ABSTRACT

Mastitis is the most common disease of dairy cows that incurs severe economic losses to the dairy industry. Currently, environmental mastitis pathogens are a major problem for most dairy farms. A current commercially available Escherichia coli vaccine does not prevent clinical mastitis and production losses, likely due to antibody accessibility and antigenic variation issues. Therefore, a novel vaccine that prevents clinical disease and production losses is critically needed. Recently a nutritional immunity approach, which restricts bacterial iron uptake by immunologically sequestering conserved iron-binding enterobactin (Ent), has been developed. The objective of this study was to evaluate the immunogenicity of the keyhole limpet hemocyanin-enterobactin (KLH-Ent) conjugate vaccine in dairy cows. Twelve pregnant Holstein dairy cows in their first through third lactations were randomized to the control or vaccine group, with 6 cows per group. The vaccine group received 3 subcutaneous vaccinations of KLH-Ent with adjuvants at drying off (D0), 20 (D21), and 40 (D42) days after drying off. The control group was injected with phosphate-buffered saline (pH 7.4) mixed with the same adjuvants at the same time points. Vaccination effects were assessed over the study period until the end of the first month of lactation. The KLH-Ent vaccine did not cause any systemic adverse reactions or reduction in milk production. Compared with the control group, the vaccine elicited significantly higher levels of serum Ent-specific IgG at calving (C0) and 30 d postcalving (C30), mainly its IgG2 fraction, which was significantly higher at D42, C0, C14, and C30 d, with no significant change in IgG1 levels. Milk Ent-specific IgG and IgG2 levels in the vaccine group were significantly higher on C30. Fecal microbial community structures were similar for both control and vaccine groups on the same day and shifted directionally along the sampling days. In conclusion, the KLH-Ent vaccine successfully triggered strong Ent-specific immune responses in dairy cows without significantly affecting the gut microbiota diversity and health. The results show that Ent conjugate vaccine is a promising nutritional immunity approach in control of E. coli mastitis in dairy cows.

Key words: enterobactin, mastitis, dairy cow, Escherichia coli, iron uptake

INTRODUCTION

Mastitis is one of the most economically devastating diseases in the dairy industry worldwide. Annual economic losses due to bovine mastitis in the United States alone are estimated at more than $2 billion (DeGraves and Fetrow, 1993). In the United States, on average, clinical mastitis cases cost about $444 per case during the first 30 d in milk (Rollin et al., 2015). Major economic losses stem from illness, death, and culling of infected cows (Gröhn et al., 1998, 2005; Todhunter et al., 1995; Oliver et al., 2005). Clinical mastitis may display severe systemic clinical manifestations (Katholm and Andersen, 1992; Cebra et al., 1996; Wenz et al., 2001), and approximately 10% of clinical mastitis may lead to death (Hogan and Larry Smith, 2003). Furthermore, the treatment of severe clinical mastitis with antimicrobials may promote the development of antimicrobial resistant bacteria, ultimately placing both animals and humans at heightened risk.

Current mastitis control programs, devised in the 1960s, are based on teat disinfection, antibiotic therapy, and culling of chronically infected cows. These strategies have led to considerable progress in controlling contagious mastitis pathogens, but they are much less effective against environmental pathogens (Oliver, 1988; Hogan et al., 1989; Todhunter et al., 1995; Oliver et al., 2005). Coliform bacteria such as Escherichia coli, Klebsiella spp., and Enterobacter spp. widely exist in the environment and are common causes of clinical mastitis in dairy cows (Hogan and Larry Smith, 2003).
The most common species of bacteria, isolated in more than 80% of coliform mastitis cases, is *Escherichia coli* (Bradley et al., 2007; Botrel et al., 2010).

*Escherichia coli* mastitis predominantly exhibits acute infections in a relatively short period (10–30 d), often between the last 2 wk of the dry period and the first weeks postpartum (Goulart and Mellata, 2022). The rapid onset is primarily due to the explosive growth of *E. coli* (Vandeputte-Van Messom et al., 1993), in milk by utilizing milk as source of nutrients to grow and multiply very efficiently. The doubling time of mastitis *E. coli* in milk can be as low as 15.2 min, reaching 10^8 cfu/mL in just 8 h (Goldberg et al., 1994). Therefore, reducing explosive growth of *E. coli* in milk during peripartum is crucial for preventing *E. coli* mastitis or improving the clinical outcomes of *E. coli* mastitis. Use of an effective vaccine to limit bacterial growth in milk will be a sustainable and cost-effective strategy.

Despite decades of research to develop effective vaccines against *E. coli* mastitis in dairy cows, only partial protection has been achieved. The only commercial vaccine is *E. coli* J5 vaccine, which is bacterin vaccine made from a mutant strain of *E. coli* O111:B4 lacking the outer O-antigen layer of lipopolysaccharide. J5 vaccine reduces clinical severity but does not prevent new cases (Tyler et al., 1990; Hogan et al., 1992a; Wilson et al., 2007, 2009). There are several reasons for the failure, such as antibody inaccessibility (O-antigens in wild type strains shield the outer membrane antigens; Takemura et al., 2002; Confer and Ayalew, 2013; Rainard et al., 2017; Rainard et al., 2021a, b, 2022a, b) and antigenic diversity/variation issues (Takemura et al., 2002; Braide et al., 2013). Meanwhile, specific outer membrane proteins [e.g., outer membrane protein A, OmpA (Confer and Ayalew, 2013; Guan et al., 2015; Rainard et al., 2017), ferric citrate receptor protein A, FecA (Lin et al., 1999a; Takemura et al., 2002), and ferric enterobactin receptor protein A, FepA (Lin et al., 1998a, b, 1999b)] have been used as vaccine antigens against *E. coli* mastitis, but none of them conferred protection against new cases of clinical disease, most likely due to the presence of redundant bacterial cellular components that compensate their functions when these cell surface proteins are blocked by immunity directed against them.

