Increasing serotonin bioavailability alters gene expression in peripheral leukocytes and lymphoid tissues of dairy calves

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Dairy calves are born with a naïve immune system. Feeding newborn calves high quality colostrum is a practice readily implemented on most U.S. dairy farms1,2. While colostrum is important for calf immune protection and survival, the remaining pre-weaning phase consists of a liquid diet of either whole milk or milk replacer. In the U.S., 40–60% of dairy farms feed milk replacers to pre-weaned dairy calves3. Dairy calves’ adaptive immune system develops gradually, and the pre-weaning phase has been shown to be critical for immune system development and maturation4. Emerging data demonstrate that milk not only delivers nutrients, but also primes the newborn’s growth and development through delivery of bioactive factors5,6. Although milk replacer formulation has improved over the years, it still lacks bioactive components naturally present in milk that could aid in the development of the dairy calf immune system. Therefore, there is a need to explore novel bioactive factors that when added to milk replacers can enhance dairy calf immune development.

Serotonin is a bioactive factor with immunoregulatory properties7–12 that is present in cow milk and is also endogenously synthesized by the calf13–15. However, little is known about the immunologic role of serotonin in milk or in cattle. Serotonin is derived from the conversion of L-tryptophan to 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme tryptophan hydroxylase (TPH1, in peripheral tissues, and TPH2, in the brain), which is subsequently converted to serotonin by the aromatic amino acid decarboxylase enzyme (AADC/DDC)16. There are 7 serotonin receptor families with more than 10 G-protein coupled receptor (GPCR) subtypes and 3 ion-gated channel receptor subtypes17. Depending on which serotonin receptor subtype (Gα, q/11 or i/o) is activated, signaling cascades including adenylyl cyclase (AC), protein kinase C (PKC), inositol trisphosphate (IP3) and mitogen and extracellular signal regulated kinase (MERK) are activated18 to modulate the activity of proteins or to regulate gene transcription. Serotonin action is terminated by the serotonin transporter (SERT), which removes circulating serotonin from the extracellular space to be recycled or degraded by monoamine oxidase (MAO).

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Peripheral serotonin (close to 95% of total serotonin in the body) is primarily synthesized by the enterochromaffin cells in the gut and is involved in the regulation of many physiological functions\(^4\)\(^-\)\(^6\)\(^-\)\(^8\), including immune modulation. Peripheral serotonin is mainly stored and transported by blood platelets\(^8\), the major source of serotonin for circulating immune cells and organs. Several studies support the immunomodulatory role of peripheral serotonin in the rodent and human model. For instance, platelet-derived serotonin in mice promotes neutrophil recruitment to inflammation sites by increasing L-selectin expression and enhancing endothelial interactions\(^25\). Research in humans and rodents show that different immune cells express one or more components of the serotonergic signaling pathway machinery (i.e., receptors, TPH1 and/or SERT, MAO\(^8\)\(^,\)\(^9\)\(^,\)\(^26\)\(^,\)\(^27\)), indicating their capacity to synthesize, metabolize, respond to, and/or transport serotonin\(^11\)\(^,\)\(^28\)\(^,\)\(^29\). Dendritic cells (DCs) do not express TPH1, however upon activation they increase SERT expression which allows them to take up serotonin from circulation\(^9\). Activation of murine T lymphocytes increases TPH1 expression and hence, endogenous serotonin production. This serotonin then acts as an autocrine-paracrine cytokine to enhance T cell proliferation or is taken up by circulating cells (i.e., DCs and platelets)\(^6\)\(^,\)\(^9\). Furthermore, in mice, serotonin can attract mast cells, which express both TPH1 and SERT\(^6\), to inflammation sites\(^26\). Studies using TPH1 knockout mice (lacking peripheral serotonin) show reduced macrophage infiltration and lower proinflammatory cytokine production (i.e. IL-1\(\beta\) and -6) compared to wild type mice\(^11\). Dendritic cells of TPH1 knockout mice produce less IL-12 following a 24 h lipopolysaccharide (LPS) challenge compared to wild type mice\(^35\). It has also been shown that isolated monocytes incubated with LPS secrete more cytokines when serotonin is present\(^35\).

Serotonin has been shown to regulates physiological functions that are relevant to lactation performance including metabolic status, milk synthesis and calcium regulation\(^15\)\(^,\)\(^23\)\(^,\)\(^33\). However, studies exploring serotonin’s immunomodulatory role are limited in the bovine. One study showed that supplementation of 5-hydroxytryptophan to newborn calves for 15 days increased blood mRNA abundance of genes related to innate and adaptive immunity, including nuclear factor kappa beta, chemokine C-C motif ligand 5, cyclooxygenase-2 and interleukin 1 beta\(^34\). However, a more thorough characterization of the bovine serotonergic pathway and its ability to modulate immunity is lacking. Herein, we characterize the expression profile of genes involved in serotonin synthesis, metabolism and signaling, and its impact on cytokine expression in leukocytes and lymphoid tissues of dairy calves supplemented with 5-hydroxytryptophan, the serotonin precursor, or fluoxetine, a selective serotonin reuptake inhibitor (SSRI). We hypothesized that increased cell and tissue serotonin bioavailability will promote the expression of genes involved in serotonergic machinery and signaling, and positively modulate the expression of immune genes in peripheral leukocytes, spleen, thymus and popliteal lymph node of pre-weaned dairy calves.

