Studies investigating the generation of specific immune responses in lambs have had varied results. In one study, lambs vaccinated at five months of age showed higher and more persistent antibody response than animals vaccinated at fifteen days, while lambs vaccinated at fifteen days of age could not sustain a cytokine response (Corpa et al., 2000). Lambs vaccinated with ovalbumin at 1 and 15 days of age displayed short-lived antibodies between days 7 and 21 followed by a marked decrease in antibody titers. Similarly, lambs vaccinated with ovalbumin at

28 and 42 days developed antibody titers that increased until 21 days after inoculation and then decreased (Lewis et al., 2017).

Other research has shown that neonatal lambs can generate independent immune responses, but the responses are highly dependent on dose of antigen, type of adjuvant, and the type of antigen presenting cell. One example was that the enteric immunization of newborn lambs induced both humoral and cell-mediated mucosal immune responses, although the ELISpot and gD-specific assays used displayed higher titers in five to six-week-old lambs (Mutwiri et al., 2000). Similarly, lambs vaccinated with KLH at 0 weeks generated anti-KLH Ab, but higher levels of Ab production were evident in older lambs (Gailor, 2007). Some researchers reported that young lambs were incapable of generating immunological memory (Corpa et al., 2000; Lewis et al., 2017)

Keyhole lymphocyte hemocyanin (KLH) is a common antigen used in immune-based studies because it has well documented immunostimulatory properties in humans and animals (Harris & Markl, 1999). Antigens are substances recognized by the host immune system as non-self. KLH is respiratory protein found in a marine mollusk, so it is foreign to passive, maternally derived immunoglobulins and complex enough to stimulate immune responses in lambs.

Enhancing complexity and degree of foreignness increase individual immune responses to antigens. A common approach to enhance immune responses to an antigen is by linking antigen to an adjuvant. Adjuvants linked to antigens in vaccines increase the complexity of the antigen and thus, immunogenicity, allowing for stronger, more sustained immune responses (Coffman et al., 2010). A common adjuvant, and the adjuvant used in this experiment, is alum, an aluminum hydroxide and magnesium hydroxide-based adjuvant. While little is known about the mechanism of action associated with adjuvants, besides their role in inducing and

maintaining a strong humoral response, recent studies have demonstrated that aluminum hydroxide plays an important part in establishing memory responses by stimulating macrophage activation, in addition to myeloid dendritic cells (Rimaniol et al., 2004).

Vaccines act to introduce novel antigens to stimulate immune cells. Macrophages ingest and display antigen(s) on major histocompatibility complex (MHC) molecules. T cells recognizes novel antigen(s) on the MHC and stimulate B cells to produce antibodies specifically for each antigen (Institute of Medicine, 1997; Day, 2007). The first time a host immune system recognizes and generates an immune response to an antigen is called the primary immune response. Generally, cytokines (soluble mediators) influence which isotype of immunoglobulin the B cells generate, but the antibody secreted in the primary immune response is IgM. This antibody is a pentamer with 10 antigen binding sites. When an immune response to an antigen is terminated, memory cells are formed. When an individual receives a booster vaccination, the antigen triggers memory cells to activate plasma B cells which secrete high levels of antibody that already have specificity for that antigen from the primary immune response for rapid clearance of antigen. The antibody primarily secreted in the secondary immune response to vaccination is IgG which contains multiple subtypes and has greater ability to bind and clear antigen (Institute of Medicine, 1997).

Introduction

While sheep were some of the first animals to be domesticated by humans, their relevance for providing both clothing fiber and meat has decreased in the United States since 1945. There are multiple explanations for this decline, but high lamb mortality is one of the major reasons (Jones, 2004).

Antibody-rich colostrum is paramount for early immune function in lambs. Vaccination of ewes prior to lambing insures that neonates receive passive immunity to diseases common in young lambs, like enterotoxaemia (De La Rosa et al., 1997). Variation of the amount of colostrum received, and genetic factors in the ewe can result varied amounts of antibody providing passive immunity for lambs (De La Rosa et al., 1997; Hernández-Castellano et al., 2014) These findings demonstrate the importance of establishing independent immunocompetence as quickly as possible to promote lamb health. However, studies investigating the development of the lamb immune system have reached varied conclusions. While some researchers have demonstrated immunocompetence in lambs as early as two days of age (Gailor, 2007; Mutwiri, Bateman, Baca-Estrada, Snider, Griebel, et al., 2000), others observed a lack of immunological memory in young lambs (Corpa et al., 2000; Lewis et al., 2017).

Recent research has suggested a possible role for commensal bacteria in early immune development in neonates (Abd El-Tawab et al., 2016; Bauer et al., 2006; Isolauri et al., 2001; Perdigon et al., 1995; Roos et al., 2010). Thus, while maternal antibodies provide a basic immune system to defend against known pathogens, the immune system of the young animal develops and learns to tolerate commensal microbiota that play a varied and important role in both mucosal and systemic immune function (Abd El-Tawab et al. 2016; Bauer et al. 2006; Belkaid and Hand 2014). This suggests that increasing early gastrointestinal tract colonization by

commensal microbiota or modulating the populations of organisms in the gut could modulate immune response in neonates, possibly even improving them.

The purpose of this experiment was to begin to identify the age at which lambs are first capable of generating an immune response and immunological memory to specific pathogens and to determine if weekly probiotic administration would influence this response. This information could contribute to strategies to decrease mortality in lambs. As globalization continues, the value of lamb, mutton, and wool in many cultures around the world will become more important. Additionally, there is likely to be increased consumer preference for meat that is more environmentally friendly than beef. This suggests that there is an opportunity for the sheep industry to grow in the United States. Further characterization of the lamb immune system would allow for the development of successful vaccination regimens, thus preventing diseases and decreasing mortality rates. Decreasing mortality rates would fundamentally improve animal welfare and increase profitability of farms.

Materials and Methods

Experimental Animals

Lambs in this experiment were born starting on October 30, 2017 and sired by ½ East Friesian x ¼ Dorset x ¼ Tunis rams and out of Finnsheep x Dorset or Dorset ewes. Lambs were artificially reared on the cold-milk lambar system (see appendices for specific lamb birthdates and sampling dates). Prior to any lambs being born in the fall lambing of 2017, they were assigned by birth order randomly to treatment groups to ensure that all treatment groups would include a minimum of 2 lambs. Additional lambs were assigned as controls (Table 1).

Table 1. Design of experiment using keyhole lymphocyte (KLH) antigen[1].

Probiotic	Vaccine		
Initial vaccination age	Control	Adjuvant	Adjuvant + KLH
No probiotic	- - - - - - - - - - - - Number of lambs - - - - - - - - - - - -		
2 days	1	2	2
16 days	1	2	2
30 days	0	3	2
Probiotic			
2 days	1	2	2
16 days	1	2	2
30 days	0	2	2

[1]Blood was collected from each lamb prior to initial vaccination (SmplD 0), prior to the booster 14 days later (SmplD 14)), and 14 days after the booster (SmplD 28).

Lambs were assigned to one of two probiotic groups (none or probiotic). Probiotic (*Probios Bovine Oral Gel for Ruminants* at not less than 10 million CFU2/g) contained lactic acid bacteria (*enterococcus faecium, lactobacillus acidophilus, lactobacillus casei, and lactobacillus plantarem)*. Lambs receiving probiotic were administered five grams at two days of age. Subsequent dosing of probiotics occurred on the same day of the week (Monday) for convenience during the experiment. Five grams of probiotics were administered to lambs when they were between days 2 and 15 of age, 10 grams between days 16 and 29 of age, and 15 grams from day 30 until completion of the experiment. Increasing dosage was to account for increasing weight of lambs.

The probiotic and no-probiotic lambs were distributed among 3 vaccination groups (control, adjuvant only, or adjuvant + KLH antibody), and 3 ages of initial vaccination (2, 16,

and 30 days) as shown in Table 1. Each lamb received the vaccination protocol to which it was assigned and was boosted 14 days later. Blood samples were collected prior to initial vaccination (SmplD 0), prior to booster (SmplD 14), and at 28 days after initial vaccination (SmplD 28).

Preparation of KLH Vaccine

To prepare the KLH vaccine, KLH, keyhole lymphocyte hemocyanin (Calbiochem) was dissolved in sterile phosphate buffered saline (PBS) to 10 mg/mL stock solution. For vaccination, 0.25 mg of KLH in a total volume 250 µL of PBS was mixed with 250 µL Imject Alum (Thermo Fisher), an aluminum hydroxide and magnesium hydroxide-based adjuvant. Imject was added dropwise with constant mixing to the KLH for final ratio of 1:1. The KLH alum solution was rocked on a medium speed rocker for thirty minutes before administration or refrigeration. If refrigerated, the vaccine solution was remixed by shaking prior to administration. Lambs were given the vaccine at the age that corresponded with their assigned start date (either 2, 16, or 30 days of age) and then received a booster vaccine two weeks later.

For each vaccination and booster, lambs received 500 µL. The lambs in groups assigned to receive adjuvant alone were given 250 µL Imject Alum mixed with 250 µL of PBS-prepared as described above. Both were administered to lambs by subcutaneous injection immediately following blood sample collection.

Blood Collection and Storage

Blood was collected from the jugular vein immediately prior to initial vaccination (SmplD 0) and the booster vaccination (SmplD 14). A final sample was obtained two weeks after the booster (SmplD 28). Blood was refrigerated overnight to allow it to clot and then centrifuged at 3,000 rpm for 15 minutes. Serum was removed and stored at -20ºC.

