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Research article

The role of anti-*E. coli* antibody from maternal colostrum on the colonization of newborn dairy calves gut with *Escherichia coli* and the development of clinical diarrhea

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ABSTRACT

Escherichia coli (***E. coli***) is an early organism in the colonization of mucosal tissue and has an influence on the development of mucosal and systemic inflammatory responses. To understand the impact of transfer of Immunoglobulin G **(IgG)**, *E. coli*-specific antibody and general immune factors to the calf on the gut microbiota, an observational study that followed the temporal colonization of the intestine with *E. coli* as correlated with the total quantity of IgG and the antibody recognizing *E. coli*, was conducted in newborn Holstein calves. The calves were subjected to the "gold standard" of colostrum management, including dam vaccination prior to delivery to prime colostrum. Twenty Holstein dams, producing an adequate quantity of good-quality colostrum following uncomplicated deliveries of healthy female offspring, were utilized in this study. Each calf was monitored and scored daily for diarrhea. Blood and fecal samples were collected from calves on day (**D**) D1 (before colostrum intake), then D3, D7, D14 and D28 after colostrum feeding. *E. coli* isolated from fecal samples of the calves were assessed for virulence genes and the quantity per gram of feces using conventional Polymerase chain reaction and Real-time polymerase chain reaction quantitative, respectively. The total quantity of plasma IgG and the titer of *E. coli*-binding antibody were measured by enzyme-linked immunosorbent assay. The peak in diarrhea in the calves was observed on D12. No IgG or *E. coli*-binding antibody were detected prior to colostrum intake (D1). Both total IgG and binding antibody sharply increased by D3. Total and binding IgG declined with calf age. The total number of bacteria (16S rRNA) in feces was similar between D1 and D14. The number of total bacteria increased over the period from D14 to D28. The number of *E. coli* was minimal on D1. The number of *E. coli* increased during the period D3-D7. A decrease in the number of *E. coli* per gram of feces declines between D14and D28. The stable toxin gene was frequently identified in the *E. coli* isolated from the calves. Further, *E. coli* K99 was not identified in this study. While the gut was colonized by *E. coli* early in life corresponding to the peak level of IgG and specific antibody, a decreasing number of *E. coli* were observed as the passive immunity waned. This suggests that the function of the passive antibody may be to control colonization and seed innate immune function that then stabilizes the level of *E. coli* in the digestive tract of calves.

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Implications

The transfer in colostrum of a significant quantity of antibodies that bind *Escherichia coli* appears to have an impact on the

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colonization and development of significant infections by *Escherichia coli* during the first 10 days of life. The majority of the diarrheal disease observed in these calves was of short duration. The average duration was 3 days. In addition, the diarrhea observed did not fit the clinical definition of septicemia or induce moderate to severe dehydration. Therefore, the disease appeared to be essentially only uncomplicated colibacillosis that was managed by antibody and other immune factors from the dam during its peak in the calf. These findings reinforce the importance of significant transfer of immunoglobulin G from the dam to avoid economic losses due to significant morbidity or mortality caused by enterotoxigenic *Escherichia coli* infections. The outcomes of this study suggest that the development of a model studying transferred anti-*Escherichia coli* antibody, *Escherichia coli* colonization of the gut and the development of clinical diarrhea should be developed as the basis to better understand *Escherichia coli* diarrhea in calves.

Specification table

Subject	Behavior, Health and Welfare – maternal factors that affect the relationship of calf and *E. coli*
Type of data	Tables and figures (graphic)
How data were acquired	Colostrum samples were harvested aseptically from the dams immediately after calving; Plasma samples from calves were harvested from the jugular using vacutainer system. Fecal samples from each calf were obtained digitally with sterile gloves from the rectum. Immediately, each was deposited in a sterile universal collector. In the laboratory, fecal samples were individually distributed into sterile microtubes under a laminar flow hood using an aseptic technique. All microbiological samples were stored at −80 °C. The laboratory analysis were performed, and data were collected and posted to Excel® files for storage and processing. All data comparisons were conducted using standard models in the Statistical Analysis System for Windows software system (SAS Institute Inc., Cary, NC, USA).
Data format	Raw, transformed, sorted or filtered data.
Parameters for data collection	Calf health parameters (diarrhea score and others), Colostrum Blood and fecal samples.
Description of data collection	The original data were collected during an experiment conducted at the Agrindus Farm. The samples were transported to and analyzed at the Department of Medical Clinics of School of Veterinary Medicine and Animal Science, University of São Paulo (USP), São Paulo, Brazil.
Data source location	Dairy herd was sampled at the time of calving. Institution: Agrindus farm. City/Town/Region: Descalvado/São Paulo state Country: Brazil. Latitude 21°57′44.9″S and longitude 47°41′44.8″W.
Data accessibility	Repository name: Zenodo Data identification number: https://doi.org/10.5281/zenodo.7530459
Related research article	Martin, C.C., De Oliveira, S.M.F.N., Costa, J.F. dos R., Baccili, C.C., Silva, B.T., Hurley, D.J., Gomes, V., 2021 Influence of feeding fresh colostrum from the dam or frozen colostrum from a pool on indicator gut microbes and the inflammatory response in neonatal calves. Research in Veterinary Science 135, 355–365. https://doi.org/10.1016/j.rvsc.2020.10.017

Introduction

E. coli is a major pathogen affecting the calf rearing system. It regularly causes diarrhea and septicemia in neonatal calves. Enterotoxigenic *E. coli* (**ETEC**) are the most significant pathogenic subtype associated with calf diarrhea during the first 4 days of age. This is due to the capacity of these bacteria to colonize the mucosal surface of the small intestine and to produce heat-stable enterotoxin (**ST**), at times in conjunction with heat-labile enterotoxin (**LT**). These two toxins that are the defining features of the ETEC pathogens individually, or together, increase the secretion of ions out of intestinal cells and reduce the reabsorption of water from the intestinal contents (Nagy and Fekete, 2005). ETEC also express F4 fimbriae that facilitate the binding and targeting of LT enterotoxin. EPEC, ETEC-producing Shiga-like toxin, and Enterohemorrhagic *E. coli* (**EHEC**) were not associated with very early calf diarrhea; however, young calves seem to be a reservoir of ETEC that produce the Shiga toxin 1 (**STX1**) and Shiga toxin 2 (**STX2**) (Kolenda et al., 2015).