**Results**

**Effects of FLX and 5-HTP supplementation on white blood cells counts and subfractions.** No differences were observed for total WBC (count/\(\mu\)L) among treatment groups before or after 10 days of FLX or 5-HTP supplementation (\(P > 0.19\); Table 1). Likewise, WBC subfractions (count/\(\mu\)L) including neutrophils, eosinophils, basophils, monocytes and lymphocytes were not different among treatment groups before or after 10 days of supplementation (\(P > 0.47\); Table 1).

**Effects of 5-HTP on peripheral leukocyte gene expression.** Serotonin synthesis, metabolism, and downstream pathways. After 10 days of 5-HTP supplementation, peripheral leukocytes were isolated for gene expression analysis, reported as fold change relative to CON saline-supplemented group. Supplementation of 5-HTP upregulated or tended to upregulate genes involved in serotonin synthesis and metabolism, including DDC and MAOA (\(P < 0.03\)) and MAOB (\(P = 0.09\)), while TPH1, SLC6A4 and IDO1 gene expression was not affected (\(P > 0.09\); Fig. 1A). Seven serotonin receptors were upregulated including 5-HT1A, -1B, -1D, -1F, -3B, -3C and -4 (\(P < 0.04\)) and 5-HT2B tended to be upregulated (\(P = 0.06\); Fig. 1B). The 5-HT3A receptor subtype was downregulated more than 8-fold following 5-HTP supplementation (\(P = 0.06\); Fig. 1B); whereas the expression of SHT2A, -5A, -6 and -7 remained unchanged (\(P > 0.13\)). Four genes downstream of serotonin GPCR signaling were significantly upregulated by 5-HTP supplementation, including ADCY1, PLCB2, MAPK3 and AKT1 (\(P < 0.05\)); while AKT2 and STAT5B tended to be upregulated (\(P < 0.10\); Fig. 1C).

| WBC count | Treatments* | \(\text{P-value}^b\) |
|-----------|-------------|---------------------|
| CON       | 5-HTP       | FLX                 |
| Neutrophils, \(10^3/\mu\)L | 3.69 ± 0.62 | 3.96 ± 0.67 | 3.69 ± 0.62 | 0.78 | 0.99 |
| Monocytes, \(10^3/\mu\)L | 1.08 ± 0.11 | 1.19 ± 0.12 | 0.97 ± 0.11 | 0.51 | 0.48 |
| Lymphocytes, \(10^3/\mu\)L | 4.37 ± 0.23 | 4.79 ± 0.24 | 4.79 ± 0.23 | 0.22 | 0.48 |
| Eosinophils, \(10^3/\mu\)L | 0.06 ± 0.02 | 0.06 ± 0.02 | 0.09 ± 0.02 | 0.96 | 0.42 |
| Basophils, \(10^3/\mu\)L | 0.01 ± 0.02 | 0.05 ± 0.03 | 0.03 ± 0.02 | 0.26 | 0.71 |

Table 1. Circulating white blood cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) in dairy calves with increased serotonin bioavailability. *Oral supplementation of milk replacer with saline (control; \(n = 8\)), fluoxetine (40 mg/dL; \(n = 8\)) or 5-hydroxytryptophan (5-HTP, 90 mg/dL; \(n = 8\)) to Holstein dairy calves for 10 consecutive days. \(^b\)Statistical significance declared at \(P\)-value \(\leq 0.05\) and tendencies at 0.05 < \(P \leq 0.10\).
Clusters of differentiation and immune related genes. The expression of several immune related genes in peripheral leukocytes was modulated by 5-HTP supplementation. Specifically, CTLA4 was upregulated (P < 0.006), and CD80 tended to be upregulated compared to CON (P = 0.10; Fig. 2A). A significant upregulation of various cytokines including IFNG, IL2, IL4, IL13, and IL17A (P < 0.02) was observed, with IL2 having the highest fold-change of 17.5 when compared to CON (Fig. 2B). Differentially expressed genes in peripheral leukocytes are summarized in Fig. 3A.