ELISA Assay

Anti-KLH IgM and IgG Ab levels were determined by ELISA. Briefly, the wells of Immulon 1 (VWR) microtiter plates were coated with 50 µL KLH diluted to 0.5 mg/well in carbonate buffer pH 9.6 and incubated overnight at 4ºC. The plates were then washed twice with 150 µL/well with PBS-1% Tween-20 pH 7.4, followed by two washes with 150 µL/well PBS pH 7.4. Non-specific binding to the wells was blocked by the addition of 50 µL/well PBS containing 1% BSA for one hour at room temperature. The plates were then washed as above and 50 µl of serum diluted in PBS with 0.1% Tween-20 pH 7.4 four-fold starting at 1:40 to 1:2560 was added to the wells in duplicates. The plates were covered with parafilm and incubated overnight at 4ºC. The plates were washed as above, and then 50 µL rabbit anti-sheep anti-IgM conjugated to horseradish peroxidase (HRP) (1:10,000 in PBS-0.1% Tween-20) or rabbit anti-sheep anti-IgG conjugated to HRP (1:9,000 in PBS-0.1% Tween-20) was added to each well. Both antibodies were obtained from BioRad. After addition of the rabbit anti-sheep Ig-HRP detector antibodies, the plates were covered with parafilm and then incubated at room temperature for two hours. Then, plates were washed as above and 50 µL of TMB substrate (Thermo Fisher) was added to the wells and the plates were incubated in the dark for thirty minutes. Color development was stopped with 2 M sulfuric acid, and the absorbance–corresponding to the amount of substrate– was read at 450 nm using a SpectraMax 190 ELISA Reader, equipped with Soft Max Pro 6.5.1 software (Molecular Devices).

Statistical analysis

Response variables included ELISA IgM and IgG optical densities with samples diluted 1:160 and the natural log transformed values for the reciprocal of the titer values (Reverberi, 2008). The statistical model included main effects of Probiotic, Vaccination, Vaccination Age, and Sampling Days relative to initial vaccination. All possible interactions were included. Lamb

within Probiotic, Vaccination, and Vaccination Age (with 13 degrees of freedom) was included as a random effect to test main effects of Probiotic, Vaccination, and Vaccination Age and their interactions. Residual variation (with 26 degrees of freedom) was used to test Sampling Day and all of its associated interactions.

Results

Effects of probiotic on the production of anti-KLH IgM after vaccination

We compared the production of anti-KLH IgM in 2-day old lambs that had received probiotics or not and were vaccinated at 2 days of age, and then received a booster vaccine at 16 days of age. Probiotic-treated lambs had received one dose at the time of vaccination. Serum samples were obtained immediately prior to vaccination (SmplD 0), immediately prior to booster vaccination (SmplD 14), and 14 days after that (SmplD 28). Serum was measured for levels of anti-KLH IgM in a direct ELISA. The results are shown in Figure 1. All sera from treatment and control groups were diluted 1:160 and the level of bound anti-KLH IgM, corresponding to the absorbance at 450 nm, was determined. As expected, the levels of anti-KLH IgM antibodies were increased at SmplD 14 and SmplD 28, after the first and second immunizations respectively, compared to SmplD 0.

Probiotic administration had no significant effect on the levels of anti-KLH specific IgM antibodies produced in 2-day old lambs compared with lambs that did not receive probiotics.. However, there was a trend toward higher levels at SmplD 14 in lambs that had received probiotics compared with lambs that had not received probiotics at the same timepoint. In both probiotic groups, the levels of anti-KLH IgM produced at SmplD 28 were slightly lower than at SmplD 14 but still increased compared to the levels that were produced at SmplD 0.

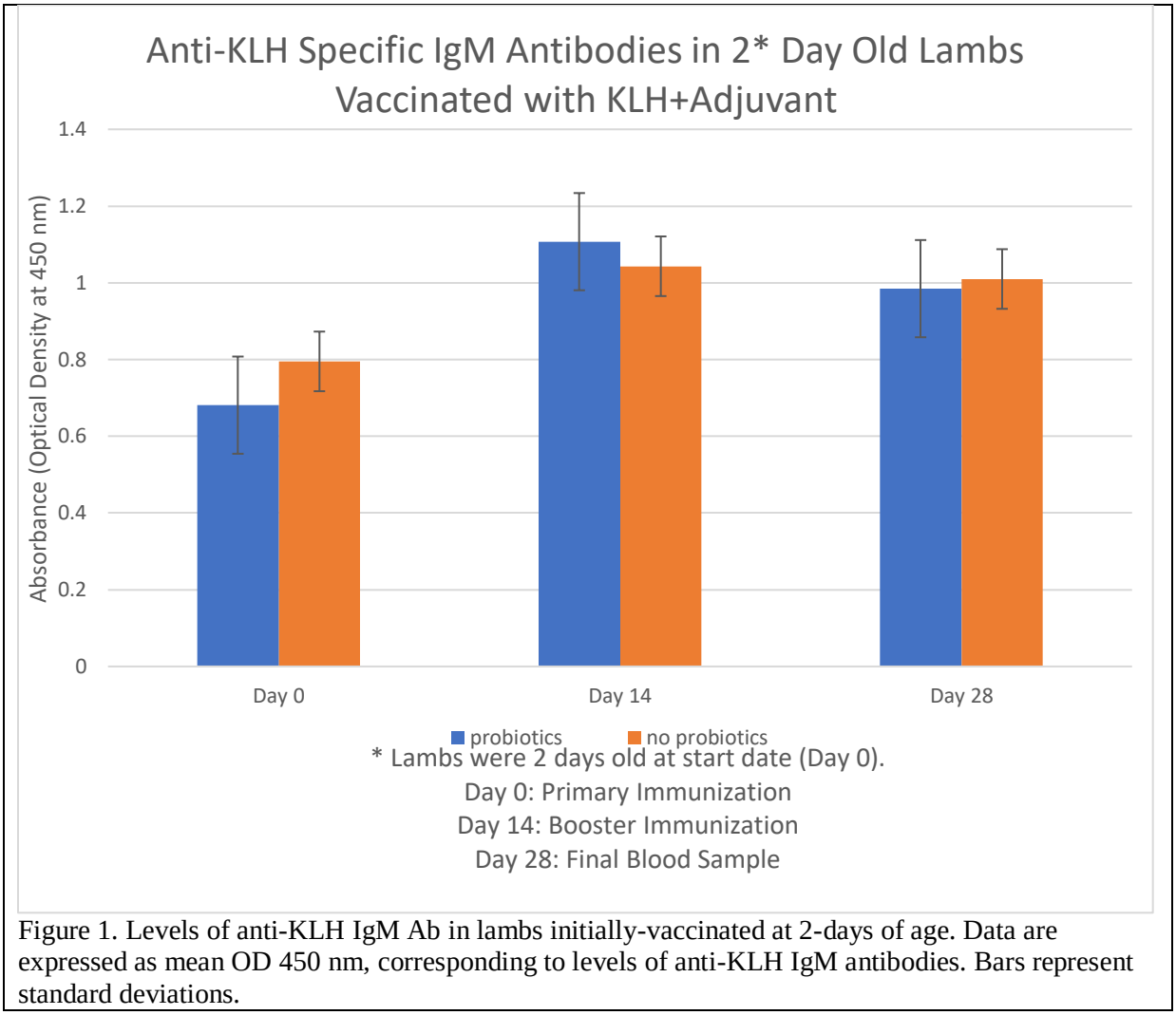

Figure 1. Levels of anti-KLH IgM Ab in lambs initially-vaccinated at 2-days of age. Data are expressed as mean OD 450 nm, corresponding to levels of anti-KLH IgM antibodies. Bars represent standard deviations.

We next determined levels of anti-KLH IgM in 16-day old lambs that had received either probiotics or not, vaccinated initially at 16 days of age and then received a booster vaccine at 30 days of age. Serum samples (from SmplD 0, 14, and 28 after initial vaccination) were measured for anti-KLH IgM by ELISA as described above, and the results are shown in Figure 2. At 16 days of age, the lambs had received two doses of probiotics. Like the data for 2-day old lambs, we found that probiotics did not modulate the production of anti-KLH specific IgM Abs in lambs that were vaccinated at 16 days of age. Relatively high ODs were recorded at SmplD 0 in both probiotic-treated and untreated lambs, which may be due to non-specific binding of serum

proteins. Absorbances for SmplD 14 samples were slightly lower than for SmplD 0, but similar for probiotic or non-probiotic treated animals. SmplD 28 samples showed increased ODs in animals that had not received probiotics, however there was variability in individual samples as indicated by large standard deviation bars (Figure 2).