Colostrum contains several protective elements that regulate the growth and colonization of *E. coli* in the neonate. *E. coli* infection during the first few days of life appears to have a complex set of factors that determine if disease will occur or not. Colostrum appears to be essential, especially in cattle, whose synneptheliochorial placenta does not allow the transfer of macromolecules or cells directly from the circulation of the dam to fetus, in the control of *E. coli* from the dam and her environment. A large quantity of immunoglobulin (**Ig**) primarily IgG1 is passively transferred from colostrum across the neonatal intestine and then to the circulation following ingestion during a period of about 18 hours after birth (Godden et al., 2019). The transferred antibodies are the major pathogen targeting mechanism for protection against systemic and enteropathogens during the few first days of life (Tyler et al., 1989; Cho and Yoon, 2014). Concurrent with, and following, the transfer of maternal IgG, a period of colonization of the intestinal tract occurs. The colonization arises from microbial ‘‘seeds” transferred from the dam during birth, initial feeding, during grooming and from the immediate environment. The initial set of microorganisms that become resident on mucosal surfaces drives an ordered and gradual recruitment of innate cells and humoral

factors that lead to local activation of the mucosal immunity. Subsequent to this process, these organisms interacting with the immune elements at the mucosal surface begin the development of the fully functional innate responses, and drive the process for initiation of building the organized centers responsible for a functional specific immune system with time (Nart et al., 2008).

The hypothesis and model under which we built this study were to attempt to examine how the colonization of the gut with *E. coli* was impacted by the level and action of total and *E. coli*-specific transferred maternal antibody. The study was done in an attempt to provide a better model of how *E. coli* diarrhea develops in calves. The objectives of the study addressed: (1) Did the colonization of the calf gut with *E. coli* represent a competitive collection of many strains of *E. coli* with different properties during early in life? (2) If so, did some of these strains carry recognized virulence genes that are known to mediate diarrheal disease in young calves? (3) Was there clear evidence that the specific and total antibodies transferred from the dam impacted the development of diarrheal disease in the neonate?

Material and methods

Animal management

This research was carried out using cows from a high-producing commercial dairy located in the state of São Paulo (Brazil), between July and December of 2018. It is located at Latitude: 21°54′14″ South, Longitude: 47°37′12″ West. For the study, we selected 20 pregnant Holstein cows vaccinated against neonatal diarrhea during the prepartum period (Rotavec®, MSD Animal Health) or against endotoxemia caused by *E. coli* and *Salmonella typhimurium* (J-VAC®, Boehringer-Ingelheim), according to farm management and record. The cows were transferred to a compost bed packing system barn (compost barn) thirty days before expected calving. The cows were transferred to a calving pen (inside the compost barn) at the first signs of labor. This area featured good drainage and fresh, clean and dry wood shavings as a floor covering.

The cows and calves included in the study were verified as those that were female from an uncomplicated birth, willing to suckle and were healthy on an immediate postdelivery exam. After birth, our team assessed the calf for vigor using the APGAR scoring system (Vannucchi et al., 2015) to exclude calves suffering neonatal asphyxia. After this exam, each calf was placed in a "cuddle box" in front of her-own dam. This was to enhance the vigor of the behavior of the newborn and to enhance the letdown of maternal colostrum. The dams were milked in the calving space using a portable milking machine. Further, only calves from dams that produced a minimum of 3 l of good-quality colostrum were included in the study.

Colostrum samples were harvested under aseptic conditions immediately after calving. For further assessment, 5 mL portions of colostrum from each quarter were combined to provide a pooled colostrum sample, distributed into four sterile PCR-grade microtubes and stored at −20 °C. The quality was determined using three standard assessment tools. Each sample was assessed using (1) colostrum balls (Ms colostrum balls, Nutri Support ©, 3705010) (density ≥ 1.045 g/L), (2) a Brix refractometer (Model Q767-1, Shangai Precision & Instrument, Shangai, China) (Brix index ≥ 22%) and (3) an optical colostrometer (Nasco Bovine Colostrometer, Nasco Farm & Ranch) (IgG ≥ 50 g/L). In general, these three methods gave good agreement on the total protein content of colostrum and the colostrum was used only if there was agreement.

Calves suckled fresh colostrum ad libitum from a bottle. The colostrum was from their own dams and collected as rapidly as possible after calving. A second feeding of colostrum was offered at about 18 hours of age. This was also from the initial collection of colostrum. The level of passive immune transfer to the calf was monitored as the increase in serum total protein (by refraction). From the 2nd to the 14th day after birth, the calves were fed daily with six liters of milk replacer (Nattimilk E Max®, Auster Animal Nutrition). The calves in this study were housed in suspended individual pens.

Blood and fecal samples were collected from each calf on days 1, 3, 7, 14 and 28. Plasma samples from anticoagulated blood were obtained from each calf in vacuum tubes (BD Vaccutainer ACD Solution A REF 364606, BD Diagnosis), stored at −20 °C. Fecal samples were aseptically obtained using a new sterile glove for each calf from the rectum and deposited in sterile containers. After transport, fecal samples were divided into sterile microtubes under a laminar flow hood using an aseptic technique and stored at −80 °C for further analysis. Frozen colostrum was thawed rapidly. It was centrifuged at 8 000*g* for 20 minutes. The fat was removed, and then, the whey was collected. The pellet was disguarded. The colostral whey was frozen at −80 °C until assessment.