Effects of FLX on peripheral leukocyte gene expression. Serotonin synthesis, metabolism and downstream pathways. After 10 days of FLX supplementation, peripheral leukocytes were isolated for gene expression analysis and reported as fold change relative to CON saline-supplemented group. Supplementation of FLX upregulated genes involved in serotonin synthesis and metabolism, including DDC and MAOA (P < 0.03) and tended to upregulate MAOB (P = 0.07), whereas TPH1, SERT and IDO1 were not differentially expressed (P > 0.12; Fig. 1D). Fluoxetine supplementation upregulated the expression of 5-HT2C (P = 0.03) and 5-HT4 (P = 0.08); while 5-HT2A and -3A were significantly downregulated (>30-fold, P < 0.006; Fig. 1E). All other serotonin receptors remained unchanged (P > 0.12). Eight genes downstream of serotonin receptor signaling were upregulated including PKA, PLCB2, MAPK3, AKT1, STAT5B and PIK3CB (P < 0.03) or tended to be upregulated such as AKT2 and REL (P < 0.10) by FLX supplementation (Fig. 1F). Meanwhile, MAPK14 was significantly downregulated (P = 0.008) and MAPK1 tended to be downregulated by FLX (P = 0.09, Fig. 1F).

Clusters of differentiation and immune related genes. The expression of various immune related genes in peripheral leukocytes was modulated by FLX supplementation. Specifically, FLX upregulated the gene expression of CD14, CD80, and TLR4 (P < 0.045), CTLA4, and SELL (P < 0.10), while it downregulated CD28 expression compared to CON (P < 0.10; Fig. 2C). Supplementation of FLX upregulated the gene expression of various cytokines, including IL1B, IL4 and IL12B (P < 0.03), TNF, IL17A and CXCL10 (P < 0.10; Fig. 2D). Only TGFB gene expression in peripheral leukocytes was significantly downregulated after the 10-d FLX supplementation (P = 0.04; Fig. 2D). Differentially expressed genes in peripheral leukocytes are summarized in Fig. 3B.

Effects of 5-HTP on Thymus, Spleen, and Lymph Node Gene Expression. Serotonin synthesis, metabolism and downstream pathways. Supplementation of 5-HTP for 10 days did not affect serotonin
metabolism related genes in spleen tissue compared to CON (P > 0.15; Supplementary Fig. S1A), but upregulated serotonin receptors 5-HT1A and -4 (P < 0.01) and tended to upregulate 5-HT3B (P = 0.09; Supplementary Fig. S1B). In the spleen, MAPK1 and AKT2 tended to be upregulated, while MAPK14 tended to be downregulated (P < 0.07; Supplementary Fig. S2A). In the popliteal lymph node, serotonin metabolism related enzyme MAOB tended to be upregulated (P = 0.06; Supplementary Fig. S1A), but no serotonin receptors were differentially expressed by 5-HTP (P > 0.11; Supplementary Fig. S1B). Additionally, in the lymph node, MAPK3 and STAT5B tended to be downregulated (P > 0.08; Supplementary Fig. S2A). In the thymus, 5-HTP supplementation upregulated DDC (P = 0.04) and tended to downregulate TPH1 (P = 0.07; Supplementary Fig. S1A), while it significantly upregulated the serotonin receptor 5-HT3B (P = 0.02; Supplementary Fig. S1B). Thymus expression of genes downstream of serotonin receptors included upregulation of AKT1 and AKT2 (P < 0.043) and STAT5A (P = 0.06; Supplementary Fig. S2A). Differentially expressed genes in tissues are summarized in Fig. 3A.

Clusters of differentiation and immune related genes. The expression of several immune related genes in lymphoid tissues was altered by 5-HTP supplementation. In the spleen, 5-HTP supplementation upregulated the surface protein CTLA4 (P = 0.01) and tended to downregulate the T cell surface marker, CD8B (P = 0.07; Supplementary Fig. S3A). Spleen expression of IL17A was upregulated (P = 0.04) and IFNG and IL13 tended to be upregulated (P < 0.09) by 5-HTP supplementation; while IL1B cytokine was downregulated (P = 0.003; Supplementary Fig. S3B). In the popliteal lymph node, CD14 was downregulated (P = 0.04; Supplementary Fig. S3A) but cytokine gene expression was not affected by 5-HTP supplementation (P > 0.16; Supplementary Fig. S3B). In the thymus, the surface protein, CTLA4, gene expression was upregulated (P = 0.025; Supplementary Fig. S3A), however cytokines were not differentially expressed after 5-HTP supplementation (P > 0.11; Supplementary Fig. S3B).