Therefore, to design an effective vaccine against *E. coli* mastitis, it is imperative that the vaccine targets an essential component that is universal to different *E. coli* serotypes and is easily accessed by specific immune responses (e.g., antibodies). One such target is the enterobactin (Ent)-mediated iron acquisition system. All gram-negative bacteria require iron to survive and adapt to their living niches. However, due to its potential to generate toxic free radicals, free ferric iron in higher organisms has been restricted to a level well below that required for growth of gram-negative bacteria (Litwin and Calderwood, 1993). For example, lactoferrin (Nuijens et al., 1996; Ward et al., 2002) and citrate (Faulkner and Peaker, 1982) are 2 iron-chelating components in milk, with the dissociation constants (KD) of ~10^{-20} M (Baker and Baker, 2004) and ~10^{-32} M at pH 7.4 (Silva et al., 2009), respectively. To overcome the limitation of iron, coliform bacteria have evolved sophisticated systems for iron uptake (Wooldridge and Williams, 1993; Braun et al., 1998; Mocek and Coulton, 1998; Clarke et al., 2001; Andrews et al., 2003; Wandersman and Delepelaire, 2004; Miethke and Marahiel, 2007). Of these, the high-affinity iron acquisition systems mediated by siderophores, the bacterial synthesized iron-chelating agents, are of particular interest (Fischbach et al., 2006; Gao et al., 2015). Ent, a representative catecholate siderophore, is synthesized by most members of *Enterobacteriaceae* (e.g., *E. coli*, *Salmonella enterica*, and *Klebsiella pneumoniae*) under iron-limited conditions, such as in intestinal tracts and mammary glands. The Ent siderophore system is the most efficient [KD = 10^{-49} M (Loomis and Raymond, 1991)] and highly conserved iron acquisition system among gram-negative bacteria. It also has been demonstrated that the Ent mediated iron acquisition system plays a significant role in bacterial pathogenesis (Miethke and Marahiel, 2007; Caza et al., 2008).

We hypothesized that Ent-specific antibody induced by keyhole limpet hemocyanin-enterobactin (KLH-Ent) conjugate vaccine is an effective nutritional immunity approach for the control of *E. coli* mastitis in dairy cows. Indeed, host lipocalins (Flo et al., 2004; Clifton et al., 2009; Santos et al., 2009; Wu et al., 2010) have been found to bind Ent and suppress bacterial growth in vitro and in vivo. Furthermore, another study (Sassone-Corsi et al., 2016) and our recent studies (Wang et al., 2019, 2020; Cui et al., 2020; Zeng et al., 2021) demonstrated that vaccination with Ent conjugate vaccine could induce Ent-specific antibodies and suppresses the uncontrolled expansion of *Enterobacteriaceae* species both in vitro and in rabbit, chicken, and mouse animal models. However, small and large animals are significantly different in genetics, physiology and immunology. The safety and immunogenicity of such Ent vaccine in dairy cows are unknown. In this study, the immunogenicity of KLH-Ent conjugate vaccine in dairy cows was evaluated, and the effects of vaccination of dairy cows with KLH-Ent vaccine on milk production and gut microbiota structure and diversity were assessed.
**MATERIALS AND METHODS**

**Enterobactin Purification, Conjugation, and Vaccine Preparation**

An Ent transport mutant *E. coli* AN102 has been used for Ent purification in our previous studies (Zeng et al., 2009, 2013a,b; Xu et al., 2010), and the procedure used for Ent purification was detailed in our recent publication (Zeng and Lin, 2017). Using this standard protocol, we prepared a high-purity Ent and conjugated Ent to KLH (Thermo Fisher Scientific Inc., Waltham, MA) and to BSA (Thermo Fisher Scientific) following optimized conditions as described in our previous publications (Wang et al., 2019; Zeng et al., 2021). We used KLH and BSA carrier proteins because of their significant difference in sequence, and antibodies induced against each conjugate lack any cross-reactivity between the 2 carrier proteins, allowing us to validate the production of Ent-specific antibodies (Wang et al., 2019). The 200 µg of KLH-Ent conjugate in 2.5 mL of sterile endotoxin-free PBS (pH 7.4) was mixed with 2.5 mL of Emulsigen-D (Em-D) adjuvant (MVP adjuvant, Omaha, NE) for first immunization and with 2.5 mL of Freund’s incomplete adjuvant (FIA; Thermo Fisher Scientific) for second and third immunizations. The KLH-Ent was used to coat plates to determine serum and milk anti-KLH-Ent antibodies. BSA-Ent was used to coat plates to determine anti-Ent antibodies in serum and milk of KLH-Ent vaccinated and unvaccinated control cows.

