Effect of colostrum feeding strategies on the expression of neuroendocrine genes and active gut mucosa-attached bacterial populations in neonatal calves

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ABSTRACT

Colostrum feeding is vital for the development of the immune system and gastrointestinal tract in neonatal calves; however, it is currently unknown whether different colostrum feeding strategies affect their neuroendocrine system and potentially the gut–brain axis. The present study investigated the effect of 3 different colostrum feeding regimens on the expression of neuroendocrine genes in adrenal glands and gastrointestinal tissues and on the abundance of intestinal commensal bacteria. Holstein bull calves were fed colostrum immediately after birth and randomly assigned to 3 groups: whole milk ($n = 8$), mixture of 50% colostrum and 50% whole milk ($n = 8$), and colostrum (CF; $n = 8$) for 72 h with 12-h intervals. Adrenal glands, ileum, and colon tissues were collected at 75 h and were subjected to the expression of 11 targeted neuroendocrine genes and the abundance of tissue mucosa-associated bacteria measurement using quantitative real-time PCR and quantitative PCR, respectively. The expressions of all targeted genes were detected, and the expression of α -adrenergic receptor (*ADRA1A*) gene was affected by CF in adrenal glands and gut tissues. In addition, CF upregulated the expression of *HTR4* (serotonin receptor) and *SLC4A4* (serotonin transporter) genes in the ileum and increased the abundance of active *Lactobacillus* spp. and *Escherichia coli* (as detected at RNA level) associated with ileum and colon tissue. Furthermore, there were positive correlations between the abundance of active *Lactobacillus* spp. and *E. coli* with expression

of *HTR2B* and *HTR4* genes in the colon, suggesting that extended colostrum feeding strategies may affect the interaction between gut microbiota and host endocrine functions in neonatal calves.

Key words: colostrum feeding, neuroendocrine, mucosa-associated microbiota, neonatal calf

INTRODUCTION

Newborn calves lack immunocompetence (Hulbert and Moisé, 2016), and their development of acquired immunity largely relies on the transfer of immunoglobulins (mainly IgG) from colostrum (Hammon et al., 2013). Colostrum also induces the maturation of the gastrointestinal tract (Hammon et al., 2013), because it contains hormones and growth factors. Recent studies have also revealed that colostrum feeding can promote bifidobacterial colonization in the ileum and reduce the colonization of opportunistic pathogenic organisms in both the ileum and colon of dairy calves (Malmuthuge et al., 2015; Song et al., 2019). In addition, the timing of colostrum is important, because delayed colostrum feeding can affect passive transfer of IgG (Fischer et al., 2018). Therefore, feeding adequate amounts of colostrum in the first hours of life has been largely recommended to support neonatal calf development and adaption to extrauterine life.

To date, most studies have focused on the effect of colostrum feeding on metabolic and hormonal changes in newborn calves. For instance, calves fed colostrum immediately after birth had higher concentrations of plasma glucose, bilirubin, insulin, and insulin-like growth factors compared with calves fed colostrum on d 2, suggesting that colostrum intake stimulates systemic metabolic regulation (Hadorn et al., 1997). Similarly, restriction of colostrum and milk feeding in preweaned calves resulted in increased concentrations

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of cholesterol, albumin, and cortisol, and reduced insulin in plasma (Hammon et al., 2002). However, it is unknown whether colostrum feeding can affect the calf neuroendocrine system, which is capable of producing hormones/neurotransmitters (Toni, 2004). The hypothalamic–pituitary–adrenal and sympathetic–adrenal–medullary axes, a major neuroendocrine system that regulates stress response (Smith and Vale, 2006), are underdeveloped at birth but develop simultaneously with the immune system to make animals immunocompetent and resilient (Hulbert and Moisés, 2016). Serotonin signaling partly mediates gut–brain axis, and promotes postnatal development of gut function, such as cell proliferation and gut motility (Tharayil et al., 2010; Spohn et al., 2016). Although the neuroendocrine system is important in the systemic regulation of metabolism and immunity via endocrine pathway and neuro-transmission, our understanding of such systems in ruminants is limited, especially regarding whether colostrum feeding strategies affect local neuroendocrine function in adrenal glands and intestinal tissues in neonatal calves.

In the gut, diverse microbial populations colonize the mucosal surface and play an important role in modulating the host's immune functions (Mulder et al., 2011). Recent emerging evidence has revealed the role of gut microbiota on the gut–brain axis, as shifts in gut microbiota can affect peripheral serotonin (5-hydroxytryptamine) production and alter cytokine production, which affects neurophysiology, and butyrate, a microbial product that directly affects cell activities involved in the brain and gut barrier (Smith, 2015). It has also been reported that the supplementation of *Bifidobacteria* to mice and humans reduces their stress and anxiety (Allen et al., 2016; Moya-Pérez et al., 2017). We hypothesized that the enhanced gut *Bifidobacteria* colonization by colostrum feeding could therefore induce proper neuroendocrine function (mainly lower the stress level) in calves. However, the relationship among different colostrum feeding strategies, gut microbiota, and host endocrine production in calves has not been studied.

Commonly, calves are fed milk or milk replacer after the first colostrum feeding on farm (Vasseur et al., 2010). Given that colostrum has positive effects on gut health of animals, as stated above, we hypothesize that extending colostrum feeding could potentially benefit calves by enhancing their neuroendocrine functions and promoting the growth of beneficial bacteria in the gut. Therefore, the primary objective of the present study was to investigate the effect of extended colostrum feeding on the expression of neuroendocrine genes in the adrenal gland and gut tissues and the mucosa-attached bacterial colonization in neonatal calves.