Immunoglobulin G measurements

The quantity of IgG in colostrum and in the plasma samples from the calves was measured using a sandwich ELISA, according to the procedures previously published by Reber et al. (2008). Briefly, rabbit anti-bovine IgG antibody (capture antibody, B5645; Sigma, St. Louis, MO) diluted 1:400 in sodium carbonate buffer was used to coat Immulon 4HBX plates (Thermo Corp., Milford, MA) at 4–8 °C overnight. After, all plates were washed three times using the wash buffer. For this assay, plasma samples were diluted 1:1 000 000 and colostral whey samples were diluted 1:1 000 000 and 1:10 000 000. The samples and a standard dilution series covering 0.39–50 ηg/mL of bovine gamma globulin preparation were placed in duplicate wells and incubated for 1 h at 20–26 °C. The plates were washed three times. Bound IgG was detected using a rabbit anti-bovine IgG conjugated to horseradish peroxidase (detection antibody, A5295; Sigma, St. Louis, MO). The detection antibody was diluted 1:1 000 and incubated for 30 min. The plates were washed three times. The quantity of bound detection antibody was measured using the substrate 2,20-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, A-9941; Sigma, St. Louis, MO) containing 20 μL of 30% hydrogen peroxide per 11 mL ABTS (preconditioned at RT for 15 minutes) and incubated 30 min. The color signal was measured using a plate reader with a 405 ηm filter. An immunoglobulin standard (commercial bovine gamma globulin, I5506; Sigma, St. Louis, MO) was prepared as a serial dilution with a range of 50–0.39 ηg/mL.

Endpoint titer of antibodies binding E. coli

The antigen used in this assay was prepared from an ETC isolate from the case of calf diarrheal feces using the protocol described by Ryman et al. (2013). Briefly, the bacteria were grown in brain-heart infusion broth overnight at 37 °C. The density was adjusted to 10^{10} CFU/mL based on an optical standard. The bacteria were pelleted (30 minutes at 8 000*g*) and then washed in PBS three times (100 volumes relative to the pellet volume). This was followed by treatment of the cells suspended to the volume yielding 10^{10} CFU at 60 °C for one hour. The cells were washed again in PBS and suspended to 10^{8} CFU equivalent in PBS for freezing at −80 °C. A sample was tested for viable organisms, and none were observed.

One hundred μL of diluted antigen (1:1 000) was added to each well of 96-well plate flat bottom ELISA plates (NUNC-IMMUNO-M9410-1CS, Sigma) and incubated for 16–24 hours at 4 °C. The plates were washed three times with PBS containing 0.5% TWEEN

20 (wash buffer). Next, 200 μL of blocking solution (PBS supplemented with 0.1% Tween 20 and 0.5% ultrapure bovine serum albumin, A9418, Sigma-Aldrich) was added to each well. The plate was incubated for 1 hour at room temperature. The plate was washed three times with the wash buffer. The plasma samples from each calf were diluted in a series of 10-fold steps in tubes to achieve 1:1 000 starting endpoint. A total of 100 μL of diluted plasma (1:1 000) was placed in duplicate wells on the assay plate. Twofold serial dilutions of each sample from 1:1 000 to 1:16 000 were prepared down the plate for measurement of binding to the *E. coli* antigen. For assessment, the whey samples were diluted 1:8 000 in tubes. A total of 100 μL of colostrum (1:8 000) was placed in duplicate wells on the plate. A set of serials 2-fold dilutions (log2) of each sample was prepared to 1:1 024 000 across the plates. Positive controls (serum from a cow after 21 days of *E. coli* infection was used at a 1:160 dilution), negative control (fetal bovine serum with very low level of IgG diluted 1:500), and a blank containing wash buffer were included in each assay. Samples were incubated for 1 hour at room temperature. They were washed three times with wash buffer. Next, 100 μL of peroxidase-conjugated rabbit anti-bovine IgG diluted 1:2 000 was added to each well and incubated for 30 minutes. The plates were washed 3× with wash buffer. Then, ABTS containing saturating hydrogen peroxide (conditioned at RT in the dark for 15 minutes) was added to each well of the plate. The plate was incubated for 30 minutes in the dark. The color developed was measured using a plate reader with a 405 ηm filter when the positive control showed the expected color development to the naked eye. The endpoint (titer) was defined as the greatest dilution of the sample that generated an average optical density value equal or greater than two times that of negative control serum. The test was considered valid if the positive control serum generated the expected value (1.1 ± 0.2 OD).

Real-time polymerase chain reaction quantitative assessment of the number of total bacteria and the number of E. coli in feces

Total DNA was extracted from fecal samples using the PowerSoil 96-well DNA Isolation Kit (MoBio Laboratories, Inc.) strictly adhering to the manufacturer's instructions. After extraction, purified DNA suspensions were evaluated for DNA concentration using a UV spectrophotometry (A_{260} = 1.8 and A_{280} = 2.0 nm) (NanoDrop 2000, Thermo Scientific, USA). Stool samples were analyzed by Real-time quantitative PCR (**qPCR**) to determine the estimated number of all bacteria present (as copies of 16S RNA) and the apparent number of *E. coli* (using primers that were genus specific). The qPCR was carried out accordingly using the technique described by Martin et al. (2021). Initially, standard curves were constructed for each bacterial based on eight serial dilutions of chromosomal DNA from reference stock cultures. The efficiency of the qPCR amplification was based on a standard curve constructed with the following reference strain to measure 16S rRNA *E. coli* ATCC 25922. The slope of the DNA standard curve for the reference strain was calculated using a linear regression based on the serial dilution of the reference microorganism with the program incorporated in the Rotor Gene 6 000 detection system (Rotor Gene Software series 1.7). The efficiency of the reaction was expressed in genomic mean copies of total bacterial or *E. coli*.

The DNA quantification was performed using a SYBR Green system with a final volume of 20 μL containing 10 μL of SYBR Green PCR Master Mix 2X (GoTaq qPCR Master Mix/Promega Corporation, Brazil), 2 μL DNA, 7 μL ultrapure water (Promega, Brazil) and 0.5 μL (10 μM) of the solution from each primer pair [16S RNA: F:ACTCCTACGGGAGGCAGCAGT, R:ATTACCGCGGCTGCTGGC; *E. coli*: F: AGAAGCTTGCTCTTTGCTGA, R:CTTTGGTCTTGCGACGTTAT] (Ellis et al., 2011; Lee et al., 2012). Amplifications were performed on a Roche Light Cycler 480II (Roche, Germany). Amplification conditions included a cycle of 95 °C for 10 minutes for DNA denaturation, followed by 40 cycles of 95 °C for 15 seconds. Oligonucleotide (Tm) hybridization temperatures were 60 °C for 30 seconds. A dissociation curve (fusion curve) was obtained to determine primer specificity. The negative control was a reaction mixture without DNA. The identification of the fluorescent signal was performed within the final 30 seconds of the extension step.