**Study Animals and Vaccination Protocol**

Cows were screened weekly starting from 4 wk before drying off (i.e., at 28, 21, 14, and 7 d before the expected drying off day). For enrolment in the study, each cow needed to be in overall good health, free of mastitis caused by major mastitis pathogens such as *Staphylococcus aureus*, streptococci (*Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*), and coliform bacteria (mainly *E. coli* and *Klebsiella* spp.). For cows to be enrolled in the study, individual quarter milk SCC of ≤100,000 cells/mL of milk and composite milk (from all quarters at equal proportions) SCC of ≤200,000 cells/mL of milk were required. Additionally, all quarters had to be free of clinical and subclinical mastitis caused by coagulase negative staphylococci (CNS), also known as non-aureus staphylococci (NAS), but in the presence of NAS, individual quarter SCC had to be less than 80,000 cells/mL of milk to ensure absence of inflammatory reaction to infection. Based on our previous Ent immunization trials in rabbits and chickens (Wang et al., 2019; Zeng et al., 2021), we estimated the mean value (OD<sub>492nm</sub>) of immune responses to Ent in the control and vaccine groups would be 0.1 and 1.2, respectively. The variance was estimated to be 0.3. When type I error was set at 5%, with 95% confidence level and type II error was set at 10% (90% power), the sample size per group was calculated as 6 (i.e., n = 6 cows per group). Twelve pregnant Holstein dairy cows in their first through third lactations housed at East Tennessee AgResearch and Education Center–Little River Animal and Environmental Unit (ETREC-LRAEU) dairy farm were randomly assigned to 2 groups of 6 cows each (Table 1). The study was conducted from November 2020 to April 2021. Cows in the vaccine group (KLH-Ent) were given 3 consecutive vaccinations of KLH-Ent with Em-D at drying off (D0), and with FIA at 21 and 42 d after drying off (D21 and D42). Cows in the control group were injected with sterile endotoxin-free phosphate-buffered saline (PBS, pH 7.4) with Em-D at D0 and with FIA at D21 and D42. Injections were given subcutaneously (SC) on alternate sides of the neck area. The first vaccination was given on the upper left side of the neck, the second on the right side, and the third on the lower left side of the neck.

Vaccines were masked to personnel administering the vaccinations and conducting subsequent assays. Treatment codes were unmasked after the completion of data collection and laboratory analyses. Injections were given using sterile disposable syringes and needles. Study cows were kept under similar management with other cows on the farm and fed a mixed total ration and other supplements required for dairy cows following the normal standard operating protocol of the ETREC-LRAEU dairy farm. The study was approved by The

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**Table 1.** KLH-Ent vaccination protocol at East Tennessee AgResearch and Education Center Little River Animal and Environmental Unit Dairy Farm during the study time of November 2020 to April 2021

<table>
<thead>
<tr>
<th>Group</th>
<th>Cows (n)</th>
<th>Vaccine/adj</th>
<th>Dose/volume</th>
<th>Route/frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Ent vaccine)</td>
<td>6</td>
<td>Ent-KLH/Em-D, FIA</td>
<td>200 µg/5 mL</td>
<td>SC/3X at D0, D21, and D42</td>
</tr>
<tr>
<td>2 (PBS Control)</td>
<td>6</td>
<td>PBS/Em-D, FIA</td>
<td>2.5 mL each/5 mL</td>
<td></td>
</tr>
</tbody>
</table>

1Ent: enterobactin; KLH: Keyhole Limpet Hemocyanin; adj: Adjuvant; Em-D: Emulsigen-D; FIA: Freund’s incomplete adjuvant; SC: subcutaneous; D0: at drying off (60 d before expected calving date); D42: 42 d after drying off (39 d before expected calving date); D40: 40 d after drying off (18 d before expected calving date).
University of Tennessee’s Institutional Animal Care and Use Committee (Registration Number 2790–1020).

**Vaccine Safety Monitoring.** Cows were monitored for any adverse reactions to the vaccine, such as measuring rectal body temperatures and injection site reaction volumes, and observation of rash and breathing difficulties. Rectal temperatures were taken 24 h before and immediately before vaccination, daily for 3 d, and at 7 and 14 d following each vaccination. Similarly, vaccine injection site reaction was measured in volume by taking 3-dimensional measurements of height (depth), width (dorsal/ventral), and length (cranial/caudal) in millimeters (mm) at similar time points. The standard ellipsoid volume \( V = \frac{\pi L W H}{6} \) was calculated in cubic centimeters (Powers et al., 2007). Cows were monitored daily by a member of the research team, who is also ETREC-LRAEU staff, for any clinical signs of mastitis and other diseases.

**Blood Sample Collection and Processing**

Blood samples were collected before vaccination on D0, D21, and D42, at calving (C0), and on 14 (C14) and 30 (C30) DIM postcalving. From each cow, 20 mL of blood was collected into two 10-mL plain uncoated tubes with clot activator (Thermo Fisher Scientific) from the jugular vein at each time point. Samples were kept on ice and transported to the laboratory, and serum was prepared within 1 to 2 h of blood collection. For serum preparation, tubes were centrifuged at 2,500 RPM at 4°C for 30 min to separate serum from blood components. The top serum layer was collected aseptically into sterile 96-well serum blocks and stored at −80°C until ELISA was conducted.

**Milk Sample Collection and Processing**

Individual quarter milk samples for bacteriological analysis (3 mL) and for SCC (50 mL) were collected on D0, C0, C14, and C30. Individual quarter milk samples (50 mL) for SCC were collected into 50-mL vials with preservative pills containing potassium dichromate (Capitol Vial, Auburn, AL).

Individual quarter milk samples for bacteriological analysis were collected aseptically with gloved hands after pre-dipping teats into antiseptic solution (OPI blue dodecyl benzene sulfonic acid solution, DeLaval Inc., Kansas, MO) and thorough cleaning with an individual towel and wiping of teat opening with 70% ethanol (disposable alcohol impregnated swabs, Covidien Webcol, Salt Lake City, UT). Milk samples collected for bacteriological culture were placed on ice and transported to the laboratory and cultured immediately or stored at −20°C until processed.

Composite milk samples (50 mL, collected from all quarters at equal proportions) for measuring antibody titers were collected into sterile 50-mL Falcon tubes immediately before first vaccine injection on D0, C0, C14, and C30. Milk samples for antibody evaluation were processed into skim milk and stored at −80°C until antibody was measured by ELISA. Skim milk was prepared from composite milk by centrifugation at 10,000 RPM at 4°C for 25 min. The solid sediments and the fat from the top layer were discarded. Skim milk was stored at −80°C until ELISA was conducted. Milk samples were not collected during the dry period at D21 and D40 to avoid the break of natural teat canal closure by keratin plug and entry of pathogenic bacteria.