Effects of FLX on Thymus, Spleen, and Lymph Node Gene Expression. Serotonin synthesis, metabolism and downstream pathways. In the spleen, FLX supplementation upregulated SLC6A4 (P = 0.02) and IDO1 (P = 0.07) and downregulated TPH1 (P = 0.06; Supplementary Fig. S1C). Additionally, serotonin receptors

Figure 2. Gene expression in peripheral leukocytes of pre-weaned dairy calves after a 10-day oral supplementation of 5-hydroxytryptophan (5-HTP, 90 mg/d; n = 8), fluoxetine (FLX, 40 mg/d; n = 8) or control (CON; n = 8). Gene expression is reported as fold change (2^(-ΔΔCt)) relative to CON saline-supplemented group. (A) Gene expression of immune surface markers and (B) cytokines after 10 days of 5-HTP oral supplementation. (C) Gene expression fold change of immune surface markers and (D) cytokines after 10 days of FLX oral supplementation. Black bars denote 5-HTP vs. CON gene expression fold change, and dotted bars denote FLX vs. CON gene expression fold change. The negative inverse of fold-change values <1 was calculated for visual representation of negative fold changes. (*) indicate significant differences (P ≤ 0.05) and (#) indicate tendencies (0.05 < P ≤ 0.10) between CON and FLX or CON and 5-HTP.
5-HT1A and 5-HT1D tended to be upregulated (P < 0.08), while 5-HT3B tended to be downregulated (P = 0.054; Supplementary Fig. S1D). Only MAPK1 gene expression was upregulated by FLX (P = 0.03), while all other downstream serotonin receptor signaling genes remained unchanged in the spleen compared with CON calves (Supplementary Fig. S2B). In the popliteal lymph node, REL gene expression was downregulated following FLX supplementation (P = 0.002; Supplementary Fig. S2B). In the thymus, FLX tended to upregulate 5-HT6 receptor (P = 0.09) and the downstream PKA (P = 0.04), but the expression of genes related to serotonin metabolism or downstream pathways was similar to CON (P > 0.11; Supplementary Fig. S1D, S2B). Differentially expressed genes are summarized in Fig. 3B.

Clusters of differentiation and immune related genes. The expression of several immune related genes in lymphoid tissues was modulated by FLX supplementation. In the spleen, FLX upregulated the surface protein CTLA4 (P = 0.03) and tended to downregulate CD28 (P < 0.10; Supplementary Fig. S3C). The gene expression of IFNG and IL17A was upregulated (P < 0.04) and IL13 tended to be upregulated (P = 0.08; Supplementary Fig. S3D). Meanwhile, the expression of CXCL10 was downregulated (P = 0.001) and IL1A, IL1B and IL10 tended to be downregulated (P < 0.08; Supplementary Fig. S3D). Fluoxetine supplementation upregulated the expression of surface protein CTLA4 (P = 0.021; Supplementary Fig. S3C) in the thymus, however, cytokine genes were not differentially expressed (P > 0.11; Supplementary Fig. S3D). In the popliteal lymph node tissue, IL17A tended to be upregulated (P = 0.10; Supplementary Fig. S3D) but no other cytokine or surface markers genes were differentially expressed following FLX supplementation (P > 0.11; Supplementary Fig. S3C, S3D).

Effects of 5-HTP and FLX on Proliferation, Apoptosis, and Cell Metabolism Genes. Peripheral leukocytes. The expression of genes related to cell proliferation, apoptosis, cell metabolism and cell cycle in peripheral leukocytes after 10-d of 5-hydroxytryptophan or fluoxetine supplementation was evaluated. Supplementation of 5-HTP upregulated PTEN (P = 0.005), whereas FLX upregulated CCND1 (P = 0.02) and tended to upregulate LAMP2, CASP8 and PCNA (P < 0.10; Supplementary Table S1).

Secondary lymphoid tissues. In the spleen, 5-HTP supplementation had no effect on cell metabolism gene expression (P > 0.12) whereas FLX supplementation downregulated CCND1 (P = 0.04; data not shown). In the popliteal lymph node, 5-HTP supplementation tended to downregulate CCND1 expression (P = 0.07), while FLX
supplementation upregulated APAF1 and CASP8 (P < 0.04; data not shown) and tended to upregulate FASLG and PCNA (P < 0.10; data not shown). In the thymus, 5-HTP upregulated BAX (P = 0.01), while FLX upregulated FASLG (P = 0.03; data not shown).

Discussion

The role of serotonin as an immunoregulatory molecule has been widely demonstrated in human and rodents\(^7\)\(^\text{--}\)\(^27\), however, evidence supporting its role in livestock species is lacking. We previously reported that increasing serotonin bioavailability in dairy calves is possible through the supplementation of 5-hydroxytryptophan or fluoxetine\(^35\). Herein, we report the effects of increased serotonin bioavailability on circulating WBC count and the gene expression of peripheral leukocytes and secondary lymphoid organs of dairy calves undergoing immune system maturation. To our knowledge, this is the first experiment to characterize how the serotonin axis regulates the bovine immune system.