MATERIALS AND METHODS

Animal Study

The animal study was performed at the Dairy Research and Technology Centre of the University of Alberta from January to August of 2017. All procedures were performed following the Canadian Council on Animal Care guidelines and were approved by Animal Care and Use Committee, University of Alberta (AUP00001595).

This is a companion study to Inabu et al. (2019) and Pyo et al. (2020), in which the management of animals and experimental design is described in greater detail. Briefly, Holstein bull calves ($n = 24$) with an average BW of 42.7 ± 1.8 kg were separated from their dams at birth and housed in individual pens. They were fed pasteurized, pooled colostrum containing 62 g of IgG/L (Saskatoon Colostrum Company Ltd., Saskatoon, SK, Canada) at 7.5% BW within 1 h of life using nipple bottles. From the second feeding, calves were randomly assigned to 1 of 3 feeding management groups: whole milk (**WM**; $n = 8$); mixture of 50% colostrum and 50% whole milk (**CM**; $n = 8$); or colostrum (**CF**; $n = 8$), with all meals fed at 5% of BW. Pooled whole milk was obtained from cows at the Dairy Research and Technology Centre. All calves received their second feeding 10 h after the first colostrum feeding, and subsequent meals were provided every 12 h until 72 h of life. All animals were euthanized at 75 h of life by administering Euthanyl (30 mg of pentobarbital/kg of 75-h BW; Vetoquinol, Lavaltrie, QC, Canada), following the procedures described by Pyo et al. (2020). Ileum, colon, and right and left adrenal gland tissues were collected within 30 min after euthanasia using sterile dissection tools and following the steps as described by Pyo et al. (2020). Samples were rinsed 3 times with sterile phosphate buffered saline (pH 7.0), immediately frozen using liquid nitrogen, and stored at -80°C until further analysis.

RNA Extraction

Tissue samples were ground into fine powder under liquid nitrogen before RNA isolation. Total RNA (~ 0.1 g) from adrenal glands, ileum, and colon tissues was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's procedures. Assessment of RNA integrity and quantity was performed using Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit 3.0 Fluorometer (Invitrogen). Samples exhibiting integrity values greater than 7.0 were used for further analysis of gene expression and gut bacterial abundance.

Gene Selection and Primer Design

Sequences of mRNA for 11 targeted genes related to the neuroendocrine system of *Bos taurus* species were obtained through National Center of Biotechnology Information (www.ncbi.nlm.nih.gov). Among them, genes encoding steroidogenic acute regulatory protein (*StAR*) and cytochrome P450, subfamily XI B, polypeptide 1 (*CYP11B1*), which are key enzymes in steroid and glucocorticoid production, respectively, were assessed (Liu et al., 2013). In addition, 8 gene-encoding receptors for glucocorticoids, catecholamines (epinephrine and norepinephrine), adrenocorticotrophic hormone (*ACTH*), serotonin, and γ -aminobutyric acid were selected: nuclear receptor subfamily 3 group C member 1 (*NR3C1*)-encoding glucocorticoid receptor (Juszczak and Stankiewicz, 2018); melanocortin 2 receptor (*MC2R*)-encoding ACTH receptor (Proudnikov et al., 2008); adrenoceptor β_2 (*ADRB2*)- and adrenoceptor α_{1A} (*ADRA1A*)-encoding adrenergic receptors for catecholamines (Li et al., 2014); γ -aminobutyric acid type A receptor β_2 subunit (*GABRB2*)- and γ -aminobutyric acid type B receptor subunit 1 (*GABBR1*)-encoding receptor for neurotransmitter (Zhao et al., 2007); and 5-hydroxytryptamine receptor 2B (*HTR2B*)- and 5-hydroxytryptamine receptor 4 (*HTR4*)-encoding serotonin receptors (Corominas et al., 2010). Additionally, solute carrier family 6 member 4 (*SLC6A4*) gene-encoding serotonin transporter was included (Nutt et al., 2015). Primers targeting the above-described genes were designed using Primer Express Software (Thermo Fisher Scientific, Waltham, MA) and were validated with in silico analysis, using the UCSC In-Silico PCR Program (Kuhn et al., 2013), followed by PCR validation (Table 1).

Detection of Gene Expression Using Quantitative Real-Time PCR

Total RNA (1 μ g) was reversely transcribed using iScript reverse transcription Supermix for quantitative real-time PCR kit (**qRT-PCR**; Bio-Rad Laboratories, Hercules, CA) to generate cDNA. Quantitative RT-PCR was performed with ViiA 7 Real-Time PCR and QuantStudio 6 Real-Time PCR (Applied Biosystems, Foster City, CA) using the Fast SYBR Green Master Mix (Applied Biosystems) chemistry. All samples were analyzed in triplicate. Each reaction contained 5 μ L of Fast SYBR Green Master Mix, 1 μ L of forward primer (20 pmol/ μ L), 1 μ L of reverse primer (20 pmol/ μ L), 2 μ L of nuclease-free water, and 1 μ L of cDNA template (2.5 ng/ μ L). The reaction was carried out using the following program: 95°C for 20 s for initial denaturation;

then 40 cycles of 95°C for 1 s, followed by annealing/extension for 30 s at 60°C; and a final melting curve stage (95°C for 15 s; 60°C for 1 min; 95°C for 15 s), with fluorescence detection at 0.05°C intervals. Melting curve analysis was performed to confirm single product amplification. Expression of 3 housekeeping genes: *ACTB*, *GAPDH*, and 18S rRNA, in the ileum, colon, and adrenal glands under each treatment were analyzed using NormFinder algorithm (Wang et al., 2012). According to the statistical analysis, there are no differences of *GAPDH* expression among tissues and treatments ($P > 0.05$), whereas there is a tissue effect on the expression of 18S rRNA gene ($P < 0.001$) and a tentative effect on the expression of *ACTB* gene ($P = 0.06$). Therefore, *GAPDH* was used as an endogenous housekeeping gene for downstream qRT-PCR analysis. For each sample, the expression of targeted genes was presented as Δ Ct, calculated by subtracting the average cycle threshold (**Ct**) of the *GAPDH* from the average Ct of targeted genes. Differences in gene expression were evaluated by calculating Δ Ct values, with higher Δ Ct being lower expression and lower Δ Ct being higher expression.