**Enzyme-Linked Immunosorbent Assay**

Milk and serum anti-KLH-Ent and anti-Ent IgG, IgG1, IgG2, and IgA were determined using indirect ELISA, as previously described (Wang et al., 2019; Zeng et al., 2021). Briefly, 96-well polystyrene Immulon 2 HB plates (Thermo Fisher Scientific) were coated with 60 ng/well of KLH-Ent or 400 ng of BSA-Ent conjugates in sodium bicarbonate (NaHCO₃) coating buffer (pH 9.6, 100 µL/well) and incubated overnight (16–18 h) at room temperature. The coating buffer was removed, and plates were washed 2× with PBS-T (phosphate-buffered saline containing 0.05% Tween 20, vol/vol; Thermo Fisher Scientific). Plates were blocked with PBS-T containing 5% skim milk (wt/vol; PBS-TSM; Thermo Fisher Scientific) for 2 h. Plates were washed again 2× with PBS-T. Serum and skim milk samples were diluted in blocking solution at the ratio of 1:64 and 1:3, respectively, and incubated with the corresponding coated plates for 1 h at room temperature. Postincubation, plates were washed 5× with PBS-T. A 1:2,000 dilution of horseradish peroxidase-conjugated polyclonal sheep antobody on IgG, IgG1, IgG2, and IgA (heavy + light chain; SeraCare Life Sciences, Milford, MA) in PBS-TSM were added (100 µL/well) and incubated at room temperature for 1 h. Postincubation, plates were washed 5× with PBS-T and 100 µL of ABTS [2,2-azinobis (3-ethylbenzthiazolinesulfonic acid)] peroxidase substrate (SeraCare Life Sciences) was added and the reaction was stopped after 30 min using 100 µL of stop solution (1% SDS). The absorbance was measured at an optical density of 405 nm using an ELX808 Spectrophotometer (BioTek Instruments, Winooski, VT), and data were collected using Gen5 software (version 2.03.1; BioTek Instruments). The wells without the addition of primary antibody served as a background control. Duplicate measurements were performed for each sample.
**Milk Production and Milk SCC**

Milk yield was recorded automatically by the BouMatic (BouMatic LLC, Madison, WI) and SmartDairy parlor system (Valley Agricultural Software, Tulare, CA) with the PCDart Interface (Dairy Records Management Systems, Raleigh, NC). The weight (kg) of milk yield per day on D0, C0, C14, and C30 was obtained during milking. The SCC was determined at the DHIA Laboratory (Knoxville, TN) using the Soma Count 300 (Bentley Instruments Inc., Chaska, MN).

**Mastitis Monitoring During Study**

To detect clinical mastitis during the study period, abnormal inflammatory changes in the udder tissue and milk were examined and recorded as previously described (Oliver et al., 2012) with slight modifications. Briefly, abnormal inflammatory changes in the udder tissue were determined by clinical evaluation of the udder for swelling, pain and redness (hyperemic), increased heat during milking, and reduction or loss in milk yield. Abnormal inflammatory changes in the mammary gland tissue were scored on a scale of 0 to 3, where 0 indicates normal, the udder was pliable when totally milked out, the animal exhibited no signs of discomfort, and heat, pain, redness, or swelling were not detectable; 1 indicates slight swelling, the udder was less pliable with some firmness as if not totally milked out, additional milking or stripping did not return the gland to normal, and redness, heat, and pain were generally not detectable, and animals generally did not exhibit signs of discomfort; 2 indicates moderate swelling, the udder was firm, reddened and warm to the touch, the udder did not return to normal size when milked out, and the animal generally exhibited signs of discomfort (irritable, performs a stepping motion with feet or kicks) during prepping and milking procedures; and 3 indicates severe swelling, the udder was very hard, red, hot, and noticeably larger than other mammary quarters before milking with little or no change in size following milking, and the animal was extremely uncomfortable and very irritable. Similarly, abnormal inflammatory changes in the milk such as clots, change in color (bloody or blood tinged and watery) and change in consistency (viscous milk or purulent inflammatory fluid with pus similar to discharge) were examined and recorded on a scale of 0 to 3 as follows 0: normal milk, 1: flakes; 2: small slugs, large slugs/clots 3: bloody, watery, stringy milk. Clinical mastitis was defined as an udder quarter with a score of 2 in both milk and udder tissue or a score of 3 either in milk or udder tissue for 3 consecutive days. Both mammary gland and milk examinations and scoring were performed by a research team member who was also stationed at ETREC-LRAEU dairy farm. Scoring was done during screening, at time of sample collections, and between sample collections during milking until C30 d in milk postcalving. Subclinical mastitis was defined as SCC of >200,000 cells/mL of composite milk from all quarters or SCC of >100,000 cells/mL of milk from the individual quarter with isolation of mastitis causing pathogens.

**Milk Culture for Isolation and Identification of Bacteria**

Bacteriological analysis was performed following National Mastitis Council guidelines (Oliver et al., 2004) with slight modification. Briefly, 100 µL of milk sample was spread onto individual tryptic soy agar with 5% sheep blood and incubated at 37°C, 5% CO2: 95% air balanced incubator for 24 to 48 h. At 24 and 48 h, plates were examined for visible growth of colonies and characteristics of each colony such as size, morphology, hemolysis, and pigment production. One colony of pure culture was gram stained and differentiated into gram-positive or gram-negative bacteria with identification of bacterial morphology as cocci or rods. Gram-positive cocci were further tested by catalase to differentiate staphylococci from streptococci. Catalase positive cocci were presumed to be staphylococci and were further tested by tube coagulase test using rabbit plasma to differentiate the coagulase positive Staph. aureus from NAS.