In this experiment, WBC and WBC subfractions including neutrophil, monocyte, lymphocyte, eosinophil, and basophil counts were within the normal physiological ranges for growing dairy calves and similar among treatment groups before and after 10 days of treatment supplementation. This indicates that increasing serotonin bioavailability for 10 days did not significantly alter immune cell populations. Even though we did not see an increase in neutrophil counts, it is possible that serotonin is improving neutrophil function. For instance, human neutrophils cultured in vitro with high concentrations of serotonin have higher motility than neutrophils grown in low serotonin conditioned media\(^36\). Platelet expression of FcγRIIA, a receptor that recognizes immune complexes, plays a role in inflammation by activating neutrophils and enhancing endothelial vasoactivity\(^22\). Furthermore, neutrophils from wild type mice have been shown to have improved tissue infiltration compared to TPH1 knockout mice\(^25\). Thus, further in vitro experiments evaluating serotonin's role in neutrophil motility and function (i.e. oxidative burst and phagocytic capacity) are needed in bovine.

For over 20 years, researchers have investigated the significance of serotonin receptors in human and murine immune cells and their possible implication in autoimmune diseases. In our experiment, the entire serotonergic machinery, including genes involved in serotonin synthesis, mechanism of action, and metabolism were expressed in the circulating leukocytes of all calves. This indicates that peripheral leukocytes of dairy calves can synthesize, metabolize, uptake and degrade serotonin. Supplementation of either 5-HTP or FLX increased serotonin bioavailability\(^35\) and upregulated several genes involved in serotonin machinery in peripheral leukocytes. The ubiquitous aromatic decarboxylase enzyme, DDC, that converts 5-HTP to serotonin, and monoamine oxidase enzyme, MAOA, that metabolizes serotonin, were significantly upregulated in the peripheral blood leukocytes of both 5-HTP and FLX fed calves. This suggests an overall increase of serotonin metabolism in immune cells of calves under supplementation.

Notably, supplementing 5-HTP upregulated the gene expression of 9 out of the 13 serotonin receptor subtypes evaluated in peripheral leukocytes compared to controls. Interestingly, all serotonin receptors from family 1 subtypes (5-HT1), including 5-HT1A, 5-HT1B, 5-HT1D, and 5-HT1F were significantly upregulated, suggesting a positive feedback loop to increase ligand binding. Serotonin receptor family 1 proteins couple mainly through G\(_i/o\) proteins to inhibit adenylyl cyclase in various cells and tissues and have high affinity towards serotonin\(^18\). Serotonin receptor 5-HT1A subtypes are expressed in various human and mouse immune cells including monocytes/macrophages, dendritic cells, neutrophils, mast cells, eosinophils, B cells and T cells\(^7\). Data in human and/or rodents shows that 5-HT1A receptor subtype modulates adhesion and chemotaxis of mast cells\(^30\) and enhances phagocytosis by murine macrophages\(^88\). Upregulation of serotonin receptors subtypes from family 1 in peripheral leukocytes after 5-HTP supplementation could support calves' immune function by promoting adhesion and chemotaxis of mast cells, and/or enhancing phagocytosis. However, future functional studies targeting this specific receptor should be conducted in the bovine.

In this experiment, the 5-HT2B receptor subtype was upregulated following 5-HTP supplementation. Activation signals among 5-HT2 family receptor subtypes are different, but mainly couple through G\(_i/o\) proteins. For instance, the 5-HT2A receptor subtype signals through the activation of PLC-\(\beta\) in tissues and cells, whereas the 45% homologous 5-HT2B receptor subtype signal through various phospholipases (i.e. PLC-\(\beta\), PLA)\(^18\). This 5-HT2B receptor subtype has been widely studied in human dendritic cells (DCs) where it is reported to promote anti-inflammatory functions. Human monocytes cultured in the presence of serotonin, as well as IL-4 and granulocyte-macrophage colony-stimulating factor, differentiate into DCs with reduced expression of co-stimulatory molecules (i.e. CD86) which are needed for antigen-presenting cells (APC; i.e. DCs) and T cell cognate interactions\(^89\). Similarly, 5-HT2B receptor activation was found to downregulate monocyte derived DC expression of co-stimulatory molecules that activate naïve T cells, and possibly preventing inflammation by regulating both innate and adaptive immune systems\(^90\). Apart from 5-HT2B, 5-HT2A expression is upregulated on activated CD4+ and CD8+ T cells\(^41\). Furthermore, 5-HT2A antagonist treatment inhibits T cell activation and diminishes IL-2 and interferon gamma (IFN-\(\gamma\)) production in a dose dependent manner\(^41\). Thus, it appears that 5-HT2A acts as a proinflammatory serotonin receptor whereas 5-HT2B acts as an immunosuppressive serotonin receptor.