Estimation of Active Mucosal Attached Bacteria

The above cDNA were also used to evaluate the abundances of active total bacteria, *Lactobacilli* spp., *Bifidobacterium* spp. and *E. coli* through measuring their respective copy number of 16S rRNA using qPCR with specific primers (Table 1). The qPCR was performed using SYBR Green chemistry with StepOnePlus Real-Time PCR System (Applied Biosystems). All reactions for total bacteria, *Lactobacillus acidophilus*, *Bifidobacterium* spp., and *E. coli*, were conducted with standard curve constructed out of 16S DNA of *Butyrivibrio hungatei*, *Bifidobacterium longum*, and *E. coli* K12, respectively. The copy number of 16S rRNA/g of tissue was calculated with an equation previously described by Malmuthuge et al. (2015). Abundance of *Bifidobacterium* spp. and *E. coli* was calculated by dividing the copy number of 16S rRNA of genus/species by the copy number of total bacteria. *Bifidobacterium* spp. and *E. coli* log-transformed data were calculated as $\log_{10}$ of the copy number of 16S rRNA/g.

Cortisol and Serotonin Circulating Concentrations

Plasma samples were collected from the calves in WM, CM, and CF before euthanasia (at 72 h of life) and stored at -20°C , as described in Inabu et al. (2019). Briefly, aprotinin (Sigma-Aldrich, St. Louis, MO) was added to blood samples (in the ratio of 500 kallikrein inhibitor units:mL of blood) and centrifuged at 3,000 $\times$

Table 1. Primers used in this study

Item	Product length (bp)	Annealing temperature (°C)	Oligo sequence (5' to 3') ¹	Accession number	Reference
Targeted bacterial groups					
<i>Lactobacillus</i>	120	62	F: GAGGCAGCAGTAGGGAATCTTC R: GGCCAGTTACTACCTCTATCCTTCTTC	—	Delroisse et al., 2008
<i>Bifidobacterium</i>	196	66	F: ATCTTCGGACCBGAYGAGAC R: CGATVACGTGVACGAAGGAC	—	Cleusix et al., 2010
<i>Escherichia coli</i>	544	62	F: GGAAGAAGCTTGTCTTCTTGCTGAC R: AGCCCGGGATTTCACATCTGACTTA	—	Sabat et al., 2000
Total bacteria	468	60	F: ACTCCTACGGGAGGCAG R: GACTACCAGGGTATCTAATCC	—	Stevenson and Weimer, 2007
Targeted genes					
<i>CYP11B1</i>	208	60	F: TCACGGACATCGTGGAGACA R: CAGACTCGGACCGCAATAA	NM_174638.3	This study
<i>GABRB2</i>	150	60	F: TACGATGGACCCCATGAGA R: AACCCGGCTTCCGATACTG	NM_001192760.2	This study
<i>HTR2B</i>	156	60	F: CGAGCCCCACATCAGTAAA R: TGAACCTCGGAGCCTCATTTG	NM_001205448.1	This study
<i>MC2R</i>	199	60	F: TGTGTGGACTCCCTGTTC R: ATGGGAGAAGGTACGATGGT	NM_174109.2	This study
<i>NR3C1</i>	280	60	F: GAGAGGGAATGTGATGGA R: CAGAGGAAAGGCTGATTTGG	NM_001206634.1	This study
<i>STAR</i>	166	60	F: CTGCCGAAGACCATCATCAA R: CTCCTCGTGAGCCCTCAAAAC	NM_174189.3	This study
<i>HTR4</i>	265	60	F: AGCCATCAGCTACCTCGAAA R: ATCAAACCCACAGCCAGACC	NM_001040485.1	This study
<i>ADRB2</i>	265	60	F: GGATTGCCTTCCAGGAGCTT R: CGGCATTACAGCAGTGAGTCA	NM_174231.1	This study
<i>SLC6A4</i>	288	60	F: GGCAGTACCACCGAAATGGA R: CGTGCCGCGGTGTAATAATTC	NM_174609.2	This study
<i>GABBR1</i>	212	60	F: GCTGTGCCCGTCAAAAACC R: GCCTCGGTCTATCTCGTCAACT	NM_001163275.1	This study
<i>ADRA1A</i>	294	60	F: CTCAAATTTTCCCGCGAGAA R: CGTGTAGCCCCAGGGTGTGTT	NM_174498.2	This study
Housekeeping genes					
<i>GAPDH</i>	119	60	F: GGCGTGAACCCACGAGAAGTATAA R: CCTTCCACGATGCCAAAGT	NM_001034034.2	Zaros et al., 2007
<i>ACTB</i>	177	60	F: CTAGGCACACGGGCGTAATG R: CCACACGGAGCTCGTTGTAG	AF191490	Charavaryamath et al., 2011
18S rRNA	151	60	F: GTAACCCGTTGAACCCCAAT R: CCATCCAATCGGTAGTAGCGG	X03205	Schmittgen and Zakrajsek, 2000

¹F = forward; R = reverse.

g for 20 min at 4°C. Plasma concentrations of cortisol and serotonin were measured using commercial ELISA kits (Cortisol ELISA, K003-H5, Arbor Assays, Ann Arbor, MI; Serotonin ELISA, ADI-900-175, Enzo Life Sciences, Farmingdale, NY), following the manual provided by the manufacturer. The intra-assay coefficients of variation for cortisol and serotonin were 4.25% and 2.11%, respectively.