**Rectal Fecal Sample Collection and Processing.** Individual cow rectal fecal samples (200 µg) were collected with gloved hand into sterile 50-mL Falcon tubes (Thermo Fisher Scientific) immediately before first vaccination on D0, D21, and D42, C0, C14, and C30. Samples were kept on ice and transported to the laboratory. Immediately upon arrival at the laboratory, 10 g of feces was added to a 50-mL sterile Falcon tube and diluted with 20 mL of sterile Hank’s balanced salt solution (Thermo Fisher Scientific) by gentle and thorough mixing. The suspension was filtered through sterile gauze into 15 mL sterile Falcon tubes. A sample of the filtrate was serially diluted 10-fold from 10⁻⁰ to 10⁻³ and 100 µL of 10⁻² and 10⁻³ dilutions were plated onto CHROMagar E. coli plates (CHRMagar, Paris, France) and incubated at 37°C for 24 h. The total number of colonies of E. coli (blue colonies) were counted and expressed in colony forming units per gram (cfu/g) of feces. The remaining fecal samples were stored at −80°C for extraction of total microbial genomic DNA for analysis of fecal microbiota community abundance.
Analysis of Fecal Microbial Community and Diversity by 16S rRNA Gene Sequencing. Individual cow rectal fecal samples collected immediately before first vaccination on D0, D21, D42, and C14 postcalving were subjected to microbial community analysis described in a previous study (Wang et al., 2021). Briefly, genomic DNA was extracted using Zymo Quick-DNA Fecal/Soil Microbe MicroPrep Kit-G505255766, (ZYMO Research, Irvine, CA) following the manufacturer’s manual. DNA concentration was measured using the NanoDrop spectrophotometer (Thermo Fisher). PCR amplification was performed using primer pair 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) with barcodes attached targeting the V4 region of the 16S rRNA gene. Each 25-µL cocktail mix contained 2 µL of DNA template (100 –150 ng), 2.5 µL of Phusion Flash PCR Master Mix (Thermo Scientific), 8.5 µL of ultrapure water, 1 µL of forward primer (10 µM), and 1 µL of reverse primer (10 µM). The PCR program was as follows: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 60 s, and extension at 72°C for 90 s; final extension at 72°C for 10 min. The PCR products were purified using ChargeSwitch PCR Clean-Up Kit (Invitrogen, Carlsbad, CA). The purified samples were then pooled in equal concentrations and further quantified using KAPA Library Quantification Universal Kit (Roche, Indianapolis, IN). A 10 pM of the final library, with PhiX library as an internal control, was loaded to MiSeq Reagent Kit v3 (Illumina, San Diego, CA). The sequencing was performed with Illumina Miseq. The amplicon sequencing data were analyzed using QIIME2 platform with DADA2 pipeline for representative sequences and Scikit-learn classifier with SILVA database release 138 for classification (Callahan et al., 2016; Glöckner et al., 2017; Bolyen et al., 2019). Shannon diversity and principal coordinate analysis based on Bray-Curtis distance were conducted based on the rarefied representative sequence table at reads of 15,000. Linear discriminant analysis effect size (Segata et al., 2011) was conducted to identify the distinguishing genomic features between control and vaccinated groups as well as between different sampling days. The raw sequencing data are publicly available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA901477) with BioSample accession numbers SAMN31715141-SAMN31715188 under BioProject ID PRJNA901477.

Statistical Analysis

The effect of vaccine was assessed on the antibody responses against KLH-Ent and Ent, both in the serum and milk. The antibodies tested were IgG and its 2 subclasses IgG1 and IgG2, and IgA. Mixed effects linear regression with random intercept was used to assess the effect of vaccine on the antibody responses by modeling the main effects of treatment group (i.e., vaccination status) and sampling day, and cow ID was included as a random effect to account for the repeated measurements within a cow over time. Somatic cells count and fecal coliform counts were log10 transformed and similarly analyzed using mixed effects linear regression models. Post-hoc pairwise comparisons were made by adjusting for multiple comparisons by the Bonferroni method. The effect of serum and milk anti-KLH-Ent and anti-Ent IgG and IgA on the fecal coliforms and SCC was analyzed by linear regression by pooling all data together regardless of treatment group and sampling time point. A summary of the measured outcomes and their level of analysis is presented in Table 2. Data were analyzed in STATA 16 (STATA Corp., College Station, TX), and $P < 0.05$ was considered significant.

Table 2. Description of cows enrolled in KLH-Ent vaccination trial during the November 2020 to April 2021 study time at East Tennessee AgResearch and Education Center Little River Animal and Environmental Unit Dairy Farm

<table>
<thead>
<tr>
<th>Variable (unit of measurement)</th>
<th>Level of measurement and unit of analysis</th>
<th>Number of observations</th>
<th>Time point Measured (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>Animals</td>
<td>Control</td>
<td>Vaccine</td>
</tr>
<tr>
<td>SCC (counts/mL)</td>
<td>Quarter</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Serum antibody levels (OD)</td>
<td>Animal</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>Milk antibody levels (OD)</td>
<td>Animal</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Fecal coliforms (cfu/g)</td>
<td>Animal</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Milk production (kg)</td>
<td>Animal</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

¹OD: optical density measurements at 405 nm for ELISA.
²Depending on the variables measured (for example, 3-6 d).
RESULTS

Overall Animal Health, Mastitis Status, and Retention in the Study

All animals completed the study. All cows were free of mastitis at the baseline (D0). One vaccinated cow developed subclinical mastitis caused by Prototheca species of algae at C14 postcalving, which became clinical at C30 postcalving.