Detection of virulence factors associated with E. coli

The fecal samples were streaked onto MacConkey medium agar plates and then incubated for 24 hours at 37 °C. Following incubation, lactose fermenting colonies were selected as coliform candidates and then subjected to the identification of the *E. coli* present using standard biochemical testing. The isolates identified as *E. coli* were grown out in broth and stored at −80 °C in BHI with 15% glycerol (Knöbl et al., 2008). The DNA extraction was performed according to the method described by Boom et al. (1990). The PCR was performed to determine if any of the five virulence factors genes (F5, ST, LT, STX1 and STX2) of *E. coli* were present among the bacteria identified (Table 1).

The PCR was carried out in a 25-μl reaction volume containing 15 mM $MgCl_2$, 200 μM dNTP, primer 20 pmol, DNA template 100 ηg, Taq DNA polymerase 0.5U and distilled water to complete a volume of 25 μL. The amplification conditions included initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at a specific annealing temperature for each target (Table 1) and 1 minute at 72 °C, and finally extension for 5 minutes at 72 °C. Positive control was included in the assay. The amplified products were visualized in 1.5% agarose gel, stained with loading dye 6X (Synapse Biotechnology, Brazil) and GelRedTM and photographed by the Kodak Scientific System. DNA ladder 100 bp was used as the molecular size marker (100–1 000 bp).

Diarrhea monitoring

Diarrhea was assessed using a fecal score system. Scores were collected daily using the protocol in the *Calf Health Scoring Criteria Manual, University of Wisconsin* – Madison (McGuirk, 2008). Those calves with fecal scores of 2 or 3 (0–3 scale) were defined to have clinical diarrhea.

Statistical analysis

Data were analyzed using standard models contained in the SAS for Windows program 9.0 (SAS Institute Inc., Cary, NC, U.S.A.). Data were subjected to analysis of residual normality (Gaussian distribution) and homogeneity of variances by the function *Guided Data Analysis*. When necessary, transformation of the variables was performed as the square root, log base 10 or as the inverse of the raw value, so that the condition of normality and homogeneity of the variances were met. Transformed data are identified in the results section with the presentation of data and comparisons. The descriptive results were expressed using the untransformed mean ± the standard deviation for each set of values.

The association of the age of the calves to the residual maternal IgG concentration and the endpoint titer for *E. coli* was analyzed using the Wilcoxon test. The association of the age of the calf in days on the DNA copy number of 16S rRNA or the number of generic *E. coli* bacteria observed was determined using PROC GLM (General Linear Model procedure). These variables were parametric and were expressed as the mean and standard deviation of the values generated. Duncan's Test was utilized to identify if there was an association between the age of the calf and the number of total or *E. coli* bacteria in the feces.

Table 1
Oligonucleotide sequences used for the detection of DNA from bacteria expressing virulence factor in calf feces by conventional PCR.

Target gene	Sequence (5′–3′)	Amplicon size (pb)	Annealing temperature	Reference
F5	F:TATTATCTTAGGTGGTATGG R:GGTATCCTTTAGCAGCAGTATTTC	314	55 °C	Franck et al. (1998)
LT	F: GCGACAAATTATACCGTGCT R: CCGAATTCTGTTATATATGT	707	50 °C	Oliveira et al. (2016)
ST	F: CTGTATTGTCTTTTCACCT R: GCACCCGGTACAAGCAGGAT	182	55 °C	Oliveira et al. (2016)
STX1	F: CAACACTGGATGATCTCAG R:CCCCCTCAACTGCTAATA	349	60 °C	Oliveira et al. (2016)
STX2	F: ATCAGTCGTCACTCACTGGT R: CTGCTGTCACAGTGACAAA	110	60 °C	Oliveira et al. (2016)

Abbreviations: F5 = virulence factor; LT = heat-labile enterotoxin; ST = heat-stable enterotoxin; STX1 = Shiga toxin 1; STX 2 = Shiga toxin 2.

Table 2
Descriptive statistics (mean ± standard deviation) obtained by analysis of (A) colostrum (n = 20) and (B) passive transfer of immunity to newborn calves (n = 20).

Parameters	Mean ± SD	Median	Min–Max
Colostrum			
Quantity of Colostrum (L)	6.18 ± 2.50	6.00	3–12
Density (mg/dL)	1 052.50 ± 9.10	1 045.00	1 045–1 075
Brix Index (%)	29.88 ± 4.19	30.00	22–38
IgG Colostrometer (mg/dL)	61.50 ± 12.37	60.00	45–90
IgG ELISA (mg/dL)	74.50 ± 20.77	80.04	37.58–109.55
E.coli bindiing titer endpoint	65 600.00 ± 53 420.28	64 000	32 000–256 000
Calves			
Colostrum fed (L)	4.09 ± 0.94	4.00	3–6.5
Mass of IgG ingested (g)	242.53 ± 59.89	225.00	150–350
Serum brix Index (%)	11.48 ± 1.08	11.65	9.40–13.50
Serum protein (g/dL)	8.00 ± 0.95	8.05	6.20–9.80

Abbreviations: ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G.
Note: The data presented in this table represent measurements made either on the calving day (colostrum) or about 48 hours after delivery. The IgG ELISA was a quantitative assay using a bovine gamma globulin standard quantity reference. The *E. coli*-binding endpoint titer was the inverse of the highest dilution that showed binding activity (OD) twice the negative control.

A non-parametric assessment (chi-square test) was used to determine if there was an association between the age of the calf and the presence or absence of diarrhea disease in calves. The associations between diarrhea and each of the virulence factors found in the *E. coli* isolates were determined using the Fisher's exact test or chi-square analysis. For all assessments, $P \leq 0.05$ was considered significant.