Statistical Analysis

The effect of treatment on the expression of genes related to the neuroendocrine system and bacterial population was evaluated using nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test in R software (<https://www.r-project.org>). The Benjamini-Hochberg method was used to adjust *P*-values. Significant differences were declared at $P < 0.05$ and tendencies were discussed at $0.05 \leq P < 0.10$. Gene expression (ΔCt) and bacterial data (copy number/g of tissue) were presented in least squares means with standard error of mean. Pearson correlation in R software using function Rcorr was used to investigate a potential relationship between neuroendocrine gene expression, plasma hormone concentrations, and gut bacteria. Level of significance for correlation coefficients were declared at $r > 0.4$ or $r < -0.4$ with $P < 0.05$.

RESULTS

Expression of Genes Involved in Neuroendocrine Functions in Adrenal Glands, Ileum, and Colon Tissues

The expression of 11 selected genes was detected in adrenal glands, ileum, and colon tissues at 75 h of life (Figure 1). Among them, expression of *CYP11B1* (indicated as ΔCt , -2.22 ± 0.14 vs. 7.14 ± 0.53 or 5.61 ± 0.64 , $P < 0.001$; lower ΔCt means higher expression), *STAR* (-3.11 ± 0.09 vs. 6.66 ± 0.15 or 6.24 ± 0.49 , $P < 0.001$), and *MC2R* (2.56 ± 0.09 vs. 5.77 ± 0.28 or 5.34 ± 0.67 , $P < 0.001$) was higher in adrenal glands compared with their expressions in the ileum and colon tissues. In contrast, higher expression of *ADRA1A*, *ADRB2*, and *HTR4* was detected in ileum (7.37 ± 0.43 , 3.9 ± 0.12 , 0.16 ± 0.15) and colon (5.61 ± 0.64 , 3.71 ± 0.23 , -1.05 ± 0.28) compared with their expression levels in adrenal gland (9.66 ± 0.56 , 5.99 ± 0.25 , 7.74 ± 0.28), respectively ($P < 0.001$ for 3 genes).

Effect of Colostrum Feeding Strategy on Neuroendocrine Hormones and Expression of Neuroendocrine Genes

Colostrum feeding strategies did not affect plasma concentrations of cortisol and serotonin (Figure 2).

Figure 1. The detected expression of targeting genes in adrenal glands, ileum, and colon tissues using quantitative real-time PCR. The expression of targeted genes was presented as ΔCt (cycle threshold) of GAPDH from the average Ct of targeted gene. Lower ΔCt value means higher expression. The lower and upper hinges correspond to the first and third quartiles. The middle lines indicate the median. The lower and upper whisker extends from the hinge to the smallest and largest value no further than 1.5-fold of the inter-quartile range from the hinge. Data falling outside the whiskers are plotted as outliers of the data.

Figure 2. Plasma concentrations of (A) cortisol and (B) serotonin in calves under different colostrum and milk feeding managements (WM = whole milk; CM = mixture of 50% colostrum and 50% milk; CF = colostrum feeding; $n = 8$ in each group). Calves were fed treatment diets at 12-h intervals after the first colostrum feeding, and blood samples were collected at 75 h of life. Data are presented as mean $\pm$ SEM.

However, the expression of certain neuroendocrine genes was altered in adrenal glands and intestinal tissues (Tables 2, 3, and 4). In adrenal glands, CF-fed calves had a higher ($P = 0.02$) expression of *ADRA1A* compared with WM- or CM-fed calves (Table 2), whereas the expression of *StAR* tended to be lower ($P = 0.02$) in CM-fed calves compared with WM- or CF-fed calves. No difference in expression of other genes among the 3 groups was detected. In ileal tissue, the expression of *NR3C1* was lower ($P = 0.01$) in CF-fed calves compared with CM- and WM-fed calves (Table 3). The expression of *ADRA1A* was higher ($P = 0.03$) in CF-fed calves compared with CM-fed calves, and a tendency of higher expression was observed in CF-fed calves compared with WM-fed calves (Table 3). In addition, the expression of *SLC6A4* in the WM-fed calves was lower ($P = 0.04$) than CM-fed and tended to be lower than CF-fed calves. Moreover, the ileal expression of *HTR4* tended to be lower ($P = 0.09$) in WM-fed calves compared with CF-fed calves. In colon tissue, ex-

pression of *ADRA1A* was higher ($P = 0.03$) in WM-fed calves compared with CM-fed calves and expression of *MC2R* was lower ($P = 0.02$) in CF-fed calves compared with CM-fed calves (Table 4).

Effect of Colostrum Feeding Strategies on Abundance of Active Tissue-Associated Bacteria

Calves fed the CF dietary treatment had higher, and a tendency for higher abundance of active mucosa-attached *Lactobacillus* in the colon ($P = 0.02$) and ileum ($P = 0.08$), respectively, compared with calves fed WM (Table 5). In addition, calves fed CF had higher abundance of active mucosa-attached *E. coli* ($P = 0.03$) in colon compared with calves fed WM and CM, but no difference was observed in the ileum. No difference was detected in abundance of active mucosa-attached *Bifidobacterium* spp. in the ileum or colon among the 3 treatments.