Herein, both IL2 and IFNG gene expression were upregulated even though 5-HT2A was not differentially expressed. Interleukin-2 is an immunoregulatory cytokine, mainly produced by CD4+ T cells, which enhances T cell proliferation, regulates T cell helper cell differentiation\(^42\), and limits immune responses by enhancing T regulatory cells function\(^43\). Moreover, IL-2 induces the transcription of IFN-\(\gamma\) in T cells\(^44\). Interferon gamma is an important inflammatory cytokine that induces maturation and licensing of APC that in turn recruit and prime T cells, and increases the expression of major histocompatibility complex\(^45\). Translation of IFN-\(\gamma\) by T cells skew B cells to enhance antibody production and induces isotype switching from IgM to IgG2a\(^46\). Upregulation of IL2 and IFNG by 5-HTP supplementation, if translated to protein, could act to support adaptive immune responses. Further studies are warranted to confirm and determine the implications of these findings.
The serotonin receptors -3B and -3C, and -4 were upregulated following 5-HTP supplementation. The 5-HT3B receptor subtype is a ligand-gated ion channel and is 41% homologous to the 5-HT3A receptor. This receptor has been linked to nausea in patients undergoing chemotherapy. To our knowledge the function of 5-HT3B receptor subtype has not been linked to immunity, probably because 5-HT3B can be expressed as a heteromeric receptor, 5-HT3A/B, with properties differing from those of 5-HT3A receptor subtype.

Gene expression of key signaling molecules downstream of serotonin receptors, including ADCY1, PLCB2, MAPK3 and AKT, was upregulated by 5-HTP supplementation. Adenylyl cyclase (ADCY1) is a major downstream signaling gene for 5-HT4, -6 and -7 following Gs coupling activation. Yet, in some cell types, activation of 5-HT1A receptor inhibits ADCY1. Since both 5-HT1 and -4 receptor families were upregulated in our experiment we cannot determine which specific serotonin receptor subtype might be upregulating the expression of ADCY1 intracellularly. Furthermore, both PLCB2 and 5-HT2B were differentially expressed and PLCB2 is known to be the major signaling downstream molecule for 5-HT2B. Yet, further investigation is needed to characterize the effects of specific serotonin receptors and downstream pathways on specific circulating immune cells in the bovine.

The gene expression of clusters of differentiation CD4, CD8 and CD14 in peripheral leukocytes were not affected by 5-HTP supplementation, suggesting that increased serotonin bioavailability had no effect on immune cell concentrations. However, serotonin has been linked to the migration of specific immune cell types. For instance, Müller et al. reported that 5-HT1B receptor induces the migration of human immature DCs. Additionally, human DCs have been shown to secret IL-1β after activation of receptors 5-HT3, -4 and -7. Interestingly, in our experiment, peripheral blood leukocytes had greater expression of serotonin receptor subtypes 5-HT3B, -3C and -4, although upregulation of IL1B was not observed. Hernandez-Castellano et al. supplemented 5-HTP to newborn dairy calves for 15 days and reported an upregulation of both IL-1β and nuclear factor kappa beta (NF-κβ) genes in blood but no differences in IgG production were observed. In our experiment, REL (NF-κβ) and CTSA4 (NF-κβ subunit) were not affected by 5-HTP supplementation, however discrepancies between Hernandez-Castellano’s and our results could be attributed primarily to calf age linked to different facets of immune system development.

Fluoxetine oral supplementation increases serotonin bioavailability by blocking SERT. Fluoxetine supplementation downregulated 5-HT2A and -3A receptor subtypes by more than 20-fold in peripheral leukocytes. Contradictory findings have been reported linking the use of SSRIs to 5-HT2A downregulation in the rodent frontal cortex. Nevertheless, the effects and implications of the downregulation of 5-HT2A receptor by SSRIs in the immune system remain unknown. Monocytes and T cells express the 5-HT3A receptor subtype, as well as naïve and activated B cells, predominantly by differentiating B cells at the germinal centers of lymph nodes. Interestingly, the use of the 5-HT3A antagonist, tropisetron, inhibits T cell activation and production of IL-2. In 1994, Fan reported that 5-HT3 receptor is a target of fluoxetine, which in turn decreases serotonin influx into the cell. Moreover, fluoxetine blocks 5-HT3 receptors by interactions during both open and closed channel states, although the clinical relevance of this effect is still unknown. Thus, further research is needed to understand 5-HT3B downregulation by fluoxetine supplementation.

The thymus is a central lymphoid organ where T cells develop, while the spleen and lymph nodes are important secondary lymphoid organs where immune responses are generated. Therefore, we sought to explore the effects of increased serotonin bioavailability in these tissues. Similar to peripheral leukocytes, all tissues, independent of treatment supplementation, expressed the serotonergic machinery indicating that serotonin could be playing a role in the development and deployment of adaptive immune responses. We demonstrated that increased serotonin bioavailability exerts a less pronounced effect in these tissues compared to peripheral leukocytes, at least at the mRNA level. However, it is important to mention that tissue data was collected from a subset of animals (n = 4/treatment) euthanized after 10 days of treatment supplementation and that the statistical power to detect significant differences in tissues was 66%. Nevertheless, it is notable that we observed a significant downregulation of CD14 in the popliteal lymph node after 5-HTP supplementation and FLX tended to downregulate CD8B in spleen tissue, while both treatments upregulated CTLA4 in the thymus and spleen tissues. CD8 downregulation has been shown to occur when CD8 effector T cells are switching functions. There is limited data exploring the effect of serotonin on thymus, lymph node, and/or spleen gene expression, thus, these results are novel and warrant deeper investigation.