Results

Colostrum and passive immune transfer

The results of the analysis of the colostrum samples and the efficacy of passive transfer of IgG to the calves as measured in plasma are shown in Table 2A and B, respectively. The quantity of colostrum collected from the dams during this study ranged from 3 to 12 l, with a mean of 6.18 ± 2.50 L. Most of the colostrum samples tested as "high-quality colostrum". This was defined as a density greater than 1 045 and a Brix index greater than or equal to 22%. Only two dams in this study had less than 50 mg/dL of IgG as measured using a colostrometer. Four dams had ≤50 mg/dL of IgG when measured by ELISA. ELISA titers tend to measure lower values due to the long dilution of samples and differences in coating efficacy of the plates involved, the endpoint titers for antibody binding *E. coli* ranged between 32 000 and 256 000 (calculated as the inverse of the endpoint dilution yielding two times the negative control value within an individual day's assay).

The volume of colostrum that was ingested by the calves ranged between 3 and 6.5 L, with an average of 4.09 ± 0.94 L (essentially meeting the management target overall). Thus, the mass of IgG received in the two feedings given on the first day was 242.53 ± 59.89 grams per calf. The quantity of plasma IgG in the calves indicated that 15 of the newborn's calves (75%) had excellent transfer of IgG (≥25 mg/dL), two calves (10%) had good transfer (18–24.9 mg/dL), two calves (10%) had fair, but sufficient transfer (10–17.9 mg/dL) and only one calf had poor transfer (<10 mg/dL) of IgG, and would be considered as having a failure of passive transfer. All calves had excellent protein transfer from the dam. The typical plasma Brix index % of ≥9.4% indicated this, and the chemical measurement of total plasma protein of ≥6.2 g/dL did as well.

Changes in total plasma Immunoglobulin G and the ELISA E. coli-binding titers of calves from samplings taken between 1 and 28 days of age

The concentration of plasma IgG (mg/dL) measured in the calves is reported in Table 3. The plasma IgG level was below the detection limit of the assays before the delivery of colostrum on day 1 (<0.39 ηg/mL). Plasma IgG values rose as expected within 72 hours in circulating after feeding colostrum as shown by the measurements conducted on day 3. The plasma concentration of IgG declined from its peak on day 3 in a stepwise manner until by day 28 of age it was less than half the level measured at day 3. It appears that 28 days is about the half-life of maternal IgG in these calves.

The *E. coli*-binding titer was below the limit of detection in samples collected prior to the feeding colostrum. Relative to the transfer of maternal colostrum, there was an increase in the endpoint titer of antibodies that bound to the *E. coli* antigen in our ELISA. This was greatest in the assessment from plasma collected on day 3. Similar to the case for total plasma IgG, we observed a reg-

Table 3
Changes observed in total plasma **immunoglobulin** G (IgG) and the binding titers for the. *E. coli* antigen in calves from 1 to 28 days of age.

Parameters	Age (days)	Mean ± SD	Median	Min–Max
IgG (mg/dL)	1	0.00 ± 0.00	0.00[c]	0.00–0.00
	3	35.80 ± 18.62	29.24[a]	6.91–76.76
	7	30.41 ± 2.26	21.23[a]	1.91–79.17
	14	23.80 ± 16.27	24.53[a]	0.60–65.44
	28	14.83 ± 11.39	12.46[b]	1.54–46.97
Antibody binding to poled *E. coli* antigen as inverse of endpoint dilution	1	0.00 ± 0.00	0[d]	0–0
	3	9 300.00 ± 4 867.70	8 000[a]	2 000–16 000
	7	7 789.47 ± 4 848.58	8 000[ab]	2 000–16 000
	14	5 555.56 ± 3 329.41	4 000[bc]	2 000–16 000
	28	3 100.00 ± 1 518.31	2 000[c]	2 000–8 000

Abbreviations: IgG = immunoglobulin G.
Note: IgG values are presented in ng/mL. Antibody binding to *E. coli* antigen is shown as the inverse of the highest dilution giving an OD at least twice the negative control for that day's assay. Different letters within rows represent significant differences between calves of different ages when assessed with the Wilcoxon test ($P \leq 0.05$).

Table 4
Mean values ± standard deviation of DNA copy number of total bacteria (16S rRNA) and *Escherichia coli* (qPCR) in feces of Holstein calves in the first month of life (1st, 7th, 14th and 28th day of life).

Sample times	Total bacteria (16S rRNA)	*Escherichia coli* (qPCR)
D1	2.53E+01 ± 3.92E+01[b]	4.01E-02 ± 5.45E−02[b]
D3	1.08E+05 ± 9.12E+04[b]	1.43E+04 ± 1.32E+04[a]
D7	1.75E+05 ± 1.51E+05[b]	9.03E+03 ± 1.46E+04[a]
D14	3.46E+05 ± 3.44E+05[b]	1.49E+03 ± 1.75E+03[b]
D28	1.22E+06 ± 1.32E+06[a]	1.93E+02 ± 3.74E+02[b]

Letters in the same column indicate a significant difference between calves of different ages for each measurement according to analysis with Duncan's test ($P \leq 0.05$).

ular decline in the binding titer for *E. coli*. The decay followed the pattern for total IgG transferred from the dam, but had a more severe decline in titer, with about a 50% reduction by day 14 and about 66% reduction two weeks later.

Quantification of bacterial DNA in the calf feces by qPCR

The mean and standard deviation of the copy number for 16S RNA (representing the total number of bacteria in the fecal sample) and the DNA copy number for a generic *E. coli* sequence that were measured in calf feces are shown in Table 4. The results of a time series analysis indicated a gradual increase in the total number of bacteria (16S rRNA) in the feces of calves. This reached its highest level on the last day of the trial, day 28 (significantly higher than on any prior days 1 to 14). Despite a gradual, stepwise increase in the number of bacteria in the feces, there were no statistical differences among the values for days 1, 3, 7 or 14 in this study relative to the initial number measured. The number of *E. coli* in feces indicated only a few of these bacteria were present in feces on day 1. The number of copies of *E. coli* DNA significantly increased on both days 3 and 7 relative to the number observed on day 1. After day 7, the number of *E. coli* in feces gradually declined in each of the remaining assessments until the end of the trial on day 28. On day 29, it appears the number of *E. coli* was essentially the same as on day 1.