Table 2. Effect of feeding management on expression of targeted genes in adrenal glands

Gene	Treatment ¹			P-value
	WM	CM	CF	
<i>ADRA1A</i>	10.97 $\pm$ 0.59 ^a	11.04 $\pm$ 0.41 ^a	7.17 $\pm$ 0.84 ^b	0.02
<i>ADRB2</i>	5.83 $\pm$ 0.25	6.23 $\pm$ 0.17	5.90 $\pm$ 0.42	0.66
<i>CYP11B1</i>	-2.12 $\pm$ 0.18	-2.02 $\pm$ 0.24	-2.50 $\pm$ 0.30	0.30
<i>GABBR1</i>	3.03 $\pm$ 0.17	3.15 $\pm$ 0.16	2.93 $\pm$ 0.26	0.99
<i>GABRB2</i>	5.68 $\pm$ 0.18	5.51 $\pm$ 0.22	5.23 $\pm$ 0.28	0.40
<i>HTR2B</i>	1.33 $\pm$ 0.49	1.83 $\pm$ 0.22	1.20 $\pm$ 0.30	0.47
<i>HTR4</i>	7.62 $\pm$ 0.41	7.78 $\pm$ 0.41	7.83 $\pm$ 0.66	0.91
<i>MC2R</i>	2.47 $\pm$ 0.21	2.64 $\pm$ 0.11	2.58 $\pm$ 0.12	0.94
<i>NR3C1</i>	1.90 $\pm$ 0.12	1.97 $\pm$ 0.12	2.16 $\pm$ 0.21	0.28
<i>SLC6A4</i>	5.92 $\pm$ 0.22	6.45 $\pm$ 0.14	6.16 $\pm$ 0.27	0.42
<i>STAR</i>	-3.26 $\pm$ 0.12 ^a	-2.76 $\pm$ 0.15 ^b	-3.26 $\pm$ 0.14 ^a	0.02

^{a,b}Different superscripts within a row indicate a significant difference among treatments ($P < 0.05$).

¹Treatment for 72 h after birth; WM = whole milk; CM = mixture of 50% colostrum and 50% milk; CF = colostrum feeding; $n = 8$ in each group. Expression levels are indicated as ΔCt mean $\pm$ SEM; Ct = cycle threshold; lower value means higher expression.

Table 3. Effect of feeding management (WM = whole milk; CM = mixture of 50% colostrum and 50% milk; CF = colostrum feeding; for 72 h after birth; n = 8 in each group) on expression of targeted genes in ileal tissue

Gene ¹	Treatment ²			P-value
	WM	CM	CF	
<i>ADRA1A</i>	7.68 ± 0.84 ^{ab}	8.40 ± 0.38 ^a	5.72 ± 0.30 ^b	0.03
<i>ADRB2</i>	3.97 ± 0.12	3.74 ± 0.24	3.98 ± 0.24	0.65
<i>CYP11B1</i>	6.88 ± 0.57	5.59 ± 0.92	4.66 ± 1.14	0.24
<i>GABBR1</i>	3.12 ± 0.21	2.70 ± 0.18	2.99 ± 0.20	0.26
<i>GABRB2</i>	6.08 ± 0.92	4.80 ± 0.69	6.57 ± 0.51	0.10
<i>HTR2B</i>	1.70 ± 0.35	1.51 ± 0.19	1.75 ± 0.22	0.50
<i>HTR4</i>	0.43 ± 0.35 ^a	0.26 ± 0.21	-0.21 ± 0.19 ^b	0.09
<i>MC2R</i>	5.53 ± 0.53	5.62 ± 0.28	6.50 ± 0.67	0.36
<i>NR3C1</i>	0.50 ± 0.12 ^a	0.52 ± 0.12 ^a	1.14 ± 0.17 ^b	0.01
<i>SLC6A4</i>	8.70 ± 0.35 ^a	6.34 ± 0.96 ^b	6.05 ± 1.38 ^b	0.04
<i>STAR</i>	6.68 ± 0.23	6.58 ± 0.18	6.72 ± 0.36	0.87

^{a,b}Different superscripts within a row indicate a significant difference among treatments ($P < 0.05$).

¹*ADRA1A* = adrenoceptor α_{1A} ; *ADRB2* = adrenoceptor β_2 ; *CYP11B1* = cytochrome P450, subfamily XI B, polypeptide 1; *GABBR1* = gamma-aminobutyric acid type B receptor subunit 1; *GABRB2* = gamma-aminobutyric acid type A receptor beta2 subunit; *HTR2B* = 5-hydroxytryptamine receptor 2B; *HTR4* = 5-hydroxytryptamine receptor 4; *MC2R* = melanocortin 2 receptor; *NR3C1* = nuclear receptor subfamily 3 group C member 1; *SLC6A4* = solute carrier family 6 member 4; *STAR* = steroidogenic acute regulatory protein.

²Expressional levels are indicated as ΔCt mean $\pm$ SEM; Ct = cycle threshold; lower value means higher expression.

Relationship Between Active Mucosal Attached Bacteria and Neuroendocrine Gene Expression and Endocrine Concentrations

Pearson correlation analysis revealed a positive correlation ($r = 0.76$; $P < 0.001$) between the abundance of active mucosa-associated *Lactobacillus* spp. and *E. coli* in colon tissue (Figure 3A). The abundance of active mucosa-associated *Lactobacillus* spp. ($r = 0.41$; $P = 0.04$, Figure 3B) and *E. coli* ($r = 0.42$, $P = 0.04$, Data not shown) in ileum was positively correlated with serum cortisol level. We also found a positive correlation between expression of *HTR2B* and *HTR4*,

and abundance of active mucosa-attached *E. coli* ($r = 0.77$, $P < 0.001$ and $r = 0.51$, $P = 0.01$; Figure 4A, B) and active mucosa-attached *Lactobacillus* spp. ($r = 0.64$, $P < 0.001$ and $r = 0.57$, $P = 0.0036$; Figure 4C, D) in colon tissue, respectively. No significant correlations were observed between abundance of active mucosa-associated bacteria and expression of targeted endocrine genes in ileum.