Vaccine Safety

There were no signs of local or systemic reactions to the vaccine throughout the vaccination and immune response evaluation period except minor local swelling at the injection site, which is expected. There was no significant ($P > 0.05$) difference in injection site reaction volume (cm$^3$) between groups. All vaccinated cows had normal rectal body temperature at 24 h before vaccination, immediately before vaccination and for 3 consecutive days and at 7 and 14 d after each vaccination (Figure 1).

Serum and Milk Anti-KLH-Ent and Anti-Ent Antibody Levels

Serum and Milk Anti-KLH-Ent IgG, IgG1, and IgG2 Antibody Levels. In the vaccinated group, the mean serum anti-KLH-Ent IgG and IgG2 levels significantly ($P < 0.001$) increased starting at D42 and continued to increase until the end of the experiment (C30) compared with those of the control group. The mean serum anti-KLH-Ent IgG1 increased at D21 and D42, then declined to the preimmunization level on C0 ($P > 0.05$) and C14, before significantly ($P < 0.001$) rising again on C30 compared with that of control group (Figure 2A–C). The mean milk anti-KLH-Ent IgG levels were significantly higher at C30 ($P < 0.001$) (Figure 2D); IgG1 levels at C30 ($P < 0.001$; Figure 2E) and IgG2 levels at C14 and C30 ($P = 0.001$) (Figure 2F) in the vaccinated group compared with the control group.

Serum and Milk Anti-Ent IgG, IgG1, and IgG2 Antibody Levels. Mean serum anti-Ent IgG levels were significantly higher at C0 ($P = 0.006$) and C30 ($P < 0.001$; Figure 3A) in the vaccinated group compared with the control group. Mean serum anti-Ent IgG1 did not significantly ($P > 0.05$) differ between vaccinated and control groups (Figure 3B) but the mean serum anti-Ent IgG2 levels were significantly ($P < 0.05$) higher from D42 to C30 than those of the control group (Figure 3C). Mean milk anti-Ent IgG levels were significantly higher at C30 ($P = 0.001$; Figure 3D) but milk anti-Ent IgG1 did not significantly ($P > 0.05$) differ between vaccinated and control groups over the study time (Figure 3E). Mean milk anti-Ent IgG2 levels were significantly ($P = 0.004$) higher in the vaccinated group compared with the control group at C30 (Figure 3F).
Serum and Milk Anti-KLH-Ent and Anti-Ent IgA Antibody Levels. In the vaccinated group, mean serum anti-KLH-Ent IgA levels significantly increased at C0 ($P = 0.001$) and C14 ($P = 0.049$) compared with the control group before returning to their baseline level at C30 ($P = 1.00$; Figure 4A). The mean serum anti-Ent IgA levels were significantly higher only at C0 ($P < 0.001$; Figure 4B) in the vaccinated group compared with the control group. The mean milk anti-KLH-Ent IgA ($P = 1.00$) and anti-Ent IgA ($P > 0.05$) levels did not significantly differ between the vaccinated and control groups at any time points tested (Figure 4C and 4D).

Effects of Vaccination on Milk Production and SCC. Vaccination did not have any statistically significant ($P = 0.242$) effect on milk production during the observation period (until 30 d after calving). However, as expected in healthy cows, milk production significantly ($P < 0.001$) increased from C0 to C14 and C30 in both groups (Figure 5A). Vaccination did not have any significant ($P > 0.05$) effects on SCC (Figure 5B).

Detection of Mastitis Pathogens in Milk. Seven different bacteria including NAS, Bacillus species, Streptococcus species, Corynebacterium species, gram-negative rods, yeast, and Prototheca species were isolated and identified. Of these bacteria NAS was the most prevalent species from both treatment groups. Prototheca species caused clinical mastitis in a cow from the vaccinated group, but the others did not cause clinical or subclinical mastitis despite being isolated from milk samples (Table 3).

Effects of Vaccination on Fecal E. coli Count and Fecal Microbial Diversity and Community. Fecal E. coli count significantly ($P = 0.009$) increased in the control group at C0 compared with its baseline (D0) count and returned to baseline count thereafter.
In the vaccinated group there was no significant ($P > 0.05$) change in the fecal $E. \text{coli}$ counts. There was no significant ($P = 1.00$) difference between vaccinated and control groups in fecal $E. \text{coli}$ count (cfu/g; Figure 6A).

The microbial diversity for both control and vaccinated groups increased from D0 to D42 then decreased from D42 to C14. However, there was no significant difference between control and vaccinated groups on each sampling day (Figure 6B). The microbiota was similar between control and vaccinated groups but different between the sampling days (Supplemental Figure S1 A, https://figshare.com/articles/figure/Supplemental_data_docx/23668794; Kerro Dego, 2023). This was further confirmed by the specific dissimilarity between sampling days for control and vaccinated groups (Supplemental Figure S1 B). Interestingly, the microbial community shifted in the same direction (Supplemental Figure S1 B). The microbial communities that contributed to the shift along the sampling days are shown in Supplemental Figure S2 (https://figshare.com/articles/figure/Supplemental_data_docx/23668794; Kerro Dego, 2023).

To identify the microbial communities that potentially contributed to the differences in the fecal microbiota between the sampling days and the control and vaccinated groups, the composition of the fecal microbiota was examined in more detail. Both bacterial and ar-
archaeal populations were observed in the fecal samples. In the control group, archaea accounted for 12.8% of the sequences on D0, subsequently decreasing to 7.8% on D21 and further decreased to around 3% on D42 and C14 (Supplemental Figure S3, https://figshare.com/articles/figure/Supplemental_data_docx/23668794; Kerro Dego, 2023). A similar trend could be observed in the vaccinated group on the same sampling day (Figure 6C). Firmicutes remained the most dominant phylum throughout the sampling days, followed by Bacteroidetes. It is worth mentioning that Bacteroidetes greatly increased on D42 for the cows in both control and vaccinated cows, indicating the similar microbiota diversity between the 2 groups (Figure 6C). The most abundant phylum in the control group on D0 was Firmicutes (an average of 57.3%), followed by Bacteroidetes (18.0%), Euryarchaeota (12.8%), Actinobacteria (6.2%), and Spirochetes (3.8%). All the other phyla were less than 1% abundant. A Similar trend was observed in the vaccinated group on the same sampling day (Figure 6C). Firmicutes remained the most dominant phylum throughout the sampling days, followed by Bacteroidetes.
vaccinated groups, which decreased the Firmicutes/Bacteroidetes (F/B) ratio.