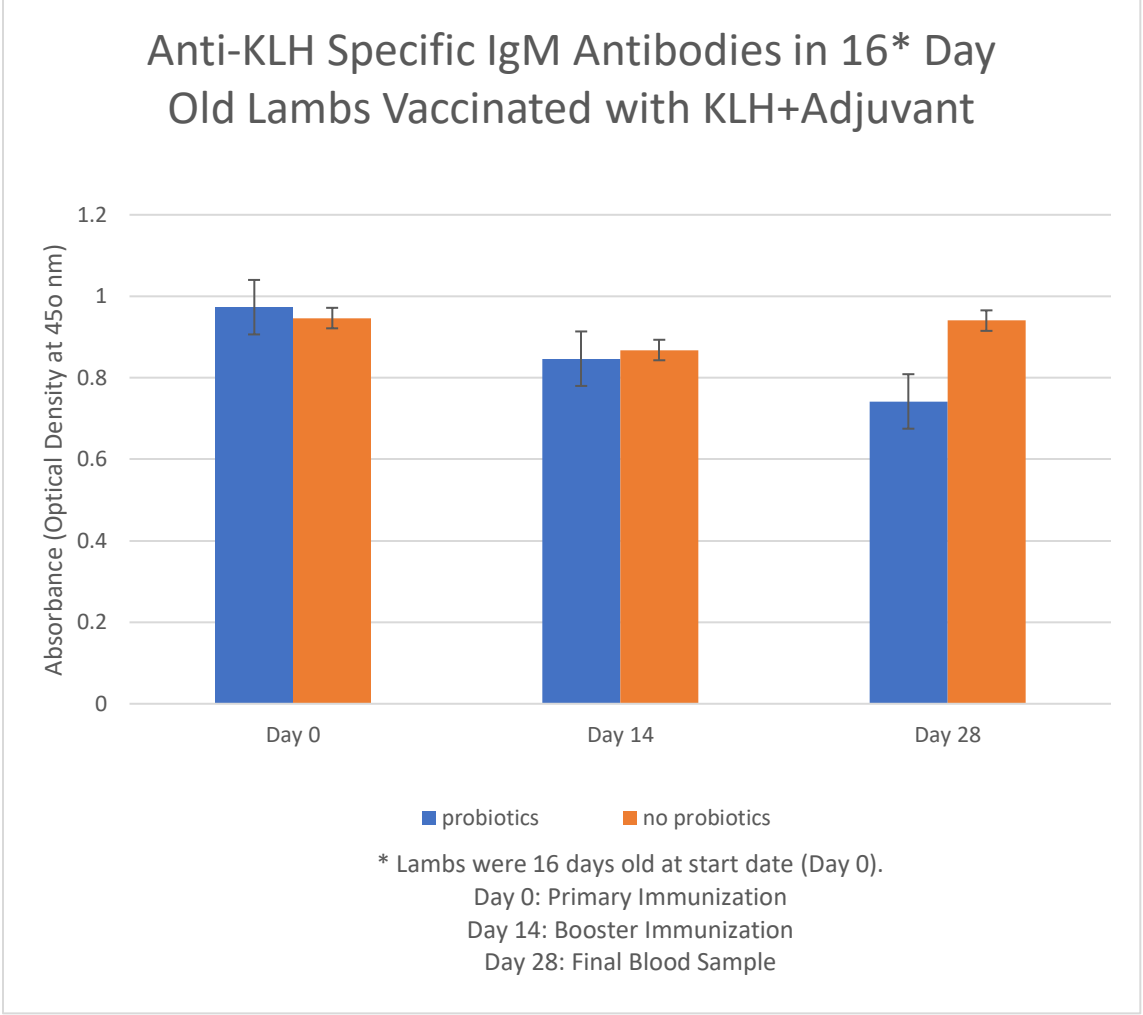

Figure 2. Levels of anti-KLH IgM Ab in 16-day-old lambs. Data are expressed as mean OD 450 nm, corresponding to levels of anti-KLH IgM antibodies. Bars represent standard deviations.

We also compared the production of anti-KLH IgM in 30-day old lambs that had received either probiotics or not, blood-sampled and vaccinated at 30 days of age (SmplD 0), and then blood-sampled and received a booster vaccine at 44 days of age (SmplD 14) and blood-sampled at 48 days of age (SmplD 28). Lambs that were 30 days old had received four doses of

probiotics. SmplD 0, 14, and 28 serum samples were measured for anti-KLH IgM by ELISA as described above, and the results are shown in

Figure 3. In contrast to the results for 2- and 16-day old lambs, lambs that had received probiotics and then were vaccinated at 30 days of age showed a trend toward an improved immune response, with increased production of anti-KLH specific IgM antibodies in probiotic-treated lambs compared with lambs that had not received probiotics at SmplD 0 and SmplD 14, and lower but still slightly higher than non-probiotic treated lambs at SmplD 28 (Figure 3).

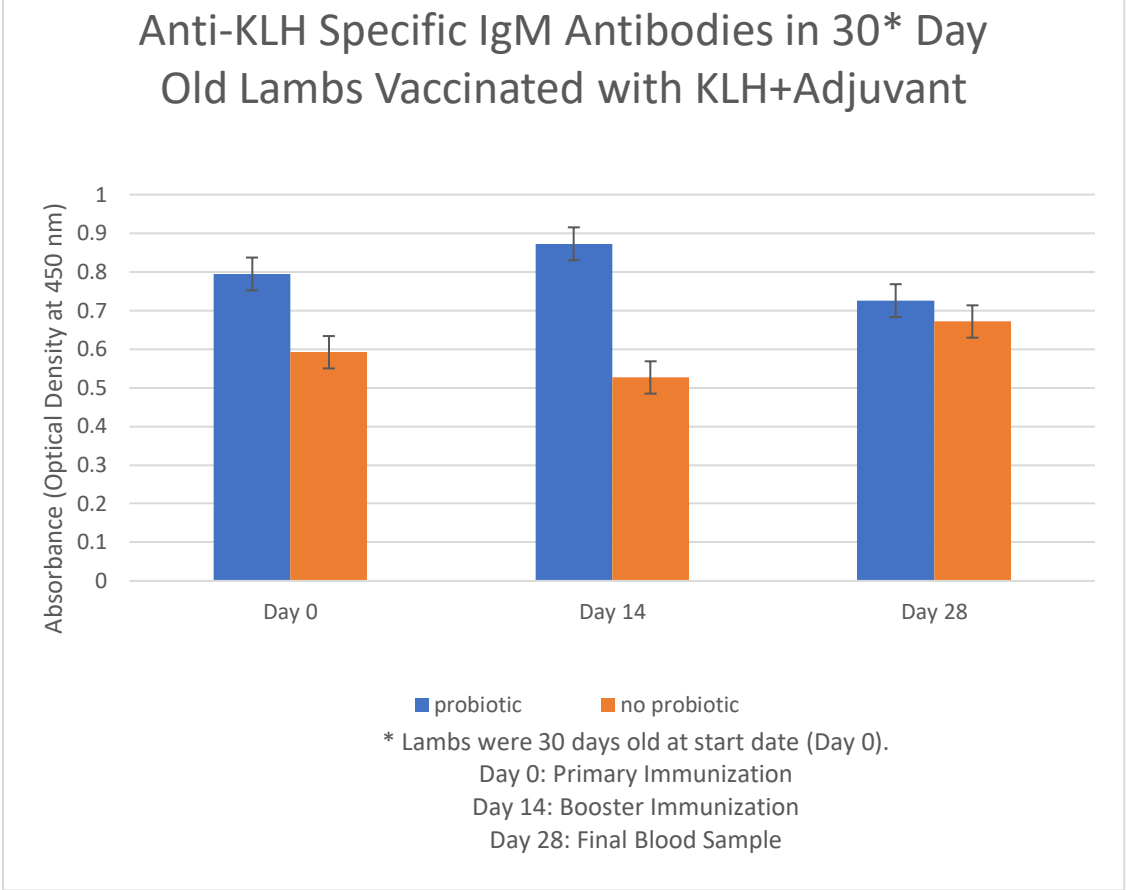

Figure 3. Levels of anti-KLH IgM Ab in 30-day-old lambs. Data are expressed as mean OD 450 nm, corresponding to levels of anti-KLH antibodies. Bars represent standard deviations.

Effects of probiotic on the production of anti-KLH IgG after vaccination

The production of anti-KLH IgG in 2-day old lambs that had received either probiotics or not and were vaccinated at 2 days of age, and then received a booster vaccine at 16 days of age was determined. Lambs had received one dose of probiotics. Serum samples at SmplD 0, 14, and 28 were measured for anti-KLH IgG levels by ELISA and the results are shown in Figure 4