The present experiment targeted candidate genes involved in serotonin production, metabolism, transport, signaling and immune regulation. We established that bovine peripheral blood leukocytes and immune tissues express components of the serotonin signaling pathway, including TPH1, SERT, DDC, MAO and serotonin receptors. This indicates that these cells and tissues have the potential to synthesize, transport, respond to and/or degrade serotonin. We demonstrated that at the mRNA level, increased serotonin bioavailability exerts a pronounced immunomodulatory response, particularly in peripheral leukocytes and spleen tissue. Indeed, specific
serotonin receptors and cytokines were differentially expressed upon 5-HTP or FLX supplementation, which could potentially influence the developmental trajectory and maturation of immune cells in dairy calves at a young age. Differences in the modulatory effects of 5-HTP and FLX in the peripheral immune system could be attributed to intrinsic differences in their molecular mechanism of action. Indeed, we previously reported that 5-HTP calves had greater circulating serotonin concentrations when compared to fluoxetine calves35, thus we hypothesize that greater serotonin bioavailability exerts different effects. While promising correlations between serotonin and the immune system exist in other species, and now in the bovine at the transcriptional level, it is currently unknown whether promoting serotonin translates into both innate and adaptive immune system orchestration. Ultimately, the fact that 5-HTP is a biogenic modified amino acid whereas fluoxetine is a synthetic drug should be taken into consideration when developing strategies to enhance livestock health and development.

Methods

Animals and experimental design. All methods and procedures performed in this study were carried out in accordance with relevant guidelines and regulations approved by the Institutional Animal Care and Use Committee at the University of Florida (protocol # 201709851). Experimental design and treatments are described in detail by Marrero et al.35. Briefly, Holstein bull calves (n = 24, 18 ± 2 d of age, 47 ± 3.2 kg) were assigned to one of three treatments in a complete randomized block design (8 pens, 2.3 m x 2.5 m; n = 3 per pen, one of each treatment). Calves received 4 L of milk replacer (Southeast Milk Inc, Okeechobee, FL) at 0700 h and 1700 h. Treatments were administered once daily (0700 h feeding) by supplementing milk replacer with 5-hydroxytryptophan (5-HTP), 90 mg/d, n = 8, Sigma, St. Louis, MO, USA; #H9772), fluoxetine (FLX), 40 mg/d, n = 8, Spectrum Chemical, Gardena, CA, USA; #F1200) or saline (CON, n = 8) for 10 consecutive days. Treatment was applied individually to each calf. Supplementing 5-HTP and FLX increases serotonin bioavailability by different mechanisms: exogenous 5-HTP bypasses the rate limiting enzyme TPH1, allowing its conversion to serotonin by AADC69, whereas FLX binds to SERT inhibiting endogenous serotonin reuptake within the cell34,70.

Hematology analysis. Whole blood samples were collected from the jugular vein before (d0) and on the 10th day of treatment supplementation 4 h after 0700 h feeding in tubes containing K2 EDTA (BD, Franklin Lakes, NJ, USA, #368047). Within 2 h of collection, blood samples were analyzed for hematology parameters including white blood cells (WBC), neutrophil, lymphocyte, monocyte, eosinophil and basophil count/µL using the Idexx ProCyte Dx analyzer (IDEEX Laboratories Inc., Westbrook, ME).

Peripheral blood mononuclear cell isolation. Blood samples were collected from the jugular vein on the 10th day of treatment supplementation 4 h after the 0700 h feeding using heparin blood collection tubes (BD, Franklin Lakes, NJ, USA, #366430) and kept on ice until laboratory arrival. Blood was centrifuged at 1,200 g for 18 min at 20°C, plasma layer was discarded, and peripheral leukocytes (i.e., buffy coat) was transferred to a 15 mL conical tube. To lyse residual red blood cells, a hypotonic solution (lyse buffer: 1.5 g Na2HPO4 (Fisher; #BP332-1) and 0.3 g NaH2PO4 (Fisher; #BP329-1) at pH 7.2) was used. To restore cells, restore buffer was used (27 g NaCl (Fisher cat. #BP358-1) added to the lyse buffer solution and adjusted to pH 7.2). To each sample, 8 mL of lye buffer and 4 mL of restore buffer were added. Tubes were centrifuged at 650 g for 5 min at 4°C to form a pellet. Pellets were resuspended in 200 µL of RNAlater (Invitrogen, Carlsbad, CA, USA; #AM7021) and stored at −80°C until RNA extraction.