Another interesting trend is that the relative abundance of Actinobacteria increased from D0 (6.2 and 7.8% for control and vaccinated groups, respectively) to D21 (12.1 and 12.8%), then dropped back on D42 and C14. Coincidently, Spirochaetes decreased from D0 to D21, then bounced back on D42 and C14. Euryarchaeota kept dropping from D0 (12.8 and 9.3% for control and vaccination groups, respectively) to C14 (3.0 and 3.7%) with an increase in Proteobacteria (0.2 and 0.5% on D0; 2.7 and 4.3% on C14). To sum up, consistent with the principal coordinate analysis, the overall trend of the microbial community shift was similar between control and vaccination groups, and it was directional for both groups.

**Figure 5.** Effects of vaccination on milk yield (A) and somatic cells count (B) of cows vaccinated with KLH-Ent and unvaccinated controls during November 2020 to April 2021 vaccination trial at East Tennessee AgResearch and Education Center Little River Animal and Environmental Unit Dairy Farm. Data are presented as mean of total milk yield in kilogram (kg) per group (A) and mean of log10 of the actual SCC counts (B). The asterisks represent statistically different values at that time point and baseline value (D0). No difference between Groups. The error bars represent 95% CI for the mean values. D0: at drying off; C0: at calving; C14: 14 d after calving; C30: 30 d after calving; V1, V2, and V3: first, second, and third vaccinations, respectively.

**Table 3.** Bacterial isolation from milk samples of KLH-Ent vaccinated and unvaccinated control cows during the vaccination trial of November 2020 to April 2021 at East Tennessee AgResearch and Education Center Little River Animal and Environmental Unit Dairy Farm.

<table>
<thead>
<tr>
<th>Day</th>
<th>CNS</th>
<th>Bsp</th>
<th>Strsp</th>
<th>Chsp</th>
<th>GNR</th>
<th>Yeast</th>
<th>Protsp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vac</td>
<td>Ctr</td>
<td>Vac</td>
<td>Ctr</td>
<td>Vac</td>
<td>Ctr</td>
<td>Vac</td>
</tr>
<tr>
<td>D0</td>
<td>5,000</td>
<td>2,120</td>
<td>80</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C0</td>
<td>330</td>
<td>70</td>
<td>70</td>
<td>40</td>
<td>2,400</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>C14</td>
<td>2,370</td>
<td>60</td>
<td>30</td>
<td>40</td>
<td>2,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C30</td>
<td>3,330</td>
<td>260</td>
<td>40</td>
<td>40</td>
<td>2000</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are presented as total bacterial count in cfu/mL of milk per group at each sampling time point. D0: at drying off; C0: at Calving; C14: 14 DIM postcalving; C30: 30 DIM postcalving; CNS: coagulase negative *Staphylococcus* species; Strsp: *Streptococcus* species, Chsp: *Corynebacterium* species, GNR: gram-negative rod; Protsp: *Prototheca* species; Vac = vaccine; Ctr = control.
DISCUSSION

Three consecutive vaccinations of dairy cows at 21 d interval with KLH-Ent conjugate vaccine at drying off, 21 and 42 d after drying off induced significantly increased serum and milk Ent-specific IgG and IgG2 antibodies postvaccination at calving and during early lactation at 14 and 30 DIM. These results are similar to our previous studies using the same KLH-Ent conjugate vaccine in other species of animals where subcutaneous vaccination of rabbits and chickens with KLH-Ent conjugate vaccine induced significantly increased (up to 4,096 fold) anti-Ent IgG antibody titers in rabbit serum (Wang et al., 2019) and IgY antibody titers in serum and egg yolk of chickens postvaccination. The anti-Ent IgG from rabbit inhibited in vitro growth of E. coli and Salmonella enterica (Wang et al., 2019, 2020). Another immunogenicity and efficacy study of KLH-Ent conjugate vaccine in chickens reported that this vaccine induced significantly increased Ent-specific antibody in serum and subsequently reduced Campylobacter jejuni colonization of the intestine (Cui et al., 2020).

Figure 6. Effects of vaccination on fecal Escherichia coli counts (A), diversity (B), and microbiota abundance at phylum level (C) of cows vaccinated with KLH-Ent and unvaccinated controls during November 2020 to April 2021 vaccination trial at East Tennessee AgResearch and Education Center Little River Animal and Environmental Unit Dairy Farm. In Figure 6B, blue boxes represent control cows while pink boxes represent vaccinated cows. Data are presented as the mean of log_{10} cfu/g of feces per group (A), and error bars represent the 95% CI of mean values.
Based on our vaccine safety evaluation by monitoring rectal body temperature and injection site reactions, KLH-Ent conjugate vaccine is safe for dairy cows and did not affect milk yield. As expected in normal healthy dairy cows, milk yield was significantly increasing in both groups at 14 and 30 d from preimmunization (D0), indicating an increasing trend toward peak lactation at around 60 DIM. Similar to our result, Cui et al. (2020) monitored the effect of KLH-Ent conjugate vaccine on BW gain and development of major immune organs in chickens and concluded that KLH-Ent vaccine is safe for chickens and did not affect weight gain or the growth of immune organs.