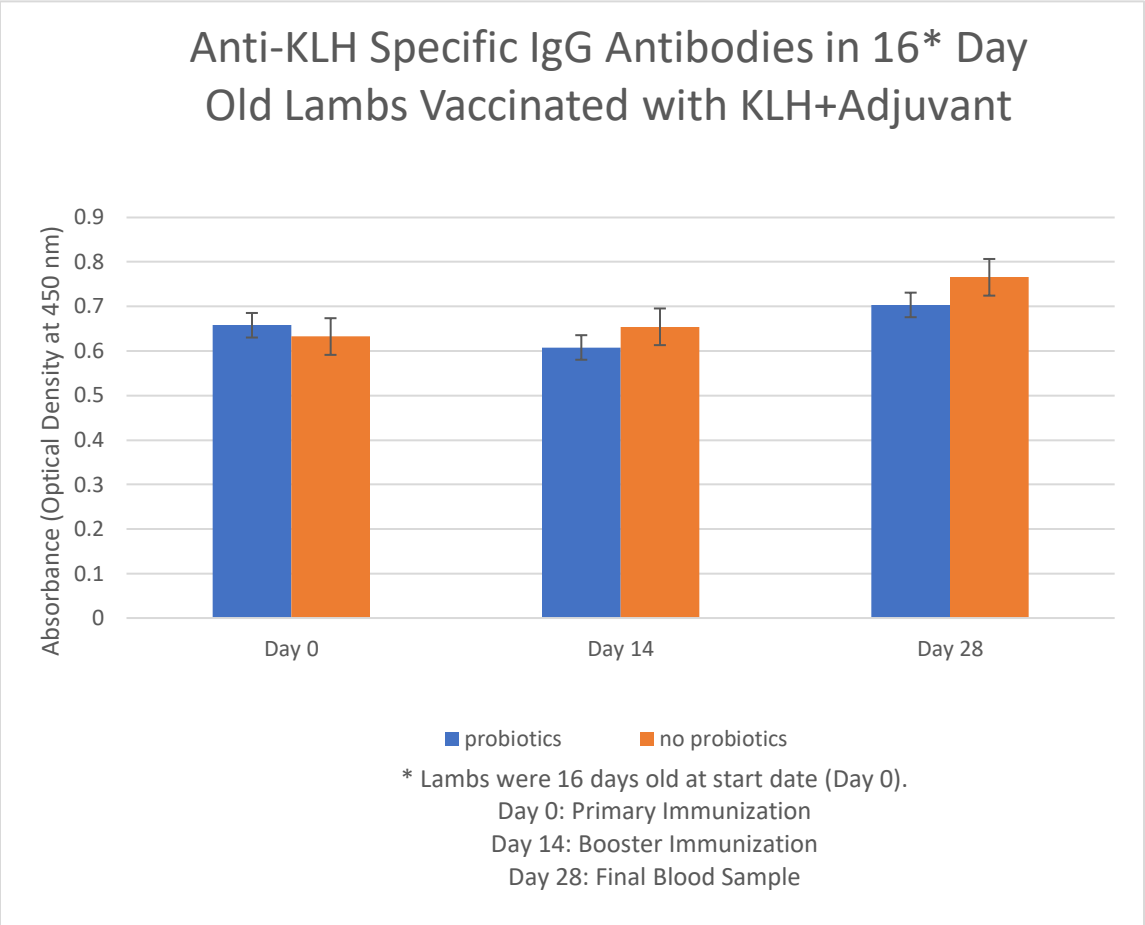

Figure 5. All sera from treatment and control groups were diluted 1:160 and the level of bound anti-KLH IgG was measured, corresponding to the absorbance at 450 nm. While no significant differences

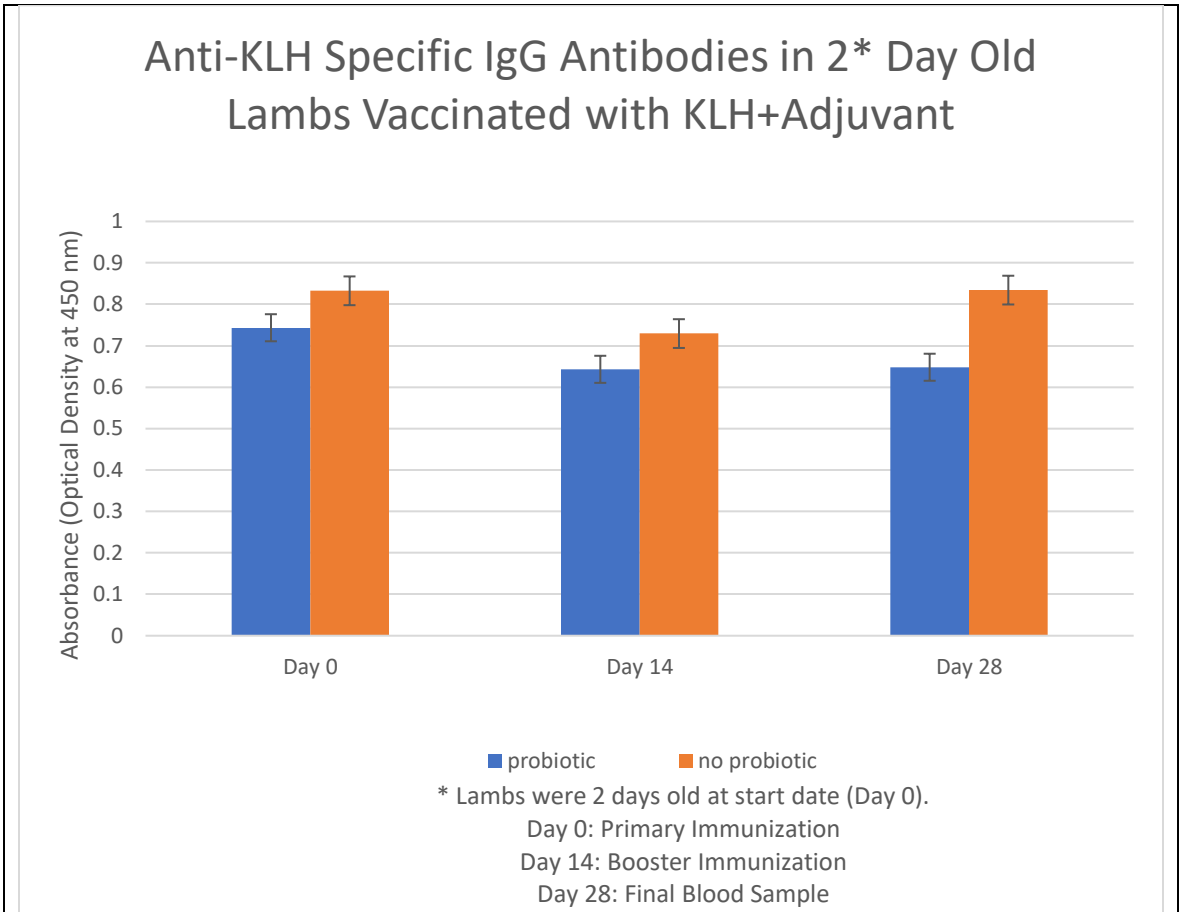

Figure 4. Levels of anti-KLH IgG Ab in 2-day-old lambs. Data are expressed as mean OD 450 nm, corresponding to levels of anti-KLH antibodies. Bars represent standard deviations.

were detected, there was a trend toward lower levels of anti-KLH IgG antibodies in lambs in treated with probiotics compared to non-probiotic treated lambs. This trend was also observed on SmplDs14 and 28. Lambs that did not receive probiotics maintained higher anti-KLH IgG Ab levels overall compared to lambs that received probiotics.

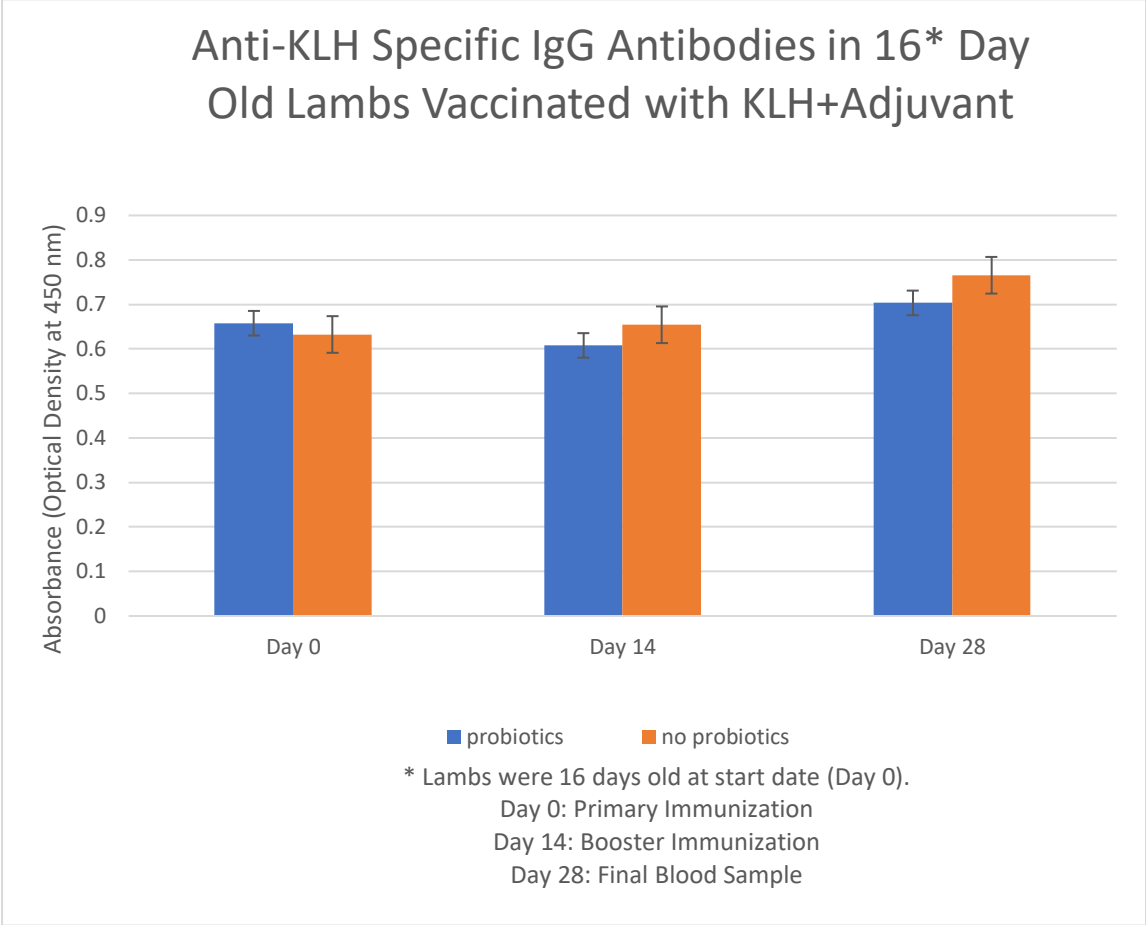

Figure 5. Levels of anti-KLH IgG Ab in 16-day-old lambs. Data are expressed as mean OD 450 nm, corresponding to levels of anti-KLH antibodies. Bars represent standard deviations.