Attempts to detection of E. coli virulence factor genes

A total of seventy-six *E. coli* isolates were isolated from 100 fecal samples over the period of the study. Thus, 76% of the samples chosen provided *E. coli* for analysis. The fraction of fecal samples yielding *E. coli* isolates was only 10% on day 1. However, the isolation frequency improved with 100% of attempts on day 3, 90% of attempts on day 7, 95% of attempts on day 14, and 85% of attempts on day 28 yielding isolates for testing. On each collection day, 20

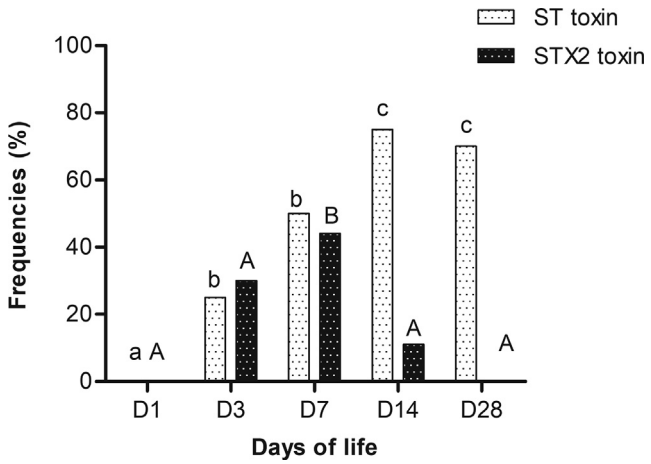

Fig. 1. The percent of *E. coli* isolates positive for heat-stable enterotoxin genes (ST) or Shiga toxin 2 genes (STX2) isolated from fecal samples of calves collected over the period from birth through 28 days of age. Note: Lowercase letters above the columns shown here indicate significant differences between samples collected at different times during the study for the gene for ST toxin. Capital letters above the columns shown here indicate significant differences between samples collected at different times during the study for the gene for STX2 toxin. Abbreviations: ST = heat-stable enterotoxin; STX2 = Shiga toxin 2.

fecal samples were utilized in attempts to isolate *E. coli* for virulence testing.

The two virulence factor genes most frequently identified among the *E. coli* isolates collected on days 3 through 28 are shown in Fig. 1. On day 1, no enterotoxin genes were identified in any of the isolates tested. However, this was the smallest sampling of isolates as only 10% of the 20 fecal samples yielded an isolate for assessment. The fraction of *E. coli* isolates carrying genes for ST toxin increased steadily over from the D3 sampling to the D14 sampling. ST-positive isolates were identified most frequently on day 14 (75%). This fraction of ST-positive isolates did not appear to change on day 28 (70%) given the sensitivity of the measurement and small n of 17. In contrast, all the *E. coli* isolates collected were negative for the LT toxin gene over the entire sampling period. This may represent that LT is not found in the immediate neonatal environment of these 20 calves.

Finally, the F5 pilus gene was only isolated once in this study. It was identified that an *E. coli* was isolated on day 14. While it is an important factor in the colonization of some pathogenic *E. coli*, it is unlikely to have played a role in the diarrhea seen in this study. The STEC STX1 gene was identified in two *E. coli* isolates from day 14, 10% of those assessed. The STX2 toxin gene was identified in 30% of the *E. coli* isolates collected on day 3, 44.4% of the isolates collected on day 7, 11.1% of the isolates collected on day 14, but none

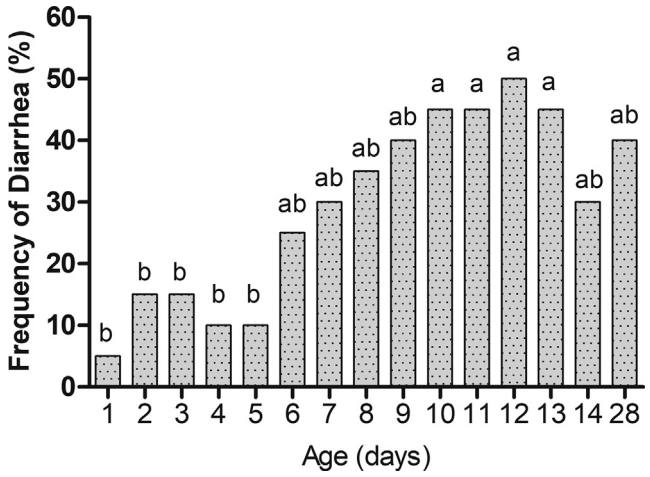

Fig. 2. Daily fraction of calves with diarrhea during the period of 28 days of age as determined by fecal scoring. Note: Different letters above the columns show significant differences between days of age using chi-square analysis ($P \leq 0.05$).

of the isolates collected on day 28. When the identity of the calves and the clinical indications of diarrhea were subjected to correlational analysis, there was no statistically significant association found between any of these virulence factors and clinical diarrhea at point this study. This was tested using Fisher's exact test or chi-square analysis depending on the relative frequency of the data ($P \leq 0.11$).

Diarrhea detection

The percent of calves with diarrhea each day is shown in Fig. 2. The percent of calves with diarrhea was quite low during the first 5 days. A small representation was seen on days 2 and 3, this is often due to K99 *E. coli*, a bacterial pathogen not tested for in this study. A gradual increase in the fraction of calves with clinical diarrhea was observed starting on day 6 that peaked on day 12 after calving. This suggests that the diarrhea was caused by *E. coli* from the calf environment that should be represented among those measured in the fecal matter. It is interesting to note that the peak number of *E. coli* copies in fecal contents roughly aligns with the initial rise in diarrhea incidence (about day 7, see Table 4 and Fig. 2). While the measurements were not well synchronized to allow a day-by-day assessment of *E. coli* number in feces, by day 14, both diarrhea incidence and *E. coli* number had declined.