Euthanasia and tissue collection. After the 10th d of treatment supplementation, 4 calves per treatment (n = 4 pens) were euthanized at the University of Florida abattoir. Calves were sedated by intravenous administration of 0.2 mg/kg xylazine and euthanized using a captive bolt pistol followed by jugular exsanguination. Spleen, popliteal lymph node and thymus tissues (approximately 1 g each) were harvested, rinsed in sterile PBS, transferred to a cryotube containing RNAlater and stored at −80°C until RNA extraction.

Peripheral Leukocyte and Tissue RNA Extraction. Peripheral leukocytes resuspended in RNAlater were centrifuged at 650 g for 10 min at 4°C to reform the pellet and RNAlater was removed. For tissues, RNA was extracted from 60 mg each of spleen, popliteal lymph node and thymus. Each sample was placed in 1 mL of QIAzol Lysis reagent (Qiagen, cat. #79306) and homogenized using a tissue homogenizer (Tissue Master 125, Omni International, GA, USA). A commercial RNA extraction kit (RNeasy Plus Universal Mini Kit, Qiagen, cat. #73404) was used according to the manufacturer's instructions. RNA concentration and quality were determined using a NanoDrop (NanoDrop Spectrophotometer, Thermo Scientific, USA; #ND-2000). RNA samples were stored at −80°C until gene expression analysis.

Primer design, validation and, gene expression analysis. We quantified the expression of genes related to serotonin machinery and signaling (i.e., synthesis and metabolism, serotonin receptors, and downstream pathways), immunomodulated genes (i.e., cytokines), and cellular processes (i.e., apoptosis, cell cycle, among others) in peripheral leukocytes, spleen, thymus and popliteal lymph node tissues. For this, high-throughput Multiplex RT-qPCR BioMark Dynamic Array Integrated Fluidic Circuits (IFCs) was used (Fluidigm Corporation, South San Francisco, CA). Briefly, 96 primers targeting 91 genes of interest, 4 reference genes (ACTB, GAPDH, RSP9 and HPRT1), and one reference structural gene were assayed (see Supplementary Table S1). An initial quantification run was performed for primer validation using an 8-point, two-fold dilution series (in triplicate) using RNA pools per tissue of interest. The linearity between RNA quantity and cycle threshold (Ct) was tested and efficiency of amplification was calculated. Primers were considered validated if they passed 5 points with an efficiency of 0.8–1.3 and an R2 ≥ 0.92. Specificity of amplification for each primer pair was evaluated by plotting the dissociation-characteristics of double-stranded DNA. A single peak following melt curve analysis indicated a pure, single amplicon. For gene expression of immune-related tissues and peripheral leukocytes, RNA
was diluted to 5–15 ng/μL. All samples were normalized to 256 pg RNA and transferred to the IFC plate with the primer-probe sets. All nanoliter reactions were performed as per manufacturer’s recommendations using the following thermal protocol: 95 °C for 1 min, followed by 30 cycles of 96 °C for 5 s and 60 °C for 20 s. The software, Fluidigm Real-Time PCR Analysis, was used to calculate Ct values for all 96 genes for each sample. Non-detectable expression was set at a Ct of 26 for spleen tissue and 28 for WBC, popliteal lymph node, and thymus tissues. The geometric mean of the four reference genes was calculated for each sample and used to normalize Ct values of genes of interest. Normalized gene expression (ΔΔCt) was used for statistical analysis.

**Statistical analysis.** Data were analyzed using linear models in R programming 3.5.1 (R Foundation for Statistical Computing; Vienna, Austria). Variables analyzed were white blood cell counts and ΔΔCt of genes evaluated by qPCR. The model included pen and treatment (CON, 5-HTP or FLX) as fixed effects. For gene expression analysis, the estimates of the model (ΔΔCt) for each gene were used to calculate fold change relative to CON (i.e. 5-HTP vs. CON or FLX vs. CON), using the 2−ΔΔCt method. The negative inverse of fold-change values < 1 was calculated for visual representation of negative fold changes. Homogeneity of variance and normality of residuals were evaluated by plotting and influential points were detected using Cook’s distance test. Peripheral leukocyte gene expression ΔΔCt averages for CON, 5-HTP and FLX, and fold changes and P values for 5-HTP vs. CON and FLX vs. CON can be found in Supplementary Table S2. Statistical significance was declared at P < 0.05 and tendencies at 0.05 < P ≤ 0.10.

**Data availability**

All data generated or analyzed during this study are included in this published article [and its supplementary figures and tables online].

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Author contributions
J.L. designed the experiment, obtained funding and supervised all aspects of the research. M.G.M., A.L.S., S.F., and B.D.-S. conducted the experiment. J.D. assisted with gene selection, data analysis and interpretation. M.G.M and J.L. wrote the initial draft. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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