The levels of anti-KLH IgG in 16-day old lambs that had received either probiotics or not and were vaccinated at 16 days of age with a booster at 30 days of age are shown in Figure 5. SmplD 0, 14, and 28 serum samples were measured in a direct ELISA as described as above. SmplD 0 samples for both probiotic and non-probiotic groups showed higher than expected but similar absorbance levels for both probiotic groups, which likely was due to non-specific binding or interactions of serum proteins with ELISA wells. The absorbance of serum samples from SmplD 14 was decreased in lambs that received probiotics but slightly increased in lambs that did not receive probiotics. Absorbances at SmplD 28 were increased for both probiotic-treated

and untreated lambs, with the lambs that had not received probiotics showing a trend to higher absorbance levels.

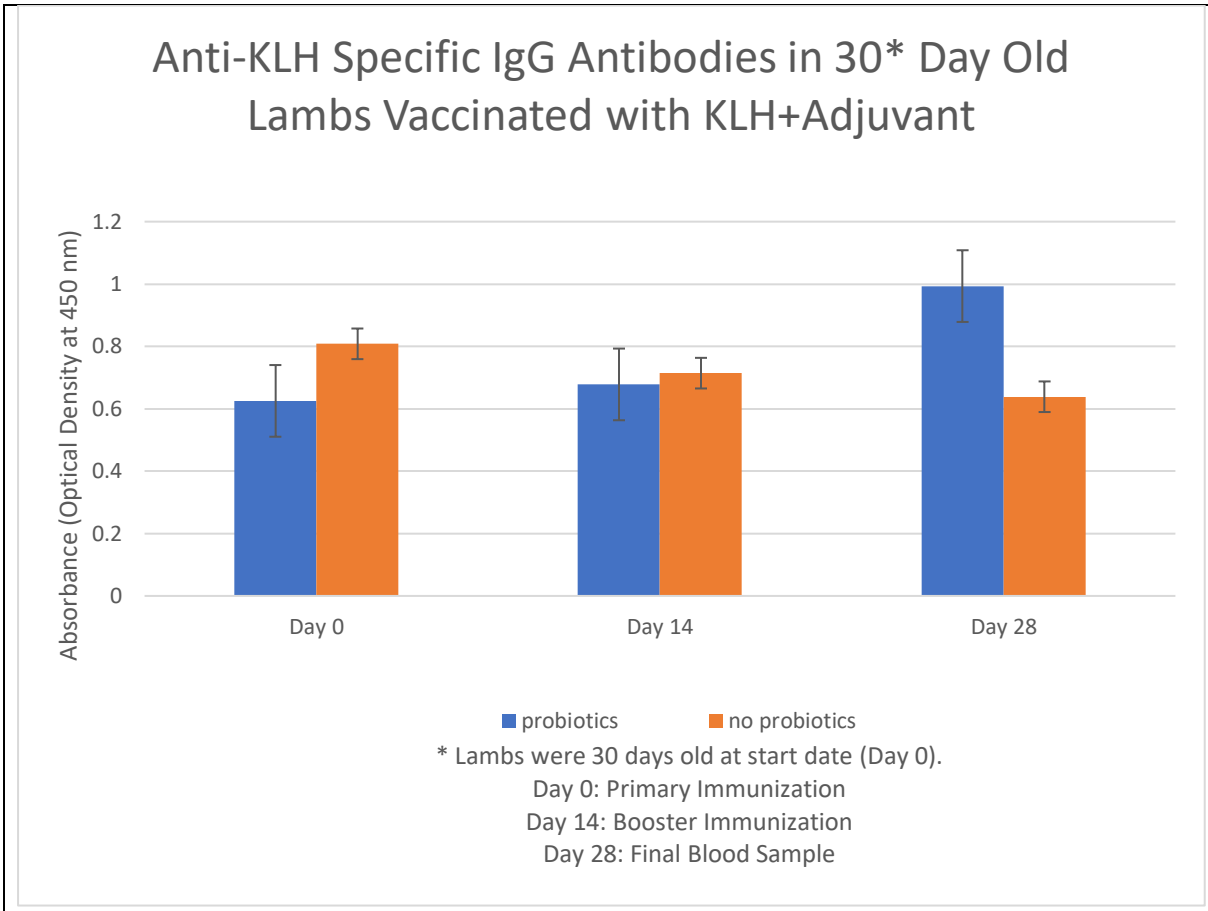

Figure 6. Levels of anti-KLH IgG Ab in 30-day-old lambs. Data are expressed as mean OD 450 nm, corresponding to levels of anti-KLH antibodies. Bars represent standard deviations.

Data for 30-day old lambs that had received either probiotics or not and were vaccinated at 30 days of age followed by a booster 14 days later are shown in Figure 6. A direct ELISA as described above was used to determine anti-KLH IgG levels in serum samples taken at SmplD 0, 14, and 28. At 30 days of age, the lambs had received four doses of probiotics. At SmplD 0, the absorbance level was lower in lambs that had received probiotics compared to lambs that had not received probiotics, while at SmplD 14 there were similar levels of anti-KLH specific IgG were in both probiotic groups. While there was variation in the level of antibody at SmplD 28 in both groups, as shown by the relatively large standard deviations, there was a trend toward increased

levels of production of anti-KLH specific IgG in lambs that had received probiotics, and relatively decreased, or perhaps less enhanced, anti-KLH specific IgG levels in lambs that had not received probiotics.

Statistical Analysis of the Data

The summary of the statistical analyses of the data is in Table 2 . There were differences in anti-KLH IgG levels that depended on the age of the lambs at vaccination (Vaccination age x Sample time interaction, $P < 0.001$). While statistical analyses did not detect a significant effect of probiotic on the levels of anti-KLH specific IgM or IgG produced by lambs vaccinated at any age in this experiment, the trends in the data indicate that probiotic treatment may modulate the immune response, suggesting that further investigation in a larger study is warranted.

Interestingly, when IgM binding to KLH over time was compared for the 2, 16 and 30-day old animals, much a lower absorbance reading was observed in the 2-day lambs compared to the 16 and 30-day old animals, that showed relatively high absorbance at SmplD 0 (Figure 7). Higher absorbance indicates greater specific antibody binding within the individual ELISA wells. This difference in the kinetics of the antibody response was not unexpected, since the immune system of newborn lambs is immature and develops as the animals age. Sex and breed differences may also contribute to differences in immune responses among the lambs.

Table 2. Effect of probiotic and vaccination on serum concentrations of IgM and IgG antibodies to KLH in lambs vaccinated at 2, 16, and 30 days of age and sampled at 0, 14 (booster given), and 28 days after vaccination.

	Optical density read at 450 nm and at 1:160 dilution		IgM		IgG	
Item	IgM	IgG	ln	Geometric mean	ln	Geometric mean
Probiotic						
No	0.87	0.67	7.28	1451	6.50	665
Yes	0.93	0.60	7.35	1556	6.50	665
SE	0.045	0.034	0.158		0.280	
P-value	0.402	0.151	0.775		1.000	
Vaccination						
Adjuvant	0.95	0.63	7.44	1703	6.50	665
Adjuvant + KLH	0.85	0.64	7.19	1326	6.50	665
SE	0.046	0.034	0.158		0.280	
P-value	0.143	0.883	0.287		1.000	
Vaccination age, days						
2	0.92	0.67	6.57699	718	6.92	1012
16	0.98	0.51	7.84776	2560	6.11	450
30	0.81	0.71	7.52044	1845	6.46	639
SE	0.05	0.042	0.193		0.342	
P-value	0.132	0.011	0.001		0.280	
Sample day, days from vaccination						
0	0.86	0.59	6.51923	678	6.46	639
14	0.93	0.64	7.63597	2071	6.27	528
28	0.92	0.67	7.79000	2416	6.77	871
SE	0.043	0.030	0.142		0.311	
P-value	0.289	0.076	< 0.001		0.493	
Vaccination age X Sample day	ns	ns	< 0.001		ns	

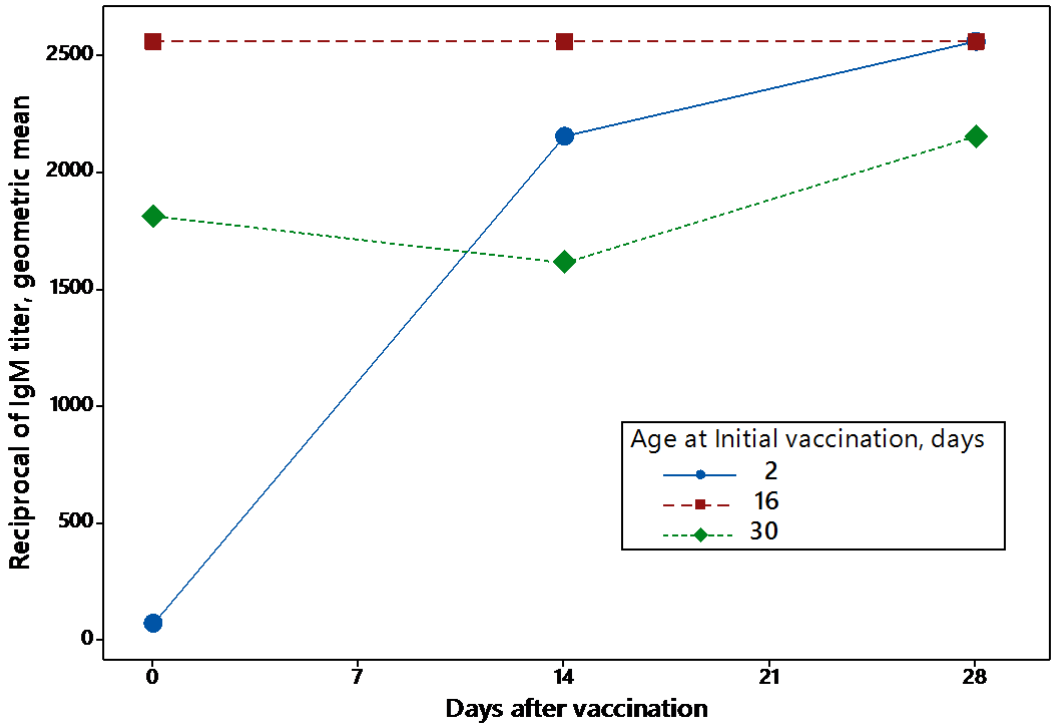

Figure 7. Effect of vaccination age on the relationship of the IgM titer to days after vaccination. Lambs received the first dose of vaccine on SmplD 0, and a booster vaccine on SmplD14 after the initial vaccination.