DISCUSSION

Colostrum plays a fundamental role in passive transfer of immunity to newborn calves, which protects the

Table 4. Effect of feeding management (WM = whole milk; CM = mixture of 50% colostrum and 50% milk; CF = colostrum feeding; for 72 h after birth; n = 8 in each group) on expression of targeted genes in colon tissue

Gene	Treatment ¹			P-value
	WM	CM	CF	
<i>ADRA1A</i>	4.70 ± 0.76 ^a	7.87 ± 0.42 ^b	6.86 ± 0.81 ^b	0.03
<i>ADRB2</i>	3.34 ± 0.35	3.41 ± 0.45	4.37 ± 0.29	0.12
<i>CYP11B1</i>	5.85 ± 0.99	2.35 ± 1.15	5.12 ± 0.91	0.10
<i>GABBR1</i>	2.82 ± 0.22	3.52 ± 0.44	2.98 ± 0.24	0.67
<i>GABRB2</i>	5.97 ± 0.58	3.77 ± 0.81	6.07 ± 0.57	0.10
<i>HTR2B</i>	0.72 ± 0.53	-0.38 ± 0.35	1.02 ± 0.39	0.11
<i>HTR4</i>	-1.44 ± 0.56	-1.13 ± 0.29	-0.58 ± 0.57	0.40
<i>MC2R</i>	5.15 ± 0.89 ^{ab}	3.27 ± 1.52 ^a	7.47 ± 0.43 ^b	0.02
<i>NR3C1</i>	2.12 ± 0.16	2.02 ± 0.97	2.49 ± 0.13	0.13
<i>SLC6A4</i>	7.11 ± 0.71	4.99 ± 1.13	7.63 ± 0.58	0.23
<i>STAR</i>	6.47 ± 0.80	5.05 ± 1.08	7.20 ± 0.47	0.40

^{a,b}Different superscripts within a row indicate a significant difference among treatments ($P < 0.05$).

¹Expressional levels are indicated as ΔCt mean $\pm$ SEM; Ct = cycle threshold; lower value means higher expression.

Table 5. Effect of feeding management on abundance (mean $\pm$ SEM of 16S rRNA copy/g of tissue) of active tissue-associated bacteria¹

Item	Ileum			Colon		
	WM	CM	CF	WM	CM	CF
<i>Bifidobacterium</i>	(4.90 $\pm$ 3.30) $\times 10^3$	(8.30 $\pm$ 4.30) $\times 10^3$	(7.50 $\pm$ 3.50) $\times 10^3$	(2.99 $\pm$ 0.71) $\times 10^8$	(1.81 $\pm$ 0.58) $\times 10^8$	(1.87 $\pm$ 0.57) $\times 10^8$
<i>Lactobacillus</i>	(2.87 $\pm$ 0.50) $\times 10^{10a}$	(3.50 $\pm$ 3.10) $\times 10^7$	(9.00 $\pm$ 6.30) $\times 10^{7b}$	(5.80 $\pm$ 2.70) $\times 10^{7a}$	(2.20 $\pm$ 1.30) $\times 10^{8ab}$	(4.20 $\pm$ 1.70) $\times 10^{8b}$
<i>Escherichia coli</i>	(3.60 $\pm$ 1.50) $\times 10^5$	(8.50 $\pm$ 3.40) $\times 10^5$	(2.42 $\pm$ 0.93) $\times 10^6$	(1.50 $\pm$ 0.46) $\times 10^{8a}$	(1.35 $\pm$ 0.58) $\times 10^{8a}$	(4.20 $\pm$ 1.70) $\times 10^{8b}$
Total bacteria	(7.16 $\pm$ 2.26) $\times 10^8$	(1.18 $\pm$ 0.76) $\times 10^9$	(3.87 $\pm$ 2.17) $\times 10^9$	(7.08 $\pm$ 4.25) $\times 10^9$	(5.20 $\pm$ 2.52) $\times 10^9$	(7.03 $\pm$ 4.74) $\times 10^{10}$

^{a,b}Different superscripts within a row indicate a significant difference among treatments ($P < 0.05$).

¹WM = whole milk; CM = mixture of 50% colostrum and 50% milk; CF = colostrum feeding.

hypoagammaglobulinemic neonatal calf against pathogens and other environmental challenges (Bendixen et al., 2011). In practice, a minimum feeding of 100 g of IgG in colostrum is recommended to ensure successful passive transfer of immunity in neonatal calves (Weaver et al., 2000). The hypothalamic–pituitary–adrenal axis serves as the primary regulator and modulator of immunity for newborn animals without a developed adaptive immune system (Hulbert and Moisé, 2016). In this regard, how colostrum feeding regimens affect expression of neuroendocrine genes may help evaluate the efficacy of newborn calf management strategies, such as extended colostrum feeding, which may be important to ensure passive transfer of immunity if the quality of colostrum is relatively low or unknown.