A model of how transferred maternal E. coli-binding antibody, the number of fecal E. coli and clinical diarrhea are related

The relationship among binding antibody (*E. coli* IgG) mean values, the number of fecal *E. coli* determined by PCR (qPCR16S) and frequency (%) of clinical diarrhea determined by fecal score of calves at D1, D3, D7, D14 and D28 of life is shown in Fig. 3. Given the reasonable relationship between the level of *E. coli* reactive antibody and the limited development of diarrhea in the face of a rapidly increasing number of enteric *E. coli*, it is reasonable to postulate that a combination of enteric action and systemic delivery of passive anti-*E. coli* antibody manages the early development of diarrhea in the calves and limits the number by clinical disease. It is clear that as the titer of anti-*E. coli* antibody drops, the frequency of clinical diarrhea rises and it is initially associated with reduced management of *E. coli* number in the enteric tract by maternal factors, probably antibody and others not measured here.

A role for systemic antibody transferred with colostrum is also reasonable. It is likely that the anti-*E. coli* antibody in circulation is released into tissues, including mucosal tissues as inflammatory insult opens the circulation to flood those tissues with protective humoral elements in an attempt to manage the developing disease. As the enteric surface if rather large in the calf, the efficacy is not complete (see Fig. 2) with the clinical diarrhea persisting past the drop in both *E. coli* number in fecal contents and the decline in anti-*E. coli* antibody we can measure. Thus, it is reasonable to suggest that failure of passive transfer or transfer of calves to an environment where maternal antibodies against local *E. coli* pathogens are not as effective would produce more cases and more severe cases of clinical diarrhea with poorer outcomes.

Author's point of view

This research represents the development of a basic model derived from the results estimating the total number of *E. coli* in the gut of very young calves over the first 28 days after birth as associated with the titer of anti-*E coli* antibody that was transferred from the dam to function both in the gut and circulation of the calf in management of *E. coli* colonization and the development of *E. coli*-mediated disease. The results of this punctate longitudinal study can be used in the context of the model suggested in the results to design further research to evaluate more fully the ecology of maternal antibody transfer designed to prevent *E. coli* disease in the calf based on more intensive sampling, including collecting both fecal samples and antibody titers at days 5, 7, 9, 11, 14, 18 and 21 to examine the dynamics against the typical diarrhea profile in such calves. Further, as we know that maternal antibody can present a barrier to modulation of the immune status of the calf, we also believe that study of prepartum vaccination, colostrum management, colostrum supplement or use of commercial colostrum substitutes should also be studied within the context of the model.

Colostrum intake and management are broadly agreed to be the single most important factor in providing a healthy calf and to mediate survival of the calf through weaning. The average level of IgG in colostrum was 74.50 ± 20.77 mg/mL in this study. This was similar to the IgG level of 70.7 ± 26.6 mg/mL that was recently reported by Derbakova et al. (2020). Our study was designed to ensure an adequate quantity and quality of colostrum intake during the first few hours of life. The volume of colostrum fed, and the typical mass of IgG ingested by the calves in our study were 4.1 L and 242.3 g, respectively. All the calves in our study ingested a minimum of 150 g of IgG as recommended in the literature (Lago et al., 2018). Only one calf was on the borderline of that expectation. This appeared to provide for successful colostrum management. The reference values for minimal transfer of protein from colostrum that were recently established by Godden et al. (2019) were exceeded in these calves.

Before colostrum intake, both the total level of plasma IgG, and *E. coli*-binding antibody activity were below the level of detection in our assay system. After the intake of colostrum, the level of circulating transferred IgG and *E. coli*-binding antibody titers reached its maximum level by the first measurement on day 3. A gradual decline in the circulating level of total IgG and the specific *E. coli* titer was observed. All the dams in our study received two commercial vaccines during pregnancy. Each contained components to stimulate a response to *E. coli*. These were used to enhance the production and transfer of antibodies to the mammary gland during the end of the prepartum period represented in the composition of the colostrum (Crouch et al., 2001).

Our data were in general agreement with an earlier study conducted by Widiasih et al. (2004). These authors evaluated the level of antibody against ST associated with Shiga toxin-producing *E. coli* (STEC) in a binding ELISA. They used samples of colostrum, transi-

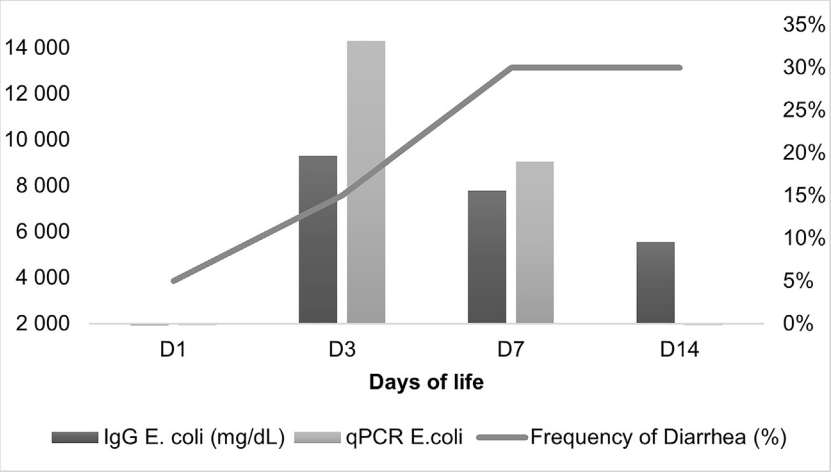

Fig. 3. Relationship among the mean of binding antibody (*E. coli* IgG) expressed in mg/dL, number of fecal *E. coli* determined by PCR (qPCR) and frequency (%) of clinical diarrhea determined by fecal score of calves at D1, D3, D7, D14 and D28 of life.

tion milk and serum from calves after colostrum intake to assess O-antigen-bearing LPS binding. The highest antibody binding titers against these LPS from STEC were in the colostrum of the dams after parturition. Further, the titers were most elevated in the serum of calves receiving colostrum from dams with high STEC antibody titers. Al-Alo et al. (2018) assessed specific antibodies recognizing whole cell ETEC antigen, and they also found similar results to those in our study (Al-Alo et al., 2018).