Discussion

The purpose of this experiment was to begin to define the age at which lambs are first capable of generating an immune response and immunological memory to specific pathogens and to determine if weekly probiotic administration would influence this response. Time of initial vaccination combined with time after vaccination influenced the production of anti-KLH-specific IgM in lambs vaccinated with KLH in alum adjuvant (Table 2). Our observation that 2-day old lambs were immunocompetent, but that as animals aged the immune system matured with increasing immune function, is in agreement with prior research (Chase et al., 2008; Corpa et al., 2000; Gailor, 2007; Lewis et al., 2017; Mutwiri et al., 2000).

In the present experiment, lambs received maternal colostrum during the first twenty-four hours of life. While this is a common and critical management practice used in sheep operations it likely contributed to some of the variability in our data. Individual ewes could produce different amounts and quality of colostrum, resulting in differences in the levels of circulating antibodies and colostrum-dependent proteins in the individual lambs. Other researchers have found that colostrum was necessary for increased plasma concentration of apolipoprotein A-IV, plasminogen, serum amyloid A, and fibrinogen levels, most of which are proteins with immune functions (Hernández-Castellano et al., 2014). In the present experiment, we would not have expected ewes to provide lambs with specific antibody protection against KLH since they did not receive the vaccine, but nonspecific Ab interaction and binding of colostrum-derived proteins might have contributed to the higher than anticipated absorbance reading for control and adjuvant lambs as well as in the SmplD 0 serum samples (see Figures 1-6; data not shown for adjuvant and control lambs). Additionally, soluble mediators such as cytokines, adipokines, or complement components produced during the innate immune response to vaccination might also have contributed to higher than expected absorbance values.

Although not statistically significant, we saw trends in an influence of probiotics on anti-KLH IgM and IgG production in animals vaccinated at 16 and 30 days of age, which would have received two and four doses of probiotics respectively, suggesting that as beneficial organisms become established in the gastrointestinal tract, they may contribute to enhanced immune responses. In nature, colonization of the gastrointestinal tract of lambs begins in the birth canal with commensal organisms unique to the ewe. While the ewes in this experiment were the similar breeds and housed under the same conditions, it is likely that lambs started life with a different and individual microbiota repertoire based on commensal organisms unique to their

dam, which may also have contributed to some of the variability that we observed in SmplD 0 serum samples. In this experiment, lambs received weekly doses of probiotics from birth, and just as natural microbial colonization takes time to become established, artificial colonization with probiotics containing bacterial strains would likely take some time to equilibrate and establish in the gut so that any potential beneficial effects on immune function may not be observed until that occurs (Roos et al., 2010; Wu et al., 2016).

IgM is synthesized in newborns shortly after birth and is followed several weeks later by the production of IgG. The ability to newly synthesize IgA and antibodies of other isotypic classes occurs even later. The results of the present experiment also showed that a lamb's ability to synthesize antigen-specific immunoglobulin increased as it aged. The trends that we observed in the production of anti-KLH IgG by 2, 16 and 30-day lambs receiving probiotics suggests that probiotics may help to increase the ability of the lamb immune system to produce antigen-specific IgG at an earlier age and/or at higher titers compared to animals that do not receive probiotics. This is in agreement with previous studies, which in addition, found that the effect of probiotics on vaccine efficacy was also dependent upon the bacterial strain of probiotic used, the number of doses of probiotic that were administered, and the timing of probiotic administration relative to vaccination (Zimmermann & Curtis, 2018). The route of vaccination was also critical, with probiotic treatment having a greater effect on the production of immunoglobulin in response to an oral vaccine compared to the production of immunoglobulin when a vaccine to the same antigen was administered subcutaneously. Thus, probiotics will likely have different effects on antibody production after vaccination and in natural infection, but the mechanisms that are involved remain largely unknown (Kukkonen et al., 2006; Zimmermann & Curtis, 2018).

We also found that, while anti-KLH IgM levels were high and unchanged in all samples from 16 and 30-day old lambs, SmplD 0 levels in 2-day old lambs were low, but then increased at the later time points. We assumed that none of the sheep in this experiment would previously have been exposed to the KLH antigen. Therefore, anti-KLH IgM levels also should have been low at SmplD 0 in lambs first vaccinated at 16 and 30 days of age. Perhaps, because the lambs were all kept in the same pen, lambs first vaccinated at 16 and 30 days of age had been exposed to the KLH antigen after the 2-day old lambs were vaccinated.

The lower levels at SmplD 0 in the 2-day old lambs might be that these lambs would have higher levels of circulating maternal antibody compared to the older lambs. The maternal antibody may have interfered with the ability of the 2-day old lambs to respond to the KLH vaccine by masking antigenic epitopes on KLH and/or down-modulating B cell function by blocking B-cell signaling (Nowak & Poindron, 2006; Sawyer et al., 1977). Another difference, as discussed previously, might also be the differences in the microflora of the younger lambs.

There were also sex and slight breed differences among lambs that may have impacted individual lambs' abilities to mount an immune response. Sex steroids have been shown to directly impact immune function and development, and in humans, estradiol provides an immune-protective role while testosterone is more immunoinhibitory. Both steroid hormones are thought to function in a similar manner in sheep, leading to sex differences in immune responses (Giefing-Kröll et al., 2015) . In the present experiment, because of the limited numbers of lambs that were available, both male and female lambs were used, and assigned randomly before birth to the treatment groups. In the older lambs, increased levels of these hormones could also have affected the immune response to KLH.

Genetic differences in the major histocompatibility complex (MHC) among the lambs may have impacted the immune response to KLH because they were sired by crossbred rams that were ½ East Friesian x ¼ Dore x ¼ Tunis breeds. Breed purity inherently results in limited genetic heterozygosity at MHC loci and results in differences in ability to mount specific immune response between breeds (Day, 2007). Presentation of antigen bound to MHC on antigen-presentation cells is required to activate T cells in the adaptive immune response that results in the production of antibody. Thus, different genetic backgrounds of the lambs may have had an impact on immunoglobulin production (Day, 2007). To account for these variables, future studies should include larger numbers of animals of the same sex and genetic background, if possible.

Finally, the probiotic that was used in this experiment was a commercial preparation that contained mainly lactic acid bacteria species, which have been shown to promote a Th1cytokine response and inhibit intestinal Th2 cytokine responses (Wu et al., 2016). The humoral immune response, which includes the production of antibodies, is mainly Th2 cytokine-driven. We chose to use Alum as an adjuvant in this experiment as there is a large body of data supporting that it skews immune responses to Th2 cytokine and antibody production (Coffman et al., 2010). However, other research has shown that Alum can stimulate both Th1 and Th2 immune responses (Brewer et al., 1999). Consequently, the differences in the cytokine profiles induced by the probiotics and the adjuvant may also have influenced antibody production.

Conclusion and Future Directions

In this pilot experiment, we found that antigen-specific antibody titers that develop after vaccination were affected by the age at vaccination and time after vaccination. Trends in the data pointed to the possibility that probiotic administration could lead to earlier and higher levels of

specific antibody production. This should be explored further. The data also suggested that more rapid establishment of the probiotic populations in the GI tract could enhance the beneficial effects of these populations on immune function. For example, early and perhaps larger and/or more frequent dosing with probiotics could have a positive effect, but the time, cost and management issues required to implement this into general lamb flock management might be prohibitive.

Future studies could also include analysis of probiotic impact on immune function in colostrum-deprived lambs. This would be extremely interesting because it could suggest ways to enhance the immunocompetence of these lambs and provide a unique look at immune system development in the absence of maternal Ab or protein interactions. Other studies could investigate immunomodulatory capabilities of different species and strains of commensal organisms to assess their potential to increase the overall immune response in lambs, in addition to antibody production.