Production of stress hormones occurs primarily in the adrenal glands, and other organs, such as the brain, thymus, skin, vascular system, lungs, and intestine, have the capacity to produce glucocorticoids and catecholamines locally (Eisenhofer et al., 1997; Noti et al., 2009; Pacak, 2011). However, it is currently unknown whether stress hormones are also produced by intestinal mucosa in neonatal calves. In the present study, the expression of *StAR* and *CYP11B1* genes that encode key enzymes in steroid and glucocorticoid production were detected in ileum and colon tissues, suggesting that intestinal mucosa may produce glucocorticoids in neonatal calves. The expression of *MC2R* (ACTH receptor) was also detected in the calf ileum and colon, which is known to mediate stress-induced cortisol secretion in response to ACTH signal (Proudnikov et al., 2008). These results correspond with previous findings of mucosal synthesis of glucocorticoids in intestinal tissues of rodents and humans (Eisenhofer et al., 1997; Cima et al., 2004; Noti et al., 2009; Kostadinova et al., 2014), suggesting that the gut of preweaned ruminants (before rumen is developed) may have similar endocrine secretion functions as those reported in monogastric animals. The expression of *StAR*, *CYP11B1*, and *MC2R* was significantly higher in adrenal glands compared with the intestinal tissues in calves. This suggests that extra-adrenal glucocorticoids production may not have a great effect on the gut endocrine functions, but rather regulates local physiological processes including the gut immune system and other metabolic pathways (Kostadinova et al., 2014). In contrast, *ADRA1A* and *ADRB2* genes, which encode α_1 - and α_2 -adrenergic receptors for catecholamines (Li et al., 2014), and *HTR4* gene, which encodes the receptor for serotonin (Corominas et al., 2010), had higher expression in the mucosa of the ileum and colon compared with those in adrenal glands. It is reported that adrenergic nerve fibers are present in Peyer's patches and in nonfollicular mucosa in close proximity to immune cells, including dendritic cells and

Figure 3. (A) Pearson correlation between log-transformed abundance of active tissue-associated *Escherichia coli* and *Lactobacillus* spp. in the colon of neonatal calves. (B) Pearson correlation between plasma concentration and log-transformed abundance of active tissue-associated *Lactobacillus* spp. in the ileum of calves. r = correlation coefficient; P = level of significance; gray area shows 95% confidence limits.

mast cells of mucosa (Lyte et al., 2011). Meanwhile, adrenergic and serotonin receptors regulate secretion of Glucagon-like peptide-2 by enteroendocrine L cells, which increases nutrient absorption and intestinal growth (Drucker, 2001; Burrin et al., 2003; Connor et al., 2015). The higher expression of *ADRA1A*, *ADRB2*, and *HTR4* in the ileum and colon tissues in the present study might indicate the importance of these receptors in the regulation of fundamental digestive functions (e.g., intestinal vermiculation), and other physiological processes associated with gut function and mucosal immunity (Santulli et al., 2012; Hodge et al., 2013; Scanzano and Cosentino, 2015). Because calves are born with undeveloped immune systems and immature intestinal morphology (Blum, 2006; Hulbert and Moisés, 2016), the stimulation of signal transduction via these neurotransmitters may play a role in adapting to environmental changes in neonatal development.

Our study also found that feeding calves with different combinations of colostrum and milk from the second meal affected the expression of neuroendocrine genes both in adrenal glands and intestine of neonatal calves. The nutritional components of colostrum and milk differ based on their qualities, including amount of lactose, protein, and fat (McGrath et al., 2016). In addition to its nutrients, colostrum contains high concentrations of immunoglobulins, growth factors, and bioactive and immunological components (Kuhne et al., 2000). Calves fed CM had lower expressions of *StAR* in adrenal glands compared with calves fed WM and CF. Because *StAR* is thought to mediate ACTH-induced cortisol production by facilitating cholesterol flux into mitochondria (Miller, 2017), the downregulated *StAR* expression in CM suggests potential changes in stress-

induced steroidogenesis in calves under such colostrum feeding management. Meanwhile, the expression of *CYP11B1* encoding rate-limiting enzyme in cortisol synthesis in adrenal glands was not affected by feeding treatments. Plasma concentrations of cortisol were also not significantly different between feeding treatments. Thus, CM feeding after the first colostrum meal was suggested to have minor effects on cortisol production detected in sera, but it is unknown whether the cortisol production might be affected in adrenal glands. Our results also showed that CF calves had a higher expression of *ADRA1A* in adrenal glands. Schäff et al. (2014) reported that calves fed colostrum tended to have higher binding capacity of glucocorticoid and α_1 -adrenergic receptors in the liver compared with calves that received milk-based formula. It has been proposed that such stimulation of adrenergic receptors via catecholamines binding in the calf's liver is involved in modulating glucose metabolism (Carron et al., 2005; Schäff et al., 2014). However, whether colostrum directly affects the expression of adrenergic receptors in adrenal glands, a main location of catecholamine production, is currently unknown. Our results suggest that extended colostrum feeding to avoid a sharp transition from a colostrum to milk may stimulate expression of adrenal α_1 -adrenergic receptors in calf adrenal glands.

In addition to the adrenal glands, different colostrum feeding strategies also changed the expression of neuroendocrine genes in the intestinal tract of calves, especially those related to glucocorticoids, catecholamine and serotonin signaling. Extended colostrum feeding tended to induce higher expression of *ADRA1A* in the ileum, which corresponds to the similar results in the adrenal glands. However, the expression of *ADRA1A* in

the colon was higher in calves that were subsequently fed WM. These results could be attributed to colostrum and milk-derived components stimulating α_1 -adrenergic receptors in the ileum and colon, respectively. Although the precise mechanism causing this inconsistent expressional change was not clear, the altered expression of *ADRA1A* in all 3 tissues suggests that colostrum feeding may affect adrenergic receptor mediated gut function, such as gut vermiculation. In addition, calves fed the CF diet tended to have increased expression of serotonin receptor (*HTR4*) and transporter (*SLC6A4*) in the ileum, whereas plasma serotonin concentration was not different. Although our data of blood serotonin concentrations seemed to be lower compared with some studies, other papers showed similar or comparative results (Watanabe et al., 2014; Weaver et al., 2016). Interestingly, serotonin concentration is reported to decrease after birth, reaching the lowest level at 2 to 3 d of age (Hernández-Castellano et al., 2018), which suggests attenuated serotonin signaling in newborn calves. Our result suggests that prolonged ingestion of colostrum may reinforce serotonin-mediated signaling in the