The number of copies of 16S RNA (indicating total bacterial load) in the feces of neonatal calves increased during the entire study. This reflected the maturation of intestinal colonization as expected. The *E. coli* DNA copy number in the fecal samples was lowest on day 1, increased to its maximum on day 7 in our calves, then declined to almost D1 levels on D28. It should be noted that the extraction of *E. coli* did not occur on D1, possibly because the sample analyzed was meconium, and it is believed that the intrauterine environment is almost sterile (Zhu et al., 2021). Therefore, intestinal colonization occurred after delivery and was confirmed by the results obtained at D3 in this study.

The abundance of *E. coli* appears to have some variation over the first seven days of life according to Lukás et al. (2007), Mayer et al. (2012) and Martin et al. (2021). There is some evidence that the initial transfer of *E. coli* at parturition and during feeding and grooming contact between dams and calves influences the colonization of the neonatal gut during early life (Laguardia-Nascimento et al., 2015; Yeoman et al., 2018). Other important sources of *E. coli* in the environment during early life included other dams in the calving pen, the bedding of the calving pen, and fomites and structure calves encounter (Matamoros et al., 2013).

In our study, all our healthy calves were born in the same environment about the same time. We used a single standard protocol for colostrum management (collection, handling and feeding) and for the feeding of the neonates. No antimicrobials were used in these calves during the first 14 days. The peak quantity of *E. coli* in feces was detected in the interval from day 3 to day 7. This was after colostrum intake, and the transfer of calf from calving pen to the calf housing system. Therefore, we believe that the colostrum-associated microbes and environmental microbes around the calves had a great influence on the initial, and transitory gut colonization with *E. coli* during the first three to seven days of life.

We saw the expect differential profiles for total bacteria in feces and the *E. coli* in the fecal samples over the first month of life. The development of the gut microbiome requires an expansion of the number of total bacteria to achieve the balance of gut development, seeding innate immune capacity and initiation of adaptive response. The number of *E. coli* in the feces is an indicator of their fraction of the total population. Its dynamics is governed by competition and host response. The analysis of *E. coli* expressing virulence gene factors investigated in this study did not yield a profile of genes generally associated with diarrhea in calves. First, the peak percent of calves with diarrhea was detected in our calves between day 12 and day 14, with a rise beginning at 7 days of age. Diarrhea in calves associated with gastrointestinal infection with *E. coli* pathotypes is most common during the first 4 days of age and often associated with *K99* organisms (McGuirk, 2008). Second, there were no associations observed between the virulence factor genes detected and the presence of clinical diarrhea during this study by the analysis we conducted. We admit that the n for the number of genes discovered on any given day of sampling was small and often not well aligned with the clinical diarrhea observed.

The peak of diarrheal disease observed in our study occurred between day 10 and day 14. This matches to some degree with the peak frequency of fecal samples having the genes for ST (indicating ETEC). However, the expression of these genes was not associated with clinical diarrhea in our study by the statistical analysis conducted. ST genes are present in many different strains of *E. coli*, and they may be detected using a standard PCR method. In addition, many of the ST genes identified do not appear to be functionally expressed by all *E. coli* in which they are detected (Osek, 2000). The expression of ST toxin protein appears to be related to the community of organisms in the intestinal bacterial microbiota, the level of competition among the bacteria in the intestinal tract, and the stress under which they are living.

We believe that it is possible that ST ETEC were involved in the dysbiosis of the gut leading to a diarrhea syndrome. This may have been caused in combination with other enteropathogens, such as *Cryptosporidium* sp. or Rotavirus that yielded clinical disease (Cruvinel et al., 2020). Fecal samples from some calves manifesting diarrhea in our study were collected for the detection of other infectious agents associated with diarrhea. Many of these samples were positive for *Cryptosporidium* sp. and/or Rotavirus (data not shown). Past studies indicated clinical diarrhea is much more common in polymicrobial infection. This investigation was conducted with a narrow focus on *E. coli* in the gut of young calves. We hoped that the data collected would help develop strategies to treat, or

prevent, neonatal diarrhea caused by *E. coli* on the commercial farm. Further research is clearly needed to understand the pathogenesis of polymicrobial diarrheal disease in the young calf.

We found STEC STX1 in fecal samples from two calves of 20 on day 14. On the other hand, the presence of STX2 gene fecal samples was found on day 3 (30%). with a peaked fraction on day 7 (44%), and then, the percent of *E. coli* isolates with STX2 declined on day 14 to 11%. The STEC bacterial pathotype was not generally associated with clinical diarrheal disease in the calves in our study. This agreed with Badouei et al. (2010). These authors identified STEC in the feces of 8% of diarrheal calves. However, they found a similar, but larger percentage in non-diarrheal calves (10.3%).

At the beginning of life, the gut of calves suffered an invasion by *E. coli* represented especially by isolates positive for ST or STX2 over the period from birth to 28 days of age. The most common *E. coli* virulence gene K99 was poorly detected in fecal samples throughout the neonatal period. The role of ST or STX2 in the literature is controversial. In addition, these strains were not associated with calf's diarrhea in this study.

The high titer of *E. coli*-binding antibodies transferred to calves in colostrum appeared to manage the colonization with *E. coli* to protect them from *E. coli* infection during the first few days. This was most effective during the first 5 or 6 days. Most of the cases of diarrhea in these calves resolved rapidly. Diarrhea lasted an average of 2–4 days. No significant systemic indicators of sepsis, or significant dehydration were observed among these calves. These observations are not consistent with classically described severe colibacillosis. We believe that the colostrum management utilized here, including prepartum vaccination of dams against neonatal diarrhea agents, and the handling and delivery of colostrum adopted from the recommendations from the Dairy and Calf Heifer Association (2016), decreased the risk of *E. coli* infection in the gut of the calves included in this study. However, we did not do a comparative study of calves fed colostrum from dams which were not vaccinated to determine the difference in plasma titers of maternal anti-*E coli* antibody or transferred *E. coli* early in life.

So, we concluded that the challenge marked by the high load of *E. coli* in the gut seems to be controlled and regulated by the specific maternal antibodies, since the calves from this study developed mild cases of diarrhea without general compromising such as sepsis caused by *E. coli.*

Ethics approval

The Animal Research Ethics Committee of the School of Veterinary Medicine and Animal Science at the University of São Paulo approved all procedures involving animals in this study (Protocol number 2329260218).

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Declaration of interest

None.

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