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Appendix Tables

Appendix Table 1. Lamb Assignment, Randomization, Birth and Sampling Dates

Lamb group	Vaccine age, days	Probiotic	Vaccine	Lamb count	Rand()	Lamb	Birth date	Initial blood & vaccine	Blood & booster	Final blood
1	30	No	Adjuvant	1	0.9256003047	1	Friday 27 Oct 2017	Sunday 26 Nov 2017	Sunday 10 Dec 2017	Sunday 24 Dec 2017
1	30	No	Adjuvant+KLH	2	0.307517531	12	Monday 30 Oct 2017	Wednesday 29 Nov 2017	Wednesday 13 Dec 2017	Wednesday 27 Dec 2017
1	30	Yes	Adjuvant	3	0.6098511838	8, 24	Sunday 29 Oct 2017	Tuesday 28 Nov 2017	Tuesday 12 Dec 2017	Tuesday 26 Dec 2017
1	30	Yes	Adjuvant+KLH	4	0.5857173183	9	Sunday 29 Oct 2017	Tuesday 28 Nov 2017	Tuesday 12 Dec 2017	Tuesday 26 Dec 2017
1	16	Yes	Control	5	0.3638005925	11	Monday 30 Oct 2017	Wednesday 15 Nov 2017	Wednesday 29 Nov 2017	Wednesday 13 Dec 2017
1	16	No	Control	6	0.3672866493	10	Monday 30 Oct 2017	Wednesday 15 Nov 2017	Wednesday 29 Nov 2017	Wednesday 13 Dec 2017
1	16	No	Adjuvant	7	0.03481769076	14	Tuesday 31 Oct 2017	Thursday 16 Nov 2017	Thursday 30 Nov 2017	Thursday 14 Dec 2017
1	16	No	Adjuvant+KLH	8	0.8385464955	3	Friday 27 Oct 2017	Sunday 12 Nov 2017	Sunday 26 Nov 2017	Sunday 10 Dec 2017
1	16	Yes	Adjuvant	9	0.8773943593	2	Friday 27 Oct 2017	Sunday 12 Nov 2017	Sunday 26 Nov 2017	Sunday 10 Dec 2017
1	16	Yes	Adjuvant+KLH	10	0.06423881815	13	Monday 30 Oct 2017	Wednesday 15 Nov 2017	Wednesday 29 Nov 2017	Wednesday 13 Dec 2017
1	2	No	Adjuvant	11	0.7558487246	5	Saturday 28 Oct 2017	Monday 30 Oct 2017	Monday 13 Nov 2017	Monday 27 Nov 2017
1	2	No	Adjuvant+KLH	12	0.6957488782	6	Saturday 28 Oct 2017	Monday 30 Oct 2017	Monday 13 Nov 2017	Monday 27 Nov 2017
1	2	Yes	Adjuvant	13	0.6918163294	7	Sunday 29 Oct 2017	Tuesday 31 Oct 2017	Tuesday 14 Nov 2017	Tuesday 28 Nov 2017
1	2	Yes	Adjuvant+KLH	14	0.7582917192	4	Saturday 28 Oct 2017	Monday 30 Oct 2017	Monday 13 Nov 2017	Monday 27 Nov 2017
2	2	No	Control	15	0.5742720214	18	Wednesday 1 Nov 2017	Friday 3 Nov 2017	Friday 17 Nov 2017	Friday 1 Dec 2017
2	2	Yes	Control	16	0.03963477679	25	Thursday 16 Nov 2017	Saturday 18 Nov 2017	Saturday 2 Dec 2017	Saturday 16 Dec 2017
2	16	No	Adjuvant	17	0.4364590074	20	Wednesday 1 Nov 2017	Friday 17 Nov 2017	Friday 1 Dec 2017	Friday 15 Dec 2017
2	16	No	Adjuvant+KLH	18	0.9841829346	15	Tuesday 31 Oct 2017	Thursday 16 Nov 2017	Thursday 30 Nov 2017	Thursday 14 Dec 2017
2	16	Yes	Adjuvant	19	0.329719127	21	Wednesday 1 Nov 2017	Friday 17 Nov 2017	Friday 1 Dec 2017	Friday 15 Dec 2017
2	16	Yes	Adjuvant+KLH	20	0.5395539654	19	Wednesday 1 Nov 2017	Friday 17 Nov 2017	Friday 1 Dec 2017	Friday 15 Dec 2017
2	2	No	Adjuvant	21	0.1812163327	22	Friday 3 Nov 2017	Sunday 5 Nov 2017	Sunday 19 Nov 2017	Sunday 3 Dec 2017
2	2	No	Adjuvant+KLH	22	0.0474172919	23	Friday 3 Nov 2017	Sunday 5 Nov 2017	Sunday 19 Nov 2017	Sunday 3 Dec 2017
2	2	Yes	Adjuvant	23	0.8795969021	16	Wednesday 1 Nov 2017	Friday 3 Nov 2017	Friday 17 Nov 2017	Friday 1 Dec 2017
2	2	Yes	Adjuvant+KLH	24	0.8380960733	17	Wednesday 1 Nov 2017	Friday 3 Nov 2017	Friday 17 Nov 2017	Friday 1 Dec 2017
3	30	No	Adjuvant	25	0.229547498	29	Sunday 19 Nov 2017	Tuesday 19 Dec 2017	Tuesday 2 Jan 2018	Tuesday 16 Jan 2018
3	30	No	Adjuvant+KLH	26	0.947283336	26	Thursday 16 Nov 2017	Saturday 16 Dec 2017	Saturday 30 Dec 2017	Saturday 13 Jan 2018
3	30	Yes	Adjuvant	27	0.8129043397	27	Saturday 18 Nov 2017	Monday 18 Dec 2017	Monday 1 Jan 2018	Monday 15 Jan 2018
3	30	Yes	Adjuvant+KLH	28	0.5768342768	28	Sunday 19 Nov 2017	Tuesday 19 Dec 2017	Tuesday 2 Jan 2018	Tuesday 16 Jan 2018
4	None	No	Control	29	0.9840324181	30	Monday 20 Nov 2017	Wednesday 20 Dec 2017	Wednesday 3 Jan 2018	Wednesday 17 Jan 2018

Appendix Table 2. Lamb Breed, Sex, Parent IDs

LambID	Sex	BirthDate	LambBreed	SireID	SireBreed	DamID	DamBreed
ST17501	R	10/27/2017	Composite	Blue54	Composite	CXB13091	Finn x Dorset
ST17502	E	10/27/2017	Composite	Blue54	Composite	CXB13091	Finn x Dorset
ST17503	R	10/27/2017	Composite	Blue54	Composite	CXB13091	Finn x Dorset
ST17504	R	10/27/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	A3755	Dorset
ST17505	E	10/28/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	CXB13044	Finn x Dorset
ST17506	R	10/28/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	CXB13044	Finn x Dorset
ST17507	E	10/29/2017	Finn x Dorset	KBK111048	Dorset	CXB13053	Finn x Dorset
ST17508	R	10/29/2017	Composite	Blue54	Composite	CXB13045	Finn x Dorset
ST17509	E	10/29/2017	Composite	Blue54	Composite	CXB13045	Finn x Dorset
ST17510	E	10/30/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	C2406	Dorset
ST17511	R	10/30/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	C2406	Dorset
ST17512	R	10/30/2017	Finn x Dorset	KBK111048	Dorset	CXB13057	Finn x Dorset
ST17513	R	10/30/2017	Finn x Dorset	KBK111048	Dorset	CXB13057	Finn x Dorset
ST17514	R	10/31/2017	Finn x Dorset	KBK111048	Dorset	CXB13055	Finn x Dorset
ST17515	R	10/31/2017	Finn x Dorset	KBK111048	Dorset	CXB13055	Finn x Dorset
ST17516	R	11/1/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	B4392	Dorset
ST17517	R	11/1/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	B4392	Dorset
ST17518	R	11/1/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	B4394	Dorset
ST17519	E	11/1/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	B4394	Dorset
ST17520	R	11/1/2017	Composite	Blue54	Composite	CXB12582	Finn x Dorset
ST17521	R	11/1/2017	Composite	Blue54	Composite	CXB12582	Finn x Dorset
ST17522	E	11/3/2017	Composite	Blue54	Composite	CXB13097	Finn x Dorset
ST17523	E	11/3/2017	Composite	Blue54	Composite	CXB13097	Finn x Dorset
ST17525	R	11/16/2017	Composite	Blue54	Composite	CXB13072	Finn x Dorset
ST17526	R	11/16/2017	Composite	Blue54	Composite	CXB13072	Finn x Dorset
ST17527	E	11/18/2017	Finn x Dorset	KBK111048	Dorset	A3725	Dorset
ST17528	E	11/19/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	A3751	Dorset
ST17529	R	11/19/2017	1/4EF	NSD1476BPC1427	EF x Dorset x Tunis	A3751	Dorset
ST17530	E	11/20/2017	Composite	NSD1476BPC1427	EF x Dorset x Tunis	CXB10447	Finn x Dorset