ileum locally. Previous reports also suggest a potential interaction between catecholamines and serotonin due to the overlapping nerve fibers of these neurotransmitters (Kim and Camilleri, 2000; Hensler et al., 2013), suggesting that serotonin-modulated signaling in the ileum could interplay with signaling induced by the α_1 -adrenergic receptor. In addition, the expression of *NR3C1* and *MC2R* was higher in the ileum and colon, respectively, under WM and CM feeding compared with that under CF. From this result, it is speculated that the higher concentration of cortisol in colostrum than milk (Shutt and Fell, 1985) decreased the expression of *NR3C1* in ileum epithelial cells, as cortisol treatment was reported to downregulate *NR3C1* mRNA level in some cell types, including enterocytes (Numakawa et al., 2009; Pang et al., 2014). Further studies are needed to compare the cortisol concentrations between milk and colostrum, and to investigate the role of these genes and their encoded proteins in affecting the physiological alterations and gut development of neonatal calves, and the possible signaling interactions of serotonin, adrenergic, and glucocorticoid receptors.

Figure 4. Pearson correlation between log-transformed abundance of active tissue-associated gut bacteria and gene expression (Δ Ct; Ct = cycle threshold) of serotonin receptors in the colon of neonatal calves. The correlation between *Escherichia coli* and *HTR2B* (A), *E. coli* and *HTR4* (B), *Lactobacillus* spp. and *HTR2B* (C), and *Lactobacillus* spp. and *HTR4* (D) are indicated. Gray area shows 95% confidence limits.

Emerging evidence has revealed the relationship between gut bacteria and host neuroendocrine function in recent studies (Sandrini et al., 2015; Farzi et al., 2018), highlighting the effect of gut microbiota on the gut–brain axis. In the present study, we observed that prolonged colostrum feeding increased the abundance of active tissue-associated *Lactobacillus* spp. in the ileum and colon, and the abundance of active tissue-associated *E. coli* in colon compared with WM and CM as subsequent meals. Colostrum generally contains higher concentrations of casein and lactoferrin than milk (McGrath et al., 2016), which have been shown to promote growth of *Lactobacillus* species (Artym and Zimecki, 2005). Fischer et al. (2018) found that calves fed colostrum immediately after birth had higher total *E. coli* prevalence compared with calves not fed colostrum until 6 h or 12 h after birth. Additionally, the identified strong positive correlation between abundance of active *Lactobacillus* spp. and active *E. coli* suggests a potential association of these 2 bacterial groups. Interestingly, the positive correlations between the expression of *HTR4* and *HTR2B* and both bacterial groups were also detected in colon tissues. A recent study using germ-free and gnotobiotic mice has shown that commensal bacteria can produce serotonin in the gut lumen in vivo (Hata et al., 2017). More specifically, it was reported that several different *Lactobacillus* spp. produce serotonin, dopamine, and other hormone-like metabolites, and this ability was enhanced in the presence of gram-negative bacteria, including *E. coli* (Özoğul et al., 2012). It has been reported that serum cortisol level significantly increased when mice were challenged with *E. coli* O157 (Forsythe et al., 2010). Indeed, moderate correlation ($r = 0.415$, $P = 0.044$) was detected between abundance of active mucosa-attached *E. coli* in the ileum and plasma cortisol, suggesting that increasing the abundance of potential pathogenic *E. coli* lead to higher stress of calves. However, the *E. coli* detected in this study is generic, and future studies to profile *E. coli* at strain/serotype level are needed to verify those that may be beneficial (stimulating growth of *Lactobacilli*) or unbeneficial (pathogenic) to newborn calves. Nevertheless, the present findings suggest a potential interaction between active tissue-associated *E. coli* and *Lactobacillus* spp. and host neurotransmitter receptor gene expression, and potentially endocrine gene expression. Future research should focus on the causal effects of region-dependent changes observed in this study to better understand the interaction between microbe–host neuroendocrine function. Because there were no significant correlations observed between gut bacteria and expression of genes encoding host neurotransmitter

receptors in the ileum, we speculate that the interactions between gut bacteria and host endocrine receptors differ among varied gut regions due to the variations in the abundance of gut microbes and gut environmental properties, such as lower pH and thinner mucus in the small intestine.

CONCLUSIONS

The present study detected and characterized the expression of genes involved in neuroendocrine functions in intestinal tissues, as well as in the adrenal gland of neonatal dairy calves. The altered expression of these neuroendocrine genes in adrenal glands and ileum and colon tissues and altered active mucosa-attached *Lactobacillus* spp. and *E. coli* were detected under different colostrum and milk feeding strategies. These suggest the effect of different colostrum feeding management on the expressional regulation of neuroendocrine genes, which is partly mediated by the alteration of gut microbiota. Future studies are required to understand the specific colostrum and milk-derived components that can influence microbiota and its production of neurotransmitter-like molecules, and the molecular mechanism in the interaction between gut-associated bacteria and host neuroendocrine function in newborn calves. In addition to the well-characterized bioactives, such as IgG, oligosaccharides, or peptides, in colostrum and milk, endocrine molecules may also differ in colostrum replacer, maternal, pasteurized, and nonpasteurized colostrum, which has not been studied to date. Such variation may also affect microbiota and gut gene expression related to gut–brain axis, which warrants further study. The knowledge obtained from this study provides a better understanding of neonatal neuroendocrine function in the gut of newborn calves, which might play an essential role in the development of gut function during early life. The knowledge generated from this study is vital to assist the industry in developing better management strategies to improve neonatal calf gut health.

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