Because Ent is produced and used by different members of the Enterobacteriaceae, it is crucial to monitor effect of Ent-specific antibodies on microbiota community composition and diversity in the vaccinated and unvaccinated control cows. Our result showed that there was no significant difference \((P > 0.05)\) in the fecal \(E. coli\) counts in cfu/gram of feces as well as no difference in microbial community structures and diversity as shown by 16S rRNA gene sequencing of fecal microbiota at all time points tested postvaccination between vaccinated and control groups (Figure 6 A, B, and C). Similar to our results, Cui et al. (2020) reported that vaccination of chicks with KLH-Ent did not affect chicken gut microbiota community structure and diversity. Our result is in line with previous studies that reported Ent-specific antibodies in mice vaccinated with CTB-Ent conjugate vaccine do not significantly affect gut microbiota in mice without intestinal inflammation (Sassone-Corsi et al., 2016).

Vaccination of dairy cows with Ent conjugate vaccine during the dry period induced significantly increased Ent-specific milk and serum IgG and IgG2 antibodies at calving and during early lactation at 14 and 30 DIM. This observation is encouraging because coliform mastitis, especially clinical mastitis, is very common during the first 30 d of lactation. If this vaccine is effective this vaccination schedule can be followed to control mastitis due to \(E. coli\) and other coliform bacteria. However, in this pilot experiment, only a small number of animals \((n = 6)\) were included, which may affect the confidence to draw a solid conclusion although the difference of Ent-specific antibodies in the serum is statistically significant. We do recognize the limitation of the small number of animals. Ent is a hapten, resulting in very low background antibody levels in the control animals. The difference of Ent-specific antibody levels between the control and vaccinated animals, especially in the serum samples, is substantial. Therefore, we only needed to include a relatively small number of animals to achieve statistical power of detecting a difference. Further controlled experimental vaccination and challenge infection or field-based efficacy studies with more animals are required to determine the efficacy of Ent conjugate vaccine against \(E. coli\) mastitis in dairy cows. Also, a longer observation time (e.g., a full lactation period) will be helpful to determine the safety of such a vaccine and duration of immunity induced by this vaccine.

Further optimization of the KLH-Ent immunization regimen, such as the choice of adjuvants and the shots of immunization, will be meaningful for practical application. In a chicken trial, a single vaccination with KLH-Ent conjugate vaccine induced significantly \((P < 0.01)\) increased anti-Ent immune response that was sustained up to 8 wk (Cui et al., 2020). The authors also reported that single immunization of 7-d old chickens with KLH-Ent conjugate vaccine with Montanide ISA VG71 adjuvant (Seppic, Paris, France) induced significantly high Ent-specific immune response that was sustained over several weeks post the immunizations. Therefore, it may be possible to reduce the number of immunizations by testing different adjuvants or by optimization of immunization regimen.

Intestinal microbiota promotes proper fermentation by increasing the surface area while decreasing the size of the feed (Xu et al., 2021a). The dominance of \textit{Firmicutes} and other bacterial and archaeal populations (Figure 6C and Supplemental Figure S3) are consistent with many other studies (Deusch et al., 2017; Xu et al., 2021b; Stockler et al., 2022). The directional shift of microbiota along with the sampling days may be caused by the change in the reproductive hormones. It is supported by the main core microbiome identified on each sampling day (Supplemental Figure S2). The decrease in the F/B ratio further confirmed this hypothesis as the F/B ratio is widely accepted to have an important effect on maintaining normal intestinal homeostasis (Stoianov et al., 2020). Notably, although the microbiota communities shifted with sampling day, the changes were similar between the control and vaccinated groups, which is consistent with previous findings in mice that showed subcutaneous vaccination with Ent conjugate vaccine did not disturb the normal intestinal microbiota (Sassone-Corsi et al., 2016; Cui et al., 2020).

We previously demonstrated that KLH-Ent conjugate vaccine can successfully trigger high levels of Ent-specific antibodies in rabbits (Wang et al., 2019) and chickens (Cui et al., 2020; Zeng et al., 2021). Unlike lipocalin, Ent-specific antibodies can even bind glycosylated Ent (i.e., salmochelins; Wang et al., 2019; Zeng et al., 2021), and inhibited in vitro growth of different gram-negative bacteria (Wang et al., 2020), most of which can synthesize salmochelins. Furthermore, both Sassone-Corsi et al. (2016) and Cui et al. (2020) demonstrated that vaccination with Ent conjugate vaccine
could suppress the uncontrolled expansion of *Enterobacteriaceae* species in vivo without disturbing the normal intestinal microbiota. In this study, we demonstrated that the KLH-Ent conjugate vaccine can trigger strong Ent-specific antibodies in dairy cows. All results strongly support the immunogenicity of the KLH-Ent conjugate vaccine, and the Ent-targeting nutritional immunity approach is a promising strategy to control *E. coli* mastitis in dairy cows.

**CONCLUSIONS**

In summary, we have successfully formulated a novel Ent conjugate vaccine and demonstrated strong and robust immunogenicity of the KLH-Ent vaccine for the induction of high levels of Ent-specific antibodies in dairy cows. Our novel KLH-Ent conjugate vaccine, based on the principle of nutritional immunity, aims at accessible and conserved Ent. It is widely acknowledged that the suppression of explosive growth of *E. coli* during early *E. coli* infection and systemic spreading of *E. coli* will be critical for the clinical outcome. Therefore, the KLH-Ent vaccine is a very promising vaccine candidate to control *E. coli* mastitis. Evaluation of efficacy under controlled experimental vaccination and challenge studies or field efficacy studies in dairy cows are required to determine its efficacy against *E. coli* mastitis in dairy cows.

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**REFERENCES**