Appendix Table 3. Anti-KLH Specific IgG Titrations

				Day 0				Day 14				Day 28			
	treatment group	probiotics?	age group	lamb ID	1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560	1:40	1:160	1:640	1:2560
4	KLH	yes	2	4	0.63685	0.67095	0.63215	0.5512	0.65545	0.63045	0.54465	0.51545	0.62395	0.49435	0.53331	0.52505
17	KLH	yes	2	17	0.84995	0.85365	0.7088	0.67425	0.63065	0.5255	0.4474	0.5984	0.67215	0.53615	0.4972	0.5384
6	KLH	no	2	6	1.007	0.99695	0.6583	0.6724	0.88865	0.82505	0.7355	0.72525	1.0492	0.9918	0.89115	0.7781
23	KLH	no	2	23	0.65815	0.62075	0.5589	0.78315	0.57	0.56935	0.75035	0.86165	0.6193	0.57735	0.57465	0.6159
22	adjuvant	no	2	22	0.6049	0.5756	0.6522	0.5985	0.6217	0.63885	0.8265	0.80405	0.8886	0.83285	0.8399	0.73035
5	adjuvant	no	2	5	0.6556	0.65515	0.91325	0.59825	0.8016	0.8785	0.3458	0.14765	0.8303	0.85185	0.38575	0.14225
16	adjuvant	yes	2	16	0.5618	0.54595	0.5594	0.76035	0.5794	0.57625	0.60575	0.78095	0.563	0.5825	0.71315	0.61555
7	adjuvant	yes	2	7	0.62275	0.5602	0.56695	0.95495	1.0168	0.5964	0.51485	0.6636	0.594875	0.558775	0.628125	0.854825
18	control	no	2	18	0.81885	1.0195	1.12155	0.70315	0.9196	0.84075	0.70625	0.63905	0.81565	0.89495	0.8708	0.5421
25	control	yes	2	25	0.83135	0.7685	0.60535	0.6752	0.88215	1.0051	0.7981	0.9801	0.90255	1.1115	0.9463	0.94815
13	KLH	yes	16	13	0.55515	0.4837	0.7336	0.6423	0.5316	0.53735	0.7037	0.50395	0.5657	0.2444	0.4251	0.6109
19	KLH	yes	16	19	0.76055	0.53435	0.58855	0.6386	0.6843	0.4171	0.6494	0.55985	0.8413	0.7645	0.6083	0.55075
15	KLH	no	16	15	0.72985	0.7642	0.5817	0.5877	0.8268	0.6875	0.74235	0.4573	0.7603	0.81635	0.80195	0.6201
3	KLH	no	16	3	0.5352	0.41175	0.48815	0.80315	0.482	0.56985	0.4388	0.7626	0.77085	0.65815	0.6587	0.75485
14	adjuvant	no	16	14	0.77205	0.5141	0.4374	0.43475	0.5802	0.56235	0.42265	0.35515	0.53425	0.61695	0.66475	0.4741
21	adjuvant	no	16	21	0.5871	0.34225	0.32515	0.68525	0.3784	0.4754	0.35505	0.3363	0.45575	0.4814	0.4145	0.5871
20	adjuvant	yes	16	20	0.3092	0.4058	0.2682	0.4126	0.3706	0.3089	0.3112	0.3443	0.28725	0.3281	0.3248	0.57755
2	adjuvant	yes	16	2	0.4488	0.17625	0.80795	0.0027	-0.05325	0.6576	0.64105	0.569	0.62455	0.56995	0.5288	-0.05325
10	control	no	16	10	0.3538	0.2719	0.30005	0.2463	0.3127	0.37285	0.4733	0.61035	0.38425	0.459	0.2653	0.3259
11	control	yes	16	11	0.31765	0.4208	0.38855	0.58255	0.33985	0.31	0.42545	0.46735	0.37275	0.3808	0.2944	0.55125
12	KLH	no	30	12	0.6568	0.46715	0.44075	0.3808	0.81805	0.73795	0.6161	0.5613	0.50625	0.71415	0.63325	0.43455
26	KLH	no	30	26	0.95965	0.59765	0.68275	0.41595	0.61035	0.64675	0.62535	0.6352	0.7709	0.99285	0.87225	0.67005
28	KLH	yes	30	28	0.666	0.46185	0.5766	0.37215	0.7013	0.557	0.6093	0.48845	0.9429	0.4999	0.685	0.51365
9	KLH	yes	30	9	0.5845	0.59525	0.7425	0.57705	0.65495	0.644	0.58945	0.47985	1.0437	0.84055	0.7747	0.6525
24	adjuvant	yes	30	24	1.0388	1.0931	0.8457	0.74635	1.1253	0.9215	0.73065	0.7006	0.89325	0.77645	0.68215	0.7184
27	adjuvant	yes	30	27	0.83375	0.7045	0.5693	0.7024	1.04755	0.77585	0.29505	0.407	0.8011	0.81795	0.81795	0.4623
1	adjuvant	no	30	1	0.6389	0.69975	0.79785	0.90425	0.83545	0.8789	0.9174	0.78475	0.88615	0.90505	0.77495	0.7847
29	adjuvant	no	30	29	0.4732	0.48605	0.58885	0.8939	0.91385	0.7532	0.81105	0.5799	0.98785	0.8031	0.79525	0.42145
30	control	no	30	30	0.46505	0.64545	0.7009	0.3769	0.6263	0.63535	0.662	0.3061	0.596	0.6398	0.58665	0.5675

Appendix Table 4. Anti-KLH specific IgM Titration

				Day 0				Day 14				Day 28			
treatment group	probiotics?	age group	lamb ID	1:40	1:160	1;640	1;2560	1:40	1;160	1;640	1;2560	1:40	1;160	1;640	1;2560
KLH	yes	2	4	0.6416	0.6761	0.7666	1.01705	0.73285	1.1984	0.85795	0.31	0.83925	**1.0374**	0.3928	0.05885
KLH	yes	2	17	0.62145	0.686	0.69955	1.00335	0.90155	1.01605	0.5411	0.23815	0.82205	0.9321	0.6691	0.2808
KLH	no	2	6	0.6312	0.6733	0.73755	0.7908	0.72655	1.00325	0.40875	0.33305	0.90295	0.8297	0.34475	0.1109
KLH	no	2	23	0.8694	0.91695	0.8797	1.1921	0.8998	1.08295	1.0086	0.3739	1.2533	1.19	0.6143	0.2305
adjuvant	no	2	22	0.8812	0.97995	1.2361	1.3328	1.2305	1.3783	1.01	0.4675	0.84265	1.1025	0.7849	0.4274
adjuvant	no	2	5	0.931	1.45255	0.96815	0.4048	0.7408	0.3332	0.11705	0.0485	1.35955	0.76295	0.32005	0.12065
adjuvant	yes	2	16	0.86305	0.9711	0.92775	1.1778	1.1018	1.1052	0.46945	0.1806	0.7489	1.1005	0.8043	0.30765
adjuvant	yes	2	7	0.69655	0.86895	1.02075	1.0628	0.9836	0.8948	0.4648	0.4666	0.87855	0.85375	0.36875	0.06105
control	no	2	18	0.7476	0.81	0.73385	0.5416	1.10265	1.055	0.67175	0.2483	0.8786	1.1742	0.62835	0.1733
control	yes	2	25	0.6197	0.5269	0.51865	0.62975	0.8866	1.0594	0.5437	0.769	1.00795	0.94485	0.61715	0.297
KLH	yes	16	13	0.9378	0.994	0.4797	0.1772	0.86095	0.87575	0.60535	0.2744	0.70025	0.6169	0.70845	0.4593
KLH	yes	16	19	0.77805	0.95265	0.69725	0.34255	0.92045	0.81805	0.6437	0.29715	0.9655	0.86705	0.84425	0.60065
KLH	no	16	15	0.80515	0.8535	0.68955	0.311	1.03685	0.8584	0.4649	0.14665	0.8163	0.81585	0.56595	0.2226
KLH	no	16	3	0.857	1.0396	0.6201	0.2181	0.9027	0.8779	0.44195	0.13185	1.0065	1.06485	0.6403	0.27675
adjuvant	no	16	14	0.8002	0.50285	0.14885	0.13925	0.69415	0.82935	0.3603	0.1699	0.8598	0.9845	0.6747	0.317
adjuvant	no	16	21	1.02695	0.97965	0.9664	0.60625	1.24935	1.08695	0.5846	0.2086	1.0626	1.1637	0.6368	0.25315
adjuvant	yes	16	20	1.14585	1.1755	0.91225	0.54035	2.05155	1.5445	0.77995	0.3788	1.1779	1.1346	0.5734	0.25735
adjuvant	yes	16	2	0.8452	0.9208	0.70485	0.2291	1.1467	0.6856	0.3293	0.089033	1.17115	0.9745	0.54005	0.25345
control	no	16	10	1.0582	1.11375	0.6291	0.38255	1.87265	1.78265	0.70085	1.02035	0.8667	0.8513	0.7265	0.80605
control	yes	16	11	1.24815	1.22855	0.55265	0.22705	1.04695	1.2304	0.85705	0.93315	0.76465	0.9528	1.14955	0.8526
KLH	no	30	12	0.5853	0.55815	0.61565	0.7154	0.6313	0.62215	0.62665	0.80355	0.59165	0.62545	0.54365	0.7147
KLH	no	30	26	0.5606	0.62575	0.25835	0.0927	0.6024	0.431	0.1935	0.0706	0.871375	0.717475	0.427325	0.168225
KLH	yes	30	28	0.68515	0.75735	0.56535	0.2173	0.6586	0.97345	0.7343	0.3532	0.7384	0.7341	0.79855	0.48815
KLH	yes	30	9	0.7416	0.72155	0.66865	0.3769	0.64345	0.77195	0.62315	0.35155	0.70025	0.71685	0.7333	0.63665
adjuvant	yes	30	24	0.95035	0.8583	0.49865	0.2106	1.0147	0.86485	0.54385	0.22895	0.8308	0.87485	0.71605	0.42995
adjuvant	yes	30	27	1.00985	0.7526	0.4229	0.1607	0.7937	0.89355	0.4234	0.1467	1.07355	0.9052	0.61025	0.2016
adjuvant	no	30	1	1.14745	0.8966	0.538	0.1993	0.90545	1.17325	0.87525	0.3716	0.84065	0.91695	0.95525	0.4695
adjuvant	no	30	29	0.9798	1.0378	0.988	0.84995	1.087885	1.18305	1.10095	0.52295	1.01645	1.4189	0.9816	0.55455
control	no	30	30	0.92345	0.69915	0.2916	0.1435	0.89745	0.8971	0.6679	0.29705	0.77525	0.887	0.87075	0